Departamento de Farmacia y Tecnología Farmacéutica
Facultad de Farmacia

Universidad de Navarra

TESIS DOCTORAL

"Polymeric devices as a platform for the delivery of actives involved in heart repair"

Trabajo presentado por Teresa Simón Yarza para obtener
el Grado de Doctor

Fdo. Teresa Simón Yarza
Pamplona, 2013

Universidad de Navarra

FACULTAD DE FARMACIA

Departamento de Farmacia y Tecnología Farmacéutica

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Certifican:

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Pamplona, 2013

Las investigaciones realizadas en el presente trabajo se han desarrollado gracias a la financiación recibida a través de la *Royal Society* (JP100124), MINECO PSE SINBAD (PSS 0100000-2008-1), *European Uninon FPVII* (INELPY, Health-2007-2.4.2.-5), Caja de Ahorros de Navarra (Programa Tu Eliges: Tu Decides) y a la Fundación Universidad de Navarra.

María Teresa Simón Yarza agradece así mismo la beca de postgrado para la formación de profesorado universitario (Programa FPU, AP2009-4556) del Ministerio de Educación y la ayuda predoctoral para la elaboración de la tesis y obtención del grado de doctor del Departamento de Educación del Gobierno de Navarra.

A mis padres, *Ana Mari y Eugenio*

AGRADECIMIENTOS

Gracias a la Universidad de Navarra y a los Departamentos de Farmacia y Tecnología Farmacéutica y al Área de Terapia Celular del Centro de Investigación Médica Aplicada, donde se ha desarrollado esta investigación, y donde, sobre todo, me he formado en estos últimos años. Gracias a la Fundación La Caixa, al Departamento de Educación del Gobierno de Navarra y al Ministerio de Educación, Cultura y Deporte, por su financiación.

Creo que no falto a la verdad si aseguro que es interminable la lista de personas a las que debo mi agradecimiento, y muchas otras cosas, después de estos años. De antemano pido disculpas: soy muy consciente de que este apartado de agradecimientos es del todo insuficiente para corresponder por todo lo que he recibido.

Muchas gracias a mis directores, la Dra. María Blanco y el Dr. Felipe Prósper. María y Felipe: gracias por acogerme en vuestros ambiciosos proyectos de investigación y por confiar en mí en todo momento. Gracias no solo por todo lo que he aprendido de sistemas de liberación y de regeneración de tejidos, que ya sería mucho, sino también porque he tenido la oportunidad de aprender cómo hacer una investigación rigurosa, exigente y sin perder nunca de vista lo más importante: el paciente. Y gracias también por vuestro ejemplo como personas. En mi memoria quedan grabados numerosos momentos concretos en los que me habéis dado ejemplo de humanidad.

Un agradecimiento especial a la Dra. Maria Jesús Renedo. Entro otras cosas, es a ella a quien en gran parte debo el defender hoy esta tesis.

Gracias a los investigadores, profesores y personal del Departamento de Farmacia y Tecnología Farmacéutica: Dña. Carmen Dios, Dña Maribel Calvo, Dña. Pilar Ygartua, Dña. Conchita Tros, Dña. María del Mar Goñi, Dña. María Jesús Garrido, Dña Socorro Espuelas, D. Iñaki Fdez. de Trocóniz, D. Juan Manuel Irache, D. Fernando Martínez, D. Félix Recarte, Dña. María Huici y D. Juan Luis Martín. Gracias porque de todos ellos he aprendido.

Gracias a John Lewis (de los Lewis de toda la vida).

A mis amigas, Bea y la ya Dra. Zinnia Parra. Han sido cinco años que espero se prolonguen para toda nuestra vida.

A mis compañeros de grupo (los blanquitos):

A Estherilla. Gracias.

A Elisa Garbayo. Intentaré no desperdiciar el gran ejemplo que me has dado.

A los que ya no están: Fabio, Edu, Ander, David, Izas. A los que seguís: Edurne, Paula, Cristina, Melissa, Ánguela… Y a los babys! Yolanda y Simón. Disfrutad de esta etapa.

A Hugo Lana, que aunque no es mi amigo en facebook ha hecho tanto por echarme una mano siempre que se lo he pedido. Y si no que le pregunten a Robin Williams.

Gracias a todos aquellos con los que he compartido poyata estos años. Sara, Patricia, Rebeca, Judit, Maite, Luisa, Koldo, Laura, Lorena, Cris, Arianna, María, Marijo, Eneko, Laura, Ana Margarita, Juana, Nekane, Nuria. Ha sido un placer.

Muchas gracias también a todos los miembros del laboratorio 1.01 del CIMA con los que he podido trabajar, y aprender tantas cosas de biología molecular. En especial al grupo de cardiovascular. Bea: muchas gracias por tu ejemplo, tu cariño y tu amistad. Mirian, he de decir que siempre que te lo he pedido me has dedicado tu tiempo y tu paciencia. Gracias también a Olalla, Manu, Sheyla, Laura, Edurne, Anama. Porque siempre, tanto en el labo como fuera, me he sabido bienvenida por vosotros.

My gratitude to Prof. Groll and all the people in the Lehrstuhl für Funktionswerkstoffe der Medizin und der Zahnheilkunde. Specially to Angella Rossi and Ilona Zilkowski. Danke Schön!

No puedo dejar de agradecer a todas las personas que han estado a mi lado estos años: Olloqui, Montserrat, Begoña, Rosario, Belén, Cristina, María...

Muchas gracias a mis amigas de Miravalles que nunca me fallan: Ana Martínez, Ana Zapata (y mi coach Juanan), Cristina Bazal, Ana Osácar y Mirian Vicente (en representación del resto).

Gracias a Mayra, Aranda, Laura, Bego, Adri, Elarre. Gracias por vuestra confianza y por todos los buenos momentos que hemos pasado juntas. Soy una gran admiradora de vuestra lealtad como amigas.

Gracias a mis amigas las marqueshitas: Ana, Majo, Elvira y Ana. No sabéis cómo os agradezco estos últimos meses. ¡Y tantos planes y conversaciones!

A mis amigos de planecillos: José, Fernando, Adri, Clara, Marisa, Marcelo, Belén, Mónica, Úriz, Maria Belén, Pablo, Ignacio y Joseba (mi dantzari preferido).

Mi segundo mayor agradecimiento se lo debo sin duda a mi familia. Nombro aquí sólo a los más cercanos, pero detrás de ellos hay muchos más. A mis padres, a los que dedico esta tesis. A mis hermanos: Ignacio y Maite, Maria Eugenia, Ana María, Fernando, Isabel y Bea. Y a las nuevas incorporaciones: Ignacio y Teresa.

Finalmente, mi mayor agradecimiento es para Dios.

What a piece of work is a man!
How noble in reason, how infinite in faculty!
In form and moving how express and admirable!
In action how like an Angel!
In apprehension how like a god!
The beauty of the world!
The paragon of animals!
And yet to me, what is this quintessence of dust?

The Tragedy of Hamlet, Prince of Denmark (Act II, Scene II, 285-300)
By William Shakespeare

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ABBREVIATIONS/ABREVIATURAS

BDGF	Brain cell-line derived neurotrofic factor Factor neurotrófico derivado de línea celular cerebral
BM-MNC	Bone marrow derived mononuclear cell Célula mononuclear derivada de médula ósea
BMP	Bone morphogenic protein Proteina morfogénica ósea
BSA	Bovin serum albumin Albúmina sérica bovina
CoQ10	Coenzyme Q10 Coenzima Q10
CPC	Cardiac progenitor cell Célula progenitora cardiaca
CT	Clinical trial Ensayo clínico
CVD	Cardiovascular disease Enfermedad cardiovascular
DCM	Dichloromethane Diclorometano
DDS	Drug delivery system Sistema de liberación
DMSO	Dimethylsulfoxide Dimetilsulfóxido
ECGS	Endothelial cell growth supplement Suplemento para el crecimiento de células endoteliales
ECM	Extracellular matrix Matriz extracelular
EF	Ejection fraction Fracción de eyección
EGF	Epidermal growth factor Factor de crecimiento epidérmico
EPC	Endothelial progenitor cell Célula progenitora endotelial
ES	Electrospinning Electro-hilado
ESC	Embrionic stem cell Célula madre embrionaria
EU	European Union Unión Europea
EVA	Ethylene-vinyl acetate Acetato de etilén vinilo
FDA	Food and Drug Administration Agencia de Alimentos y Medicamentos
FGF	Fibroblast growth factor Factor de crecimiento de fibroblastos
GDNF	Glial cell-line derived neurotrophic factor Factor neurotrófico derivado de la glía
GF	Growth fator Factor de crecimiento

HD	Huntington's disease Enfermedad de Huntington
HEMA	Poly(2-hydroxyethyl methacrylate) Poli(2-hidroxietil metacrilato)
HGF	Hepatocyte growth fator Factor de crecimiento de hepatocitos
HSA	Human serum albumin Albúmina sérica humana
HUVEC	Human umbilical vein endotelial cell Célula endothelial de vena de cordón umbilical humano
IGF	Insulin-like growth factor Factor de crecimiento insulínico
IL	Interleukine Interleuquina
iPSC	Induced Pluripotent stem cell Célula madre pluripotencial inducida
LDC	Lipid drug conjugates Conjugados lípido-fármaco
MEM	Microelectromechanical Microelectromecánico
MI	Myocardial ischemia Isquemia de miocardio
MP	Microparticle Micropartícula
MSC	Mesenchimal stem cell Célula madre mesenquimal
MTS	Cell proliferation assay Ensayo de poliferación celular
NEM	Nanoelectromechanical Nanoelectromecánico
NGF	Nerve growth fator Factor de crecimiento nervioso
NLC	Nanostructured lipid carriers Vehículos nanoestructurados lipídicos
NP	Nanoparticle Nanopartícula
Nrg	Neuregulin Neuregulina
NSC	Neural stem cell Célula madre neural
NT	Neurotrophin Neurotrofina
OP-1	Osteogenic protein-1 Proteína osteogénica-1
PAM	Pharmacological active microcarriers Microvehículos farmacológicos activos
PANI	Polyaniline Polianilina
PBS	Phosphate buffered solution Solución tampón fosfato
PCL	Poly-e-caprolactone Poly-e-caprolactona

PD	Parkinson disease Enfermedad de Parkinson
PDGF	Platelet derived growth fator Factor de crecimiento derivado de plaquetas
PEG	Poly (ethylene glycol) Polietilén glicol
PLA	Polylactid acid Ácido poliláctico
PLGA	Poly(lactic co-glycolic acid) Ácido poliláctico-co-glicólico
Ppy	Polypyrrole Polipirrol
PVA	Polyvinyl alcohol Alcohol polivinílico
R&D	Research and development Investigación y desarrollo
RT	Room temperature Temperatura ambiente
SC	Scaffold Andamio
SD	Standar deviation Desviación estándar
SEM	Scanning electron microscopy Microscopio electrónico de barrido
SLN	Solid lipid nanoparticle Nanopartícula lipídica sólida
SMSC	Synovium mesenchimal stem cell Célula madre mesenquimal sinovial
SSC	Side scatter Luz dispersada lateralmente
TBS	Tris buffered solution Solución tampón tris
TE	Tissue engineering Ingeniería de tejidos
TFA	Tri-fluoro-acetic acid Ácido trifluoroacético
TGF	Transforming growth factor Factor de crecimiento transformante
TROMS	Total recirculation one machine system
VEGF	Vascular endotelial growth factor Factor de crecimiento del endotelio vascular

INTRODUCTION

1. DRUG DELIVERY IN TISSUE ENGINEERING: GENERAL CONCEPTS

2. APPLICATION OF DRUG DELIVERY IN CARDIOVASCULAR DISEASE

DRUG DELIVERY IN TISSUE ENGINEERING: GENERAL CONCEPTS

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Nanostructured Biomaterials for Overcoming Biological Barriers, 2012, 501-526. RSC Drug Discovery Series No. 22

Edited by Maria Jose Alonso and Noemi S. Csaba

ISBN: 978-1-84973-529-2

DOI:10.1039/9781849735292

APPLICATION OF DRUG DELIVERY IN CARDIOVASCULAR DISEASE

Non-communicable diseases are the main cause of death in the world, and are regarded by the WHO as a major health challenge of the 21st century. Among them, cardiovascular diseases (CVD) are responsible for 48% of deaths, and it is estimated that in 2030 the number of deaths due to CVD will increase by 50% in relation to registered data for 2008.[1] The principal healthcare strategy to reduce this mortality rate is to focus on preventive actions, by the promotion of healthy habits: reducing tobacco use, alcohol abuse, obesity and overweight, avoiding physical inactivity, etc.[2] However, preventive actions are not enough, and conventional therapy to face CVD includes the use of numerous drugs. Some of the most widely employed drugs are angiotensin-converting-enzime inhibitors, angiotensin II receptor blockers, antiplatelet drugs, aspirin, β-blockers, calcium channel blockers, thrombolytic therapy, digoxin, nitrates and antiocoagulants. Myocardial ischemia (MI) frequently requires surgical procedures such as stent implant procedure and angioplasty or bypass implantation. In this way coronary circulation is restored, stopping hypoxia in the cardiac cells. Nevertheless, in many cases, the weakness of cardiac patients makes it impossible to apply these procedures. Heart transplants have obvious limitations such as the low number of donors and the immunological requirements to avoid rejection.

In spite of the great advances made in both pharmacological treatment and cardiac surgery, the large number of deaths resulting from heart hypoxia means that it is imperative to find alternative therapies that can not only tackle the symptoms, but also achieve regeneration of the damaged tissue. Tissue engineering (TE) came into being many years ago with this purpose in mind. In the cardiovascular framework it is necessary to know in depth all the processes that are triggered due to arterial blockade. Cells and growth factors (GFs) acting in these responses are candidates to be incorporated into the constructs that will be implanted in the patients. To date, several clinical trials have been carried out both in cell

and protein therapy.[3,4] Within the most widely assayed GFs, vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2) and granulocyte-colony stimulating factor (G-CSF) can be highlighted. They are involved in the neovascularization process, in which three sub-processes can be distinguished.[5] Firstly, vasculogenesis takes place, with the formation of new vessels, via angioblast differentiation into endothelial cells. In the next step, named angiogenesis, the vascular network is amplified and then, during arteriogenesis, these immature vessels are surrounded by pericytes and smooth muscle cells. Both VEGF and FGF-2 are active actors during all three steps.[6] G-CSF is known to promote bone marrow progenitor cell (BMPC) mobilization to the damaged tissue.[7] In several studies the ability of these cells to differentiate into other cell types implied in the neovascularization process, such as endothelial cells and smooth muscle cells, has been demonstrated. The potential ability to differentiate towards cardiomyocytes is a controversial issue since the experiments demonstrating this fact have not yet been reproduced.[8] In recent years a new factor has been gaining relevance in clinical trials: Neuregulin-1 (Nrg), with demonstrated activity promoting cardiomyocyte replication and myocardial regeneration.[9] Moreover, its indirect effect on neovascularization has been described, through the promotion of VEGF secretion (Annex IV).

In a previous study carried out by our group (Annex I) a VEGF microparticulated system was developed. This GF was chosen taking into account clinical trials performed up to that date. The VIVA study was the largest trial in which VEGF was directly administered into the myocardium of the patients.[10] The results of the study, however, were not as positive as expected. Lack of efficacy in this study, and in protein therapy in general, has been associated with the short half-life of GFs when they are directly administered. To overcome this limitation, drug delivery systems (DDS) have been proposed, since they protect proteins from degradation.[11] Other advantages are the controlled release of the protein, procuring a long lasting effect, as well as the lower toxicity consequences resulting from the presence of the protein away from the site of action. VEGF has been the GF primarily chosen to incorporate into DDS with the aim of promoting tissue neovascularization. Annex II includes a review of

preclinical studies published between 2006 and 2011 using DDS and VEGF in the cardiac regeneration context. The large number of publications included in that review reveals the great interest that the incorporation of VEGF in DDS has sparked.

Going back to the work in Annex I, VEGF was encapsulated in poly (lactid-co-glycolic) acid (PLGA) microparticles (MPs), a biocompatible and biodegradable polymer with the Food and Drug Administration's approval for its use in humans. Encapsulation efficiency was high and proangiogenic activity was demonstrated in an animal model of ischemia-reperfusion. Nevertheless, as has been shown in another paper recently published by our group (Annex III), PLGA MPs injected in the myocardium activated an immune response against the polymeric implant. This was confirmed after studying the presence of macrophages surrounding the particles by immunofluorescence against CD68, a macrophage marker. It is known that macrophages act in the organism clearing potentially harmful material. The activity of macrophages surrounding the implant site could be affecting the efficacy of the treatment, since these cells will remove the particles preventing the cytokine from exerting its therapeutic action.

Therapy with cytokines is not the only one that has been assayed to fight against ischemic heart disease. In antioxidative therapy the goal is to alleviate the negative effect of the formation of free oxygen radicals in the tissue. The role of the reactive oxygen species in the development of CVD has been gaining interest in recent years since studies demonstrate that when deregulated they are essential in the progression of the disease.[12,13] On the molecular level, their activity has been described in the control of phosphatases, kinases and transcription factors. In the CVD, their role in the pathogenesis of endothelial disfunction, atherosclerosis, hypertension, cardiac hypertrophy and heart failure has recently been reviewed.[14] This review describes in detail how mechanisms involved in cardiac hypertrophy, fibrosis and cardiomyocyte apoptosis are boosted by the presence of reactive oxygen species. All this data justifies the hypothesis that administration of antioxidants will be beneficial to treat CVDs. Within these antioxidants, Coenzyme Q10 (CoQ), also named ubiquinone, has

been administered in several clinical trials as a coadjuvant of conventional therapy. It is a molecule with a key role in the electron transport chain in the mitochondria. In a meta-analysis carried out recently, the authors concluded that CoQ administration can be beneficial for patients.[15] Nevertheless, they also mentioned the need to perform more studies with a better design in order to provide results that can be interpreted more easily. Remarkably, due to design deficiencies, only 13 out of 120 studies could be included in the mentioned meta-analysis. Besides, CoQ is considered as a nutritional supply, and is therefore, administered orally. Taking into account its lipophilic condition, its oral bioavailability is very low. It is reasonable to suppose that its therapeutic potential when orally administered will be very limited because of reduced gastrointestinal absorption. To improve bioavailability, this antioxidant was encapsulated by Prof. Ravi Kumar in PLGA nanoparticles (NPs), showing greater activity in several pathologies than commercially available CoQ formulations.[16,17,18]

Demonstrated beneficial effects of VEGF and the cardioprotective activity of CoQ suggest that the benefit for the patient could be greater if they are given concurrently.

A different strategy to increase the benefits of cytokine deliver from DDS is to combine them with cells. As mentioned before TE tends to include in the same system cells, biomaterials and proteins, this combination being known as the TE triad. Polymeric scaffolds are suitable for this purpose. Other scaffolds were made of collagen, taking advantage of its natural and biodegradable conditions.[19] Collagen sheets succeeded in increasing cell survival in the heart compared with direct cell injection in a model of MI. However these fibers are not able to encapsulate actives. Therefore, a polymeric scaffold able to incorporate molecules within the fibers and at the same time to allow cell attachment on its surface will constitute a more complete approach.

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OBJECTIVES

OBJECTIVES

1. To design, prepare and characterize PEG-PLGA microparticles loaded with VEGF with the aim of decreasing particle phagocytosis and consequently improving protein delivery using stealth technology.

2. To compare the efficacy of particulate forms of VEGF and CoQ10 and to investigate the synergistic possibilities of this combination in an animal model of myocardial ischemia.

3. To design and characterize polymeric electrospun scaffolds loaded with Neuregulin-1 and to test their *in vivo* biocompatibility in an animal model of myocardial ischemia.

CHAPTER 1

PEGYLATED-PLGA MICROPARTICLES CONTAINING VEGF FOR LONG TERM DRUG DELIVERY

CHAPTER 1

PEGYLATED-PLGA MICROPARTICLES CONTAINING VEGF FOR LONG TERM DRUG DELIVERY

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Int J Pharm. 2013 Jan 2; 440(1):13-18

Keywords: PEG, PLGA, Macrophage uptake, VEGF, Protein delivery.

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ABSTRACT

The potential of poly(lactic-co-glycolic) acid (PLGA) microparticles as carriers for vascular endothelial growth factor (VEGF) has been demonstrated in a previous study by our group, where we found improved angiogenesis and heart remodeling in a rat myocardial infarction model (Formiga *et al*., 2010). However, the observed accumulation of macrophages around the injection site suggested that the efficacy of treatment could be reduced due to particle phagocytosis.

The aim of the present study was to decrease particle phagocytosis and consequently improve protein delivery using stealth technology. PEGylated microparticles were prepared by the double emulsion solvent evaporation method using TROMS (Total Recirculation One Machine System). Before the uptake studies in monocyte-macrophage cells lines (J774 and Raw 264.7), the characterization of the microparticles developed was carried out in terms of particle size, encapsulation efficiency, protein stability, residual poly(vinyl alcohol) (PVA) and *in vitro* release. Microparticles of suitable size for intramyocardial injection (5 μm) were obtained by TROMS by varying the composition of the formulation and TROMS conditions with high encapsulation efficiency (70–90%) and minimal residual PVA content (0.5%). Importantly, the bioactivity of the protein was fully preserved. Moreover, PEGylated microparticles released in phosphate buffer 50% of the entrapped protein within 4 h, reaching a plateau within the first day of the *in vitro* study. Finally, the use of PLGA microparticles coated with PEG resulted in significantly decreased uptake of the carriers by macrophages, compared with non PEGylated microparticles, as shown by flow cytometry and fluorescence microscopy.

On the basis of these results, we concluded that PEGylated microparticles loaded with VEGF could be used for delivering growth factors in the myocardium.

1. INTRODUCTION

The concept of stealth technology came into being during the World War II in the attempt to escape from radar control. Ever since then, stealth strategy has included two different approaches: the development of radar absorbing paints, and novel designs in terms of shape and size. In the field of drug delivery systems (DDS), this concept has been applied to the ability of these carriers to avoid immunological recognition (Wassef *et al*., 1991). As in the military context, the shape (Lin *et al*., 2011), size (Maldiney *et al*., 2011) and material properties (Essa *et al*., 2011; Zhu *et al*., 2011) of the delivery system are crucial.

Recently, some research showing how macrophages have a higher affinity for specific shapes and sizes has been published (Doshi and Mitragotri, 2010). In this paper the authors conclude that particles with a size greater than 4 μm suffer less protein adsorption, which is the stage prior to macrophage phagocytosis. Interestingly, when comparing these results with the size distribution of bacteria, they found that most of these have a size between 2 and 3 μm, which favors their opsonization.

In 1978, Van Oss (Van Oss, 1978) described the phagocytosis process as a surface phenomenon, demonstrating how bacteria that are more hydrophobic than phagocytes readily become phagocytized, whereas bacteria that are more hydrophilic than phagocytes resist phagocytosis. At that time, researchers proposed the surface modification of molecules, by making them more hydrophilic, as a strategy to reduce phagocytic removal. In the 1970s, pegnology, the art of surface-modifying proteins, drugs or DDS by attaching molecules of poly(ethylene glycol) (PEG) was proposed by Abraham Abuchowski and Frank F. Davis (Abuchowski *et al*., 1977), and this has been applied effectively in protein therapies, obtaining increased stability (Khondee *et al*., 2011), increased resistance to proteolytic inactivation (Turner *et al*., 2011), decreased immunogenicity (Milla *et al*., 2012), increased circulatory half-lives (Maleki *et al*., 2012), and reduced toxicity (A. Jain and S.K. Jain, 2008),

thus improving the delivery and efficacy of proteins. To date, incorporating PEG seems to hold the most promising benefits while showing the lowest harmful effects (Owens and Peppas, 2006) and modified drugs are already on the market, most of which are PEGylated proteins (Pasut *et al*., 2008), such as interferon alpha (Fried *et al*., 2002), l-asparaginase (Abuchowski *et al*., 1984), granulocyte colony-stimulating factor (Tanaka *et al*., 1991) and uricase (Davis *et al*., 1981). However, despite the advances in the field of protein therapy, stealth technology is still emerging within the area of DDS. In fact, just one PEGylated delivery system has come onto the market (Knop *et al*., 2010): a PEGylated liposome containing doxorubicin for the treatment of cancer.

Our group recently published a study in which poly(lactic-co-glycolic) acid (PLGA) microparticles encapsulating the vascular endothelial growth factor (VEGF) were intramyocardially implanted in an ischemia–reperfusion animal model (Formiga *et al*., 2010). Benefits of the therapy were observed in terms of enhanced angiogenesis and notable reduction of negative remodeling, but when we studied the continued presence of the particles at the injection site over time, a macrophage accumulation around the particles depot was observed, which could limit the efficacy of the treatment. To overcome this challenge, in the present study our aim was to develop and characterize *in vitro* PEG-PLGA microparticles loaded with VEGF for their subsequent use in cardiovascular disease. The uptake of VEGF-PEGylated microparticles was studied by flow cytometry and fluorescence microscopy using two different monocyte-macrophage cell lines. Non PEGylated PLGA microparticles were used for comparison.

2. MATERIALS AND METHODS

2.1 Materials

Human recombinant VEGF was from R&D Systems (Minneapolis, MN, USA). PLGA with a lactic:glycolic ratio of 50:50 Resomer® RG 503H (MW 34 kDa), poly[(d,l-lactide-co-glycolide)-co-PEG] diblock Resomer® RGP d 5055 (5% PEG) and Resomer® RGP d 50105 (10% PEG) were provided by Boehringer-Ingelheim (Ingelheim, Germany). PEG 400, sodium azide, Rhodamine B isothiocyanate and human serum albumin (HSA) were provided by Sigma–Aldrich (Barcelona, Spain). Dichloromethane and acetone were obtained from Panreac Quimica S.A. (Barcelona, Spain). Poly(vinyl alcohol) (PVA), 88% hydrolyzed (MW 125,000), was from Polysciences, Inc. (WA, USA). Rabbit polyclonal anti-human VEGF-A (clone A-20, sc-152) was supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). ECLTM anti-Rabbit IgG horseradish peroxidase-linked whole antibody was from Amersham Biosciences (Buckinghamshire, UK). Mouse monoclonal anti-rat CD68 was provided by AbD Serotec (Oxford, UK). All the Western blot reagents were purchased from BioRad unless specified in the text.

The murine monocyte-macrophage cells lines J774 and Raw 264.7 were provided by Dr. Latasa (CIMA, University of Navarra). Human umbilical venous endothelial cells (HUVECs) were extracted from umbilical cords from donors, after informed consent according to the guidelines of the Committee on the Use of Human Subjects in Research at the Clinic Universidad de Navarra. CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) was obtained from Promega.

2.2 Preparation of PLGA and PEG-PLGA microparticles

VEGF-loaded microparticles were prepared by the double emulsion solvent evaporation method using TROMS (Formiga *et al*., 2010; Garbayo *et al*., 2009). Briefly, the organic solution composed of 4 ml of a mixture of dichloromethane/acetone (3:1) containing 50 mg of Resomer RG 503 was injected into the inner aqueous phase, which consisted of 50 µg of VEGF in 10 mM phosphate, 50 mM sodium chloride (PBS), 5 mg of HSA and 5 µl of PEG400. The primary emulsion (W_1/O) was recirculated through the system for 90 s under a turbulent regime at a flow rate of 38 ml/min. The first emulsion was injected into 20 ml of the external aqueous phase (W_2) composed of 20 ml of a PVA solution resulting in the formation of a double emulsion ($W_1/O/W_2$) which was homogenized by circulation through the system for 45 s. The resulting double emulsion was stirred at room temperature (RT) for at least 3 h to allow solvent evaporation and microparticle formation. Finally, microparticles were washed three times with ultrapure water and lyophilized (Genesis 12EL, Virtis). For PEGylated microparticles, 50 mg of a mixture of Resomer® 503H and Resomer® RGP d 5055 or Resomer® RGP d 50105 (1:1) were dissolved in the organic phase and microparticles were prepared as described above.

The composition of the different phases and TROMS parameters were varied to achieve an adequate particle size (of around 5 µm) for intramyocardial administration (Formiga *et al*., 2010).

2.3 Microparticle characterization

2.3.1 Particle size, size distribution and zeta potential

The mean particle size and size distribution of the microparticles were determined by laser diffractometry using a Mastersizer-S® (Malvern Instruments, Malvern, UK). Microparticles were dispersed in distilled water and analyzed under continuous stirring. The results were expressed as mean volume, in micrometers. Samples were measured in triplicate.

The zeta potential was measured using Zetaplus® (Brookhaven Instruments, NY, USA). Samples were diluted with distilled water and each experiment was repeated three times.

2.3.2 Residual PVA

The residual PVA associated with microparticles was determined by a colorimetric method based on the formation of a colored complex between two adjacent hydroxyl groups of PVA and an iodine molecule (Joshi *et al*., 1979). Briefly, 2 mg of lyophilized microparticles were resuspended in 2 ml of NaOH 0.5 M for 15 min at 60 °C. Each sample was neutralized with 900 µl of 1 N HCl and the volume was adjusted to 5 ml with distilled water. Next, 3 ml of a 0.65 M solution of boric acid, 0.5 ml of a solution of I2/KI (0.05 M/0.15 M) and 1.5 ml of distilled water were added. After 15 min of incubation, the absorbance of the samples was measured at 690 nm using an Agilent 8453 UV–visible spectrophotometer (Agilent Technologies, Palo Alto, CA, USA). A standard plot of PVA was prepared under identical conditions. Measurements were performed in triplicate.

2.3.3 Drug loading and encapsulation efficiency

The amount of VEGF encapsulated in the microparticles was determined by dissolving 1 mg of microparticles in 50 µl of DMSO. VEGF containing samples were diluted in 350 µl of PBS for western blot analysis. SDS-PAGE was performed onto 12% polyacrylamide gels. Following electrophoresis the proteins were transferred onto nitrocellulose membranes which were then blocked using 5% nonfat dried milk in Tris Buffered Saline (TBS) with 0.05% Tween 20, for 1 h RT. Membranes were incubated for 2.5 h at RT with rabbit antihuman VEGF-A antibody (A-20: sc- 152, 1:2000 dilution). The bounded antibody was detected with horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG antibody (1 h, RT, 1:2000 dilution). Chemiluminescence detection was performed using LumiLight Plus western blotting substrate (Roche Diagnostics, Mannheim, Germany). The VEGF signal was quantified by densitometry using the Quantity One software (Bio-Rad Laboratories, Inc., Munich, Germany). Samples containing defined quantities of VEGF were diluted under the same conditions (PBS and DMSO) and used as standard curve.

2.4 *In vitro* release studies

VEGF loaded microparticles (1 mg, n = 3) were resuspended in 0.25 ml PBS pH 7.4 with 0.02% (w/v) sodium azide used as a bacteriostatic agent. Incubation took place under orbital shaking in rotating vials (FALC F200, Falc Instruments, Treviglio, Italy) at 37 °C. At predefined times, the tubes were centrifuged (20,000 × g, 10 min) and the supernatant was removed and frozen at −80 °C until it was analyzed by western blot. The removed solution was replaced with an equal volume of fresh release buffer to maintain sink conditions. Release profiles were expressed in terms of cumulative release and plotted versus time.

2.5 VEGF bioactivity

The bioactivity of the VEGF released from the microparticles was evaluated *in vitro* by determining the proliferative capacity of a human umbilical vein endothelial cell (HUVEC) after VEGF treatment. Cells were obtained from human umbilical cord by 0.1% collagenase II digestion (Jaffe *et al*., 1973) and expanded in F12K medium (ATCC 30-2004) supplemented with 30 μg/ml endothelial cell growth supplement (ECGS, BD Biosciences), 10% fetal bovine serum, 1% sodium heparin and 1% penicillin/streptomycin.

For the proliferation assay, the cells were plated into 96-well culture plates at a density of 3×10^3 cells/well. After 12 h, cells were treated with 10 and 25 ng/ml of free VEGF or released from the microparticles. Culture medium and release medium from non-loaded microparticles were used as control. After 72 h incubation time under normal culture conditions, proliferation in each group was measured using MTS assay

2.6 Uptake of microparticles by macrophages

The microparticle (PEGylated and non-PEGylated microparticles) uptake study was analyzed in two different monocyte macrophage cell lines by fluorescence microscopy and flow cytometry.

2.6.1 Fluorescence microscopy

Fluorescent-labeled microparticles with Rhodamine B isothiocyanate (0.5 mg/ml) were prepared by adding the marker to the inner aqueous phase. Microparticles were prepared as described above. The uptake of fluorescence particles was evaluated in the monocyte-macrophage J774 cell line. Cells were plated into a 6-well culture plate at a 70%

confluence in serum free RPMI medium containing 1% penicillin/streptomycin. Four hours later culture medium (control), Rhodamine B isothiocyanate PLGA or PEG-PLGA microparticles were added at a final concentration of 0.33 mg/ml. After 3 h, culture supernatant containing microparticles was removed and the wells were washed three times with PBS. Fluorescence microparticles inside the cells were visualized using an EVOSfl fluorescence microscope (Euroclone, Milan, Italy). The fluorescent signal (corresponding to particle uptake) was quantified using the ImageJ software. Ten fields per well were randomly analyzed (experiments performed in triplicate). The signal emitted was normalized to the cell number in each field.

2.6.2 Flow cytometry

For flow cytometry studies, RAW 264.7 cells were seeded at a 30% confluence in DMEM 10% serum at 37 °C and allowed to adhere to the 6-well plate for 48 h. Then the medium was removed and cells were incubated with serum free DMEM for 4 h. PLGA or PEG-PLGA microparticles previously suspended in DMEM were then added (0.33 mg/ml), whereas the control group received only DMEM. At different time intervals (from 30 min up to 3 h) the medium was removed, cells were detached, collected and washed three times with PBS. After centrifugation (1500 × g, 5 min), the cells were suspended and fixed with 2% formaldehyde solution for their analysis. Cell complexity or cell granularity was studied by flow cytometry analysis using a BD FACSCalibur flow cytometer for the acquisition of samples. The side scatter cell (SSC) parameter was recorded as reflecting internal properties of cells (e.g. granularity and refractive index). Data were analyzed using the CellQuest software.

2.7 Statistics

Results are expressed as mean ± SD. Statistical significance was tested on the basis of Student's t test at 95% confidence intervals.

3. RESULTS AND DISCUSSION

3.1 Preparation of PLGA and PEG-PLGA microparticles

Among the different methods available for protein encapsulation, TROMS was selected because it is a semi-industrial technique capable of encapsulating fragile molecules while maintaining their native properties (Formiga *et al*., 2010; Garbayo *et al*., 2008). Since our final goal is to inject the microparticles in the ischemic heart, the aim when preparing the polymeric microparticles was to obtain a size between 5 and 8 μm, which has been shown to be compatible with intramyocardial administration (Formiga *et al*., 2010).

During the manufacturing process, size was shown to be affected by polymer composition. The best results, in terms of feasibility, reproducibility and adequate particle size distribution for intramyocardial injection were obtained with the polymer containing 10% of PEG, and so this polymer was selected for the subsequent experiments.

Using the same formulation and maintaining the TROMS parameters to prepare PEGylated and non-PEGylated microparticles, the size was increased for the PEG-PLGA co-polymer. Therefore TROMS parameters, mainly first and second emulsion circulation times, were studied in order to achieve PEGylated particles with the desired diameter (Table 1). The circulating times selected were 90 s for the first emulsion and 45 s for the second emulsion, obtaining a particle size of approximately 6.6 μm (Table 2). Other factors were also studied,

such as TROMS needle inner diameter, polymer % (w/v) in the organic phase and PVA % (w/v) in the external aqueous phase. Selected parameters are resumed in Table 2.

Table 1 Influence of the TROMS parameters on microparticle size.

Size (µm)		2nd emulsion circulating time (s)					
		30	60	90	120	150	180
1st emulsion circulating time (s)	30	-	12.75±9.39	5.79±4.26	20.7±20.7	4.14±1.94	14.89±13.45
	60	-	13.42±10.59	15.49±13.77	10.91±13.84	4.68±3.29	23.35±20.84
	90	13.56±1.61	3.72±1.99	1.56±0.15	1.04±0.12	1.12±0.34	0.69±0.02
	120	17.31±1.24	12.77±0.45	2.61±0.04	1.1±0.14	1.51±0.75	-

Table 2 TROMS selected parameters for microparticle formulation

Inner size of the needles (mm)		*Emulsion circulating times (s)*		*Polymer % in the organic phase*	*PVA % in the external aqueous phase*	*Microparticle size (µm)*
Needle 1	Needle 2	1st emulsion	2nd emulsion			
0.25	0.25	90	45	1.25	0.5	6.61±0.35

3.2 Microparticle characterization

3.2.1 Particle size and zeta potential

As stated above, particles with an average size close to 6 µm were obtained for both types of microparticles, which have been demonstrated to be compatible with intramyocardial injection (Formiga *et al*., 2010).

As shown in Table 3, surface charge values were negative for PEGylated and non-PEGylated particles. However, PEGylated microparticles showed a decreased negative charge (−8.79 ± 0.61 mV vs. −18.10 ± 0.71 mV). This may be attributed to the presence of the PEG chains in the surface of the particle (Essa *et al*., 2010). Moreover, it has been previously described that a higher PEG chain density on the surface of the particles decreases the mobility of the PEG chains and thus decreases the steric hindrance properties of the PEG layer (Owens and Peppas, 2006). On the other hand, if the PEG concentration is too low,

opsonins will attach to the surface and the stealth effect will be decreased. Therefore, in order to achieve an intermediate surface chain concentration between the "mushroom" and the "brush" conformation (low and high PEG concentration respectively), a ratio composition of 1:1 (w/w) of polymers Resomer 503H:Resomer RGP d 50105 was finally selected (Table 3).

Table 3 Final composition and characteristics of the selected microparticles

Polymer composition	*Encapsulation efficiency*	*Zeta potential, ζ (mV)*	*Residual PVA (%)*
50 mg PLGA (Resomer 503H)	92±7	-18.10±0.71	1.57
25 mg PLGA (Resomer 503H) 25 mg PLGA-PEG (Resomer d 50105)	85±5	-8.79±0.61	0.50

3.2.2 Residual PVA

PVA contained in the two types of microparticles was less than 2%, being lower for the PEGylated microparticles (Table 3). This lower adsorption of PVA in the surface modified particles could be explained as a consequence of the increased degree of hydrophilicity due to PEG chains, reducing PVA interaction (Essa *et al*., 2010). In any case, these concentrations are much lower than those reported in the literature for PLA microparticles (Gref *et al*., 2001).

3.2.3 Drug loading and encapsulation efficiency

In a previous study, VEGF-PLGA microparticles with high encapsulation efficiency were obtained (Formiga *et al*., 2010). In the present paper, this growth factor was entrapped into PEG-PLGA microparticles obtaining very high encapsulation values, between 80 and 100% for both PLGA and PEG-PLGA particles, as determined by western blot. Moreover, western blot allowed us to confirm that no degradation of VEGF occurred during the encapsulation process, showing a single characteristic band corresponding to 21 kDa (results not shown).

The ability to quantify proteins by light emitting chemiluminescence detection has been previously studied, highlighting the hotspots which have been taken into account in this research (Dickinson and Fowler, 2002). The results of encapsulation efficiency obtained were also indirectly confirmed in the bioactivity assays (Section 3.5). In the cell proliferation study, when treating cells with the protein released from the particles, VEGF concentration was calculated considering the encapsulation values. If cell proliferation is in accordance with the expected VEGF concentration, it is possible to confirm the encapsulation efficiency values, and in this sense the obtained results allow us to consider the western blot technique as a reliable method to measure the encapsulation efficiency.

3.4 *In vitro* release studies

The amount of VEGF released from the microparticles was measured by an *in vitro* assay, to confirm that the particles really retain the protein for a period of time and allow a sustained release.

When comparing both types of microparticles, the burst effect was higher for the PEGylated ones, which released approximately 50% of VEGF within the first 4 h, while 30% of the entrapped peptide was released from the PLGA particles (Fig. 1). Moreover, the plateau was reached after 24 h for the surface modified particles, whereas for the PLGA particles it occurred after three days. This different release behavior is attributable firstly to the fact that burst effect is mainly due to the protein located in the surface of the particle (Essa *et al*., 2010; Yoncheva *et al*., 2009). The presence of PEG chains increases the surface of the particle and, as a consequence, a greater amount of protein attaches to it. Secondly, as PEG chains are hydrophilic, when they are in an aqueous medium, like the release buffer, they are dissolved and this makes it easier for the buffer to get into the matrix, allowing the protein to be released. In any case, it has to be taken into account that a slower protein release is expected

in vivo, as previously demonstrated (Blanco-Prieto *et al*., 2004). The main reason for the slower *in vivo* kinetics is the low availability of water in the tissue compared with the *in vitro* conditions, in which the PLGA microparticles are incubated in PBS at 37 °C and shaken. Moreover, the tissue environment surrounding the microparticles will slow the release of VEGF *in vivo*.

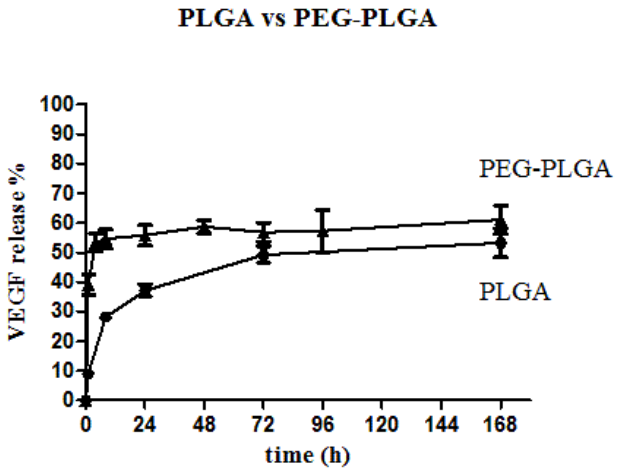


Fig. 1. *In vitro* release profiles. VEGF released from PLGA and PEG-PLGA microparticles is represented as a % of the total VEGF load in the particles.

3.5 VEGF bioactivity

VEGF is a growth factor well known for its angiogenic activity (Carmeliet and Jain, 2011; Formiga *et al*., 2012). In this sense, it has been demonstrated to promote proliferation of endothelial cells.

In order to confirm that VEGF bioactivity was preserved during the encapsulation/release processes, we tested the ability of VEGF released from the PEGylated particles to stimulate proliferation of HUVEC. Considering protein load and in vitro release profile, cells received the same dose of free VEGF and VEGF released from PEGylated microparticles (10 and 25 ng/ml). Both treatments induced the same degree of cell proliferation when compared to control groups (Fig. 2). These results allow us to conclude

that the presence of PEG in the polymer matrix does not alter the biological properties of the encapsulated VEGF, as it has been previously demonstrated for PLGA microparticles (Formiga *et al*., 2010). Furthermore, it indicates that this method is useful to encapsulate labile molecules (such as growth factors), retaining their activity, independently of the polymer matrix used.

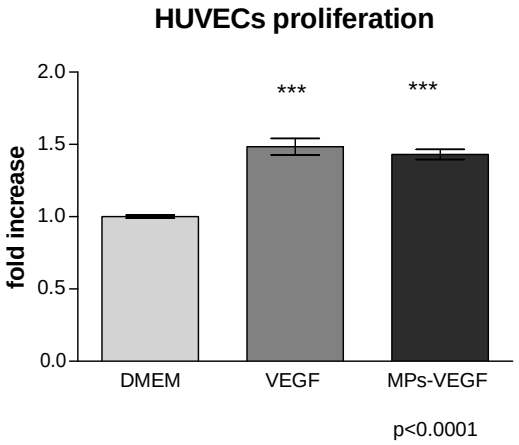


Fig. 2. VEGF bioactivity: HUVECs treated with VEGF and VEGF released from the microparticles (MPs-VEGF) at the same concentration (10 and 25 ng/ml) proliferate in the same ratio when compared to control groups. ***$p < 0.001$.

3.6 Microparticle macrophage clearance

Phagocytosis is a process in which macrophages destroy foreign particles in the body. Macrophages (phagocytic cells) are an important part of the immune system and also an important limitation for drug delivery using polymeric microparticles. In order to improve the delivery of VEGF in the ischemic heart, in the present work we prepared VEGF-PEGylated microparticles to avoid the clearance of the microparticles by the phagocytic cells.

3.6.1 Fluorescence microscopy

After we incubated J774 cells with particles loaded with Rhodamine B isothiocyanate, the uptake of microparticles by macrophages was clearly observable under fluorescent microscope. Indeed, significant differences in the fluorescent signal inside the cells were detected. In Fig. 3 representative images of cells three hours post-treatment with PEG-PLGA microparticles (A) and PLGA microparticles (B) are shown. When quantifying fluorescence we observed a four-fold increase in particle uptake in the case of the PLGA spheres compared to the PEGylated ones (Fig. 3C). These results confirmed the efficacy of the surface modification in the reduction of the macrophage internalization of the PEGylated microparticles.

3.6.2 Flow cytometry

Microparticle uptake by macrophages induces changes in cellular granularity that can be monitored by flow cytometry. Indeed, cells treated with PLGA microparticles showed high granularity levels over incubation time (up to 3 h), indicating that a large number of particles had been internalized during that period. However, coating the microparticles surface with PEG significantly influenced the uptake of the microparticles by the macrophages. Cells receiving surface modified particles maintained cell complexity in the same way as the non treated cells (control). The differences became significant after incubating the particles for 2 h in the culture medium (Fig. 4). Results obtained using flow cytometry confirmed the observation made by fluorescence microscopy, demonstrating that PLGA microparticles suffer phagocytosis in a more rapid way than PEGylated ones, and consequently confirming that particles have been successfully PEGylated.

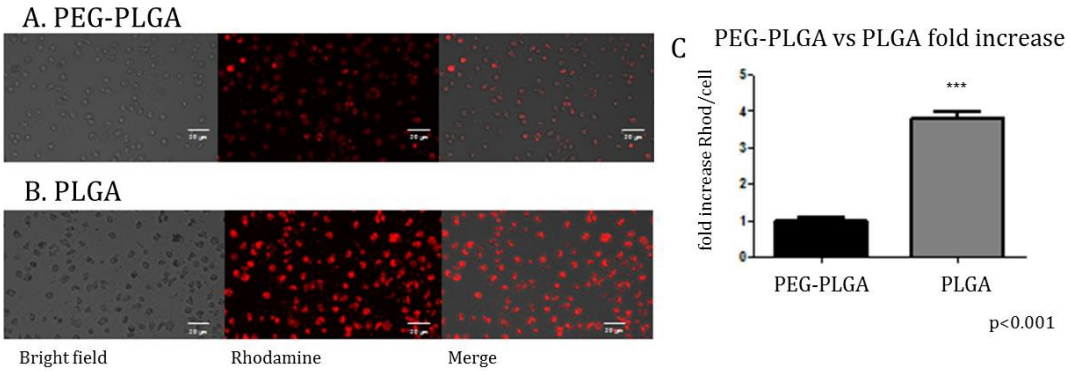
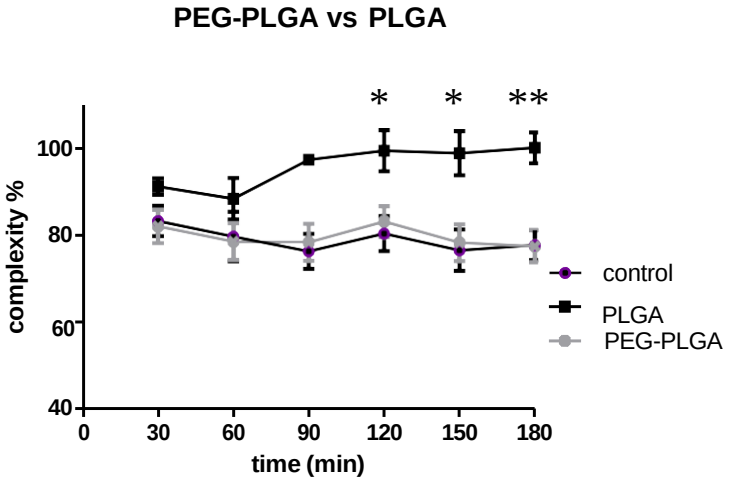


Fig. 3. Macrophage uptake studied by fluorescent microscopy. Cells observed under fluorescent microscope after incubating them with PEG-PLGA (A) and PLGA (B) microparticles containing Rhodamine isocyanate. More Rhodamine is visualized inside the cells treated with the PLGA microparticles. These differences are significantly different when quantified (C), indicating that these have been phagocyted in a larger number than those with the PEG chains in the surface. ***p < 0.001.



*p<0.05, **p<0.001

Fig. 4. Macrophage uptake studied by flow cytometry. The highest observed cell granularity value (measured as side scatter cell) has been assigned 100%. Cell complexity increases when cells are treated with PLGA microparticles, whereas cells treated with culture medium or PEG-PLGA microparticles did not have altered complexity three hours after the treatment. *p < 0.05 and **p < 0.001.

4. CONCLUSION

In this study we encapsulated VEGF in stealth microparticles, using a co-polymer of PEG and PLGA, with a percentage of PEG adequate to reduce macrophage phagocytosis.

PEGylated microparticles with high encapsulation efficiency and suitable size to be implanted in the myocardium were developed. Importantly, the bioactivity of the loaded therapeutic protein was fully preserved. Microparticles whose surface was modified by the incorporation of PEG in the formulation illustrated a significantly decreased uptake by phagocytic cells.

In summary, PEGylation could be a useful approach to obtain growth factor-loaded microparticles for myocardial administration, minimizing their local clearance and enhancing the efficacy of the protein therapy in cardiovascular disease. Consequently, the next step will be to test the developed microparticles *in vivo*, in a rat model of myocardial infarction.

ACKNOWLEDGMENTS

This work was supported by MICCIN PLE2009-0116, PSE SINBAD (PSS 0100000-2008-1) ISCIII PI10/01621, CP09/00333, University of Navarra (FUN), Caja de Ahorros de Navarra (Programa Tu Eliges: Tu Decides) and the "UTE project CIMA". We thank Dr. Estella-Hermoso de Mendoza for the critical reading of the manuscript.

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CHAPTER 2

FUNCTIONAL BENEFITS OF PLGA PARTICULATES CARRYING VEGF AND COQ10 IN AN ANIMAL MODEL OF MYOCARDIAL ISCHEMIA

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FUNCTIONAL BENEFITS OF PLGA PARTICULATES CARRYING VEGF AND COQ10 IN AN ANIMAL OF MYOCARDIAL ISCHEMIA

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Int J Pharm. 2013 Oct 1; 454(2): 784-790

Keywords: VEGF, CoQ10, protein delivery, angiogenesis, tissue engineering, myocardial ischemia.

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ABSTRACT

Myocardial ischemia (MI) remains one of the leading causes of death worldwide. Angiogenic therapy with the vascular endothelial growth factor (VEGF) is a promising strategy to overcome hypoxia and its consequences. However, from the clinical data it is clear that fulfillment of the potential of VEGF warrants a better delivery strategy. On the other hand, the compelling evidences of the role of oxidative stress in diseases like MI encourage the use of antioxidant agents. Coenzyme Q10 (CoQ10) due to its role in the electron transport chain in the mitochondria seems to be a good candidate to manage MI but is associated with poor biopharmaceutical properties seeking better delivery approaches.

The female Sprague Dawley rats were induced MI and were followed up with VEGF microparticles intramyocardially and CoQ10 nanoparticles orally or their combination with appropriate controls. Cardiac function was assessed by measuring ejection fraction before and after three months of therapy.

Results demonstrate significant improvement in the ejection fraction after three months with both treatment forms individually; however the combination therapy failed to offer any synergism. In conclusion, VEGF microparticles and CoQ10 nanoparticles can be considered as promising strategies for managing MI.

Abbreviations:

CVD: Cardiovascular disease

VEGF: Vascular endothelial growth factor

CoQ10: Coenzyme Q10

DDS: Drug delivery system

MP: Microparticle

NP: Nanoparticle

TROMS: Total recirculation one machine system

PEG: Poly (ethylene glycol)

PLGA: Poly (lactic-co-glycolic) acid

PVA: Poly (vinyl alcohol)

DMSO: Dimethylsulfoxide

DCM: Dichloromethane

HUVEC: Human umbilical vein endothelial cell

EF: Ejection fraction

MI: Myocardial ischemia

1. INTRODUCTION

Cardiovascular diseases (CVD) continue being the leading cause of death worldwide in spite of increasing efforts to improve its management. World Health Organization estimates that by 2030 almost 25 million of all global deaths will be due to CVD. Within this group of disorders coronary heart disease and stroke will be the main cause. Both, myocardial ischemia and strokes are principally caused by the accumulation of an atherosclerotic plaque in an artery. In the heart tissue ischemia is followed by a complex process involving cells (Tucka *et al*., 2012), growth factors (GFs) (Gullestad *et al*., 2012) and the extracellular matrix (Eckhouse and Spinale, 2012) that ends in the remodeling of the ventricle and heart failure.

To avoid consequences of ischemia in the heart the idea of growing new blood vessel for increased supply blood and better heart function has been proposed (Mitsos *et al*., 2012). Vascular endothelial growth factor (VEGF) is endogenously released by cells after myocardial ischemia and its supplementation to help damaged tissue to efficiently complete vessel formation has been studied in depth (Wang *et al*., 2013, Formiga *et al*., 2012). However, one of the major drawbacks of the therapy with GFs is its short half-life, being good candidates to be incorporated into drug delivery systems that protect them from degradation while extending its performace by sustaining the release (Simon-Yarza *et al*., 2012).

Cardioprotection is more of a prophylactic measure to prevent or reduce myocardial damage and the use of antioxidants is gaining importance in this area (Burgoyne *et al*., 2012, Maksimenko and Vavaev, 2012). Coenzyme Q10 (CoQ10), also known as ubiquinone, is a molecule that is responsible of the electron flow between complexes one and two with complex three in the electron transport chain (Sohal and Forster, 2007). It is well known because of its antioxidant role in several pathologies and it has been administered in coronary artery disease due to its cardioprotective role (Littarru *et al*., 2011). It is used as

adjunctive therapy combined with different drugs and/or clinical interventions (Pepe *et al*., 2007; Fotino *et al*., 2012). However, CoQ10 is still seeking solutions to overcome poor oral bioavailability problems (Beg *et al*., 2010). Strategies to improve gastrointestinal absorption have been previously studied with proven success in several pathologies (Ankola *et al*., 2007; Ratnam *et al*., 2009).

The present study is an attempt to compare the efficacy of particulate forms of VEGF and CoQ10 and to investigate the possibilities of synergism of the respective combination in an animal model of MI.

2. MATERIALS AND METHODS

2.1. Chemicals

Human recombinant VEGF was from R&D Systems (Minneapolis, MN, USA) and CoQ10 was a gift from Tishcon Corp. (Westbury, NY). All chemicals were provided by Sigma-Aldrich (Barcelona, Spain), organic solvents by Panreac Quimica S. A. (Barcelona, Spain) except high-performance liquid chromatography-grade methanol, ethanol, and acetonitrile that were procured from J.T. Baker (now Avantor Performance materials, Phillipsburg, NJ). Polymers were purchased from Boehringer-Ingelheim (Ingelheim, Germany) and western blot reagents from BioRad, unless specified in the text. Rabbit polyclonal anti-human VEGF-A (clone A-20, sc-152) was supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). ECL™ anti-Rabbit IgG horseradish peroxidase-linked whole antibody was from Amersham Biosciences (Buckinghamshire, UK). Human umbilical venous endothelial cells (HUVECs) were obtained from umbilical cords from donors, after informed consent according to the guidelines of the Committee on the Use of Human Subjects in Research at the Clinic

Universidad de Navarra. Components of cell culture media were purchased by ATCC-LGC. CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) was provided by Promega. Isofluorane employed for animal anaesthesia was IsoFlo® from Abbot Laboratories S.A. and sutures were from Ethicon (Johnson & Johnson, Brussels).

2.2. Preparation of Coenzyme Q10 encapsulated nanoparticles

CoQ10 encapsulated nanoparticles were prepared as reported previously in our laboratory (Ratnam *et al*., 2011) however with slight modifications that in the current study large scale batches were made instead of lab scale preparations (50 mg). The procedure in brief goes as follows: PLGA (Resomer RG 50:50 H; intrinsic viscosity 0.32-0.44 dl/g) (3 g) is dissolved in 150 ml of ethyl acetate under stirring and CoQ10 (1.5 g) was added to the polymer solution and stirring continued for one hour. In a separate bottle, PVA (PVA) (Mol. Wt. 30,000-70,000) (3 g) was dissolved in 300 ml of distilled water over a period of one hour. The CoQ10 containing polymer solution (25ml x 6 lots) was emulsified into (50 ml x 6 lots) of PVA solution over 30 min stirring (1000 rpm). This emulsion is then homogenized for 30 min at 15000 rpm and added to 250 ml of distilled water and left for overnight stirring to ensure complete evaporation of ethyl acetate. The suspension was centrifuged at 14,000 g for 30 min and the pellet collected was then re-suspended in 4 ml of distilled water either by vortex or occasionally probe sonication. Particle size was measured using zeta sizer (Malvern Zata sizer, UK) before and after centrifugation. The suspension was divided into two equal parts (2 ml each) in a 5 ml vial and 5% (w/v) trehalose was added and lyophilised using a bench top freeze dryer (Martin Christ, Germany) using the following condition 8 h freezing (-80 °C and safety pressure 1.650); followed by main drying for 48 h (-50 °C, 0.0035 mBar vacuum and safety pressure 1.650) and final drying of 12 h +20 °C 0.0035 mBar vacuum and safety pressure 1.650). The freeze dried particles were characterized for particle size and CoQ10

entrapment using reported HPLC method in our laboratory (Ratnam *et al*., 2011). Nanoparticles without CoQ10 (NL-NPs) were prepared following similar procedure without adding CoQ10 to the preparation.

2.3. VEGF Microparticle preparation and characterization

VEGF microparticles (VEGF-MPs) were prepared by double emulsion solvent evaporation method, as previously described (Simon-Yarza *et al*., 2013). Briefly, VEGF (50 µg) was included in the aqueous solution. On the other hand, 25 mg PLGA Resomer® RG 503 and 25 mg poly [(d,l-lactide-co-glycolide)-co-PEG] diblock Resomer® RGP d 50105 were dissolved in a mixture of DCM and Acetone (3:1). By using TROMS® technology (Garbayo *et al*., 2008; Formiga *et al*., 2010; Simon-Yarza *et al*., 2013), organic phase was incorporated into the inner aqueous phase to form the first emulsion, that was then incorporated into the PVA 0.5% external aqueous phase, to form the multiple emulsion. Particles were then formed by solvent evaporation during 3 hours under constant stirring (300 rpm). Freeze-drying was employed to store particles until its use.

Non-loaded microparticles (NL-MPs) to be used in the control groups were prepared in the same way without VEGF.

Particle size was determined by laser diffractometry using a Mastersizer-S® (Malvern Instruments, Malvern, UK). Sample preparation was done by dispersing 3 ml of particle suspension into the small volume dispersion unit. To do the measurements Mastersizer-s v2.19 software was used.

Microparticle (MP) residual PVA content was studied by a colorimetric assay based on the reaction occurring between two adjacent hydroxyl groups of PVA and iodine molecule

leading to the formation of a colored complex (Joshi *et al*., 1979). Measurements were performed in triplicate.

To determine MP protein content 0.5 mg of loaded MPs were dissolved in 20 µl DMSO. Incubation with rabbit antihuman VEGF-A antibody (1:2000) lasted 2.5 h at RT. Horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG antibody (1 h, RT, 1:2000) was performed to detect antibody binding. Chemiluminescence detection was performed using LumiLight Plus western blotting substrate (Roche Diagnostics, Mannheim, Germany). The VEGF signal was quantified by densitometry using the Quantity One software (Bio-Rad Laboratories, Inc., Munich, Germany).

For the *in vitro* release studies, 1 mg of VEGF-MPs were introduced in 1.5 ml tubes and dispersed in 300 µl PBS pH 7.4 with 0.02% (w/v) sodium azide, incorporated as a bacteriostatic agent. Samples were incubated under orbital shaking at 37 °C. At different time points, tubes were centrifuged (20,000 g, 10 min) and 60 µl of supernatant were removed for its analysis and replaced with the same volume of fresh release medium. VEGF concentration in the supernatants was measured by western blot, using the protocol described in the previous section. Release profile was expressed as cumulative release during a period of time. All measurements were done in triplicate.

2.4. Bioactivity of encapsulated VEGF

To confirm that protein bioactivity was not affected during the microencapsulation process a proliferation cell culture assay was done (Hervé *et al*., 2005). HUVECs were obtained from human umbilical cords. Tissue digestion was done with 0.1% collagenase II (Jaffe *et al*., 1973). Afterwards, cells were expanded in F12K medium (ATCC 30-2004) supplemented with 30 µg/ml endothelial cell growth supplement (ECGS, BD Biosciences), 10% fetal bovine serum, 1% sodium heparin and 1% penicillin/streptomycin.

For the proliferation study, cells were plated into 96-well culture plates (3×10^3 cells/well). 12 h after seeding, cells were treated with 10 ng/ml of non-encapsulated VEGF or released VEGF from the MPs. After 72 h incubation time under normal culture conditions proliferation in each group was measured using MTS assay.

2.5. *In vivo* experiments

The experiments were conducted according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All animal procedures were approved by the University of Navarra Institutional Committee on Care and Use of Laboratory Animals as well as the European Community Council Directive Ref. 86/609/EEC.

Female Sprague-Dawley rats (40) weighing approximately 240-260 g were provided by Harlan-IBERICS (Barcelona, Spain) and housed in conventional animal quarters. Environment was controlled (12 h light/dark cycle at 21°C) and all animals received a standard diet and water ad libitum.

2.5.1. Animal model

Anesthesia was firstly induced in an induction chamber with 2% Isoflurane. Then animals underwent tracheal intubation for ventilation that was maintained during the procedure (10-15 ml/kg, 80-90 respiratory rate). Anesthesia was maintained with 0.5% isoflurane during the surgery. Before the heart operation, animals received analgesic drug ketoprofen 5 mg/Kg subcutaneously and fentanyl 0.15 mg/kg intraperitoneally. The surgical approach consisted of a left thoracotomy through the fourth intercostal space. After pericardium was opened, left anterior descending (LAD) coronary artery was blocked 2–3

mm distal from its origin with a ligature with an 7/0 polypropylene suture. The chest was closed in layers using resorbable suture Vicryl® 4/0 and rats were allowed to recover. Surgical procedure total time was 30 minutes. Only those rats with an EF equal or below 55% (as determined by echocardiography) 4 days after MI were included in the study, since it is established that an EF under 55% is an indicator of heart damage. Animals were then divided into eight groups (n=5) as showed in Table 1.

Table 1. Animals were divided in eight different groups (n=5/group). Three efficacy studies were performed to observe effects of CoQ-NPs, VEGF-MPs and combination of both.

STUDY		GROUP	INTRAMYOCARDIAL ROUTE	ORAL ROUTE
	CoQ-Nps efficacy study	1	-	CoQ-NPs (100 mg/kg every 3 days during the 1st month; 200 mg/kg weekly up to 3rd month)
		2	-	Commercial CoQ (100 mg/kg every 3 days during the 1st month; 200 mg/kg weekly up to 3rd month)
VEGF-MPs efficacy study		3	-	-
		4	VEGF-MPs (0.6 mg)	-
		5	VEGF-MPs (1.6 mg)	
	Combined therapy efficacy study	6	NL-MPs (1.6 mg)	NL-NPs (every 3 days during the 1st month; weekly up to 3rd month)
		7	VEGF-MPs (0.6 mg)	CoQ-NPs (100 mg/kg every 3 days during the 1st month; 200 mg/kg weekly up to 3rd month)
		8	VEGF-MPs (1.6 mg)	CoQ-NPs (100 mg/kg every 3 days during the 1st month; 200 mg/kg weekly up to 3rd month)

2.5.2. Intramyocardial administration of the microparticles

One week after the LAD occlusion, MPs were intramyocardially administered using a 29 gauge needle in four points surrounding the ischemic area, evidenced by the scar formation. Intramyocardially treated groups consisted of 0.6 or 1.6 mg of VEGF-MPs (0.85 μg/mg). For its administration, particles were dispersed in a sterile buffered solution composed of 0.1% (w/v) carboxymethylcellulose, 0.8% (w/v) polysorbate 80 and 0.8%

(w/v) mannitol in PBS, pH 7.4. All animals received 80 µl total volume. After injection chest was closed and animals were allowed to recover.

Intramyocardial injection is becoming one of the main routes of administration of growth factors and cells in acute myocardial infarction preclinical and clinical trials. This administration path allows local delivery of the treatment with a better dose control and avoiding adverse effects resulted from the dissemination of the growth factor through the organism. Despite being more invasive than other routes, for the large animal models and for the clinical application sophisticated devices have been constructed (Sherman *et al*. 2006). With this intramyocardial catheter-based delivery systems open chest surgery is no longer necessary, and intramyocardial injection has become a less invasive technique with excellent safety profiles in the clinical trials.

2.5.3. Oral administration of the nanoparticles and commercial CoQ_{10}

Oral administration of the CoQ-NPs, NL-NPs or CoQ10 commercial formulation started 24 h after LAD occlusion and continued every three days during the first month and subsequently switched to weekly once until the end of the study. Animals treated with the nanoparticulate or with the commercial CoQ10 received a 100 mg/kg dose every three days throughout the first month and the 200 mg/kg dose once per week up to three months. Animals treated with NL-NPs received the equivalent amount of NPs. For oral administration, freeze dried particles were dispersed in distilled water using a sonicator prior administration that was performed with an oral rigid dosing cannula.

2.5.4. Echocardiographical evaluation

The Echocardiographic analysis was performed using a Vevo770 high-resolution ultrasound system (Visualsonics, Toronto, Canada) as previously described (Benavides-Vallve C. *et al*., 2012)

The left ventricular (LV) systolic function of the heart was evaluated by measuring the EF, calculated using the Simpson's rule, from a parasternal long axis view and four parasternal short axis views at different levels of the LV. At the long axis view the left ventricle length was measured from the aortic annulus to the endocardial border at the apex level in both diastole and systole. At the parasternal short axis view, the endocardium was traced at four different levels in both systole and diastole, to calculate the areas required to calculate the Simpson's value.

All measurements were performed offline using dedicated Vevo770 quantification software (Vevo 770 v. 3.0.0).

2.5.5. istological studies

Three months after surgery animals were sacrificed and hearts were collected for subsequent histological analysis. Harvested hearts were fixed and sliced in three 4-mm-thick segments from apex to base. The hearts were dehydrated and embedded in paraffin. Sections (5 μm) were cut from each slice.

To quantify the small caliber vessel density and area, anti-caveolin-1α antibody (diluted 1:50) was used as marker, and 2 peri-infarct and 2 intra-infarct images per section were analyzed. Secondary antibody was Alexa Fluor 488 goat conjugated anti-mouse IgG (diluted 1:100). Images were acquired using the Axio Cam MR3 video camera at 20× connected to the Zeiss Axio Imager M1 microscope equipped with epifluorescence optics. Digital images were analyzed using MatLab® software platform (Mathworks Inc., Natick, MA, USA).

3. Statistical analysis

Data are expressed as mean ± SEM. Student's *t*-test was used to analyze statistical significance. *P*-values corresponded to a two-tailed unpaired *t*-test for the group comparison. A $p < 0.05$ was considered statistically significant using Prism software (GraphPad software, San Diego, CA, USA).

4. RESULTS AND DISCUSSION

4.1.Preparation and characterization of Coenzyme Q10 encapsulated nanoparticles

The procedure was highly reproducible and we were able to produce large quantities of CoQ10 containing nanoparticles. The key variation we made from the small scale batch preparation was the homogenization duration which was increased to 30 min from 15 min used for 50 mg batch size (Ratnam *et al*., 2011). The fresh CoQ10 containing particles were of 147±9 nm (fresh), however a slight increase in the size 156±9 nm was observed after centrifugation to separate the free CoQ10 and surfactant which is expected and this increase is due to aggregation upon centrifugation. However, freeze drying process did not affect the particle characteristics and remained same due to inclusion of cryoprotectant trehalose. The entrapment efficiency was found to be about 70% of initial CoQ10 entrapment (~35 mg CoQ10/100 mg polymer). CoQ10 entrapped PLGA nanoparticles were proven efficacious in diabetic and renal hypertensive models by preventing the free radicals, inflammation and lipid abnormalities (Ankola *et al*. 2007, Ratnam *et al*. 2008, Ratnam *et al*. 2011).

4.2. VEGF Microparticle preparation and characterization

The process led to uniform sized particles of about 4.89 μm with a VEGF content of 0.85 μg/mg equivalent to 85% EE. Our previous studies suggest the size of the particles is within the range for intramyocardial administration with a 29G syringe (Formiga *et al.*, 2010). Residual PVA associated with the MP preparation was less than 1% of the initial amount used and this concentration should not cause any adverse reactions either by local injections or oral/systemic administration (DeMerlis and Schoneker, 2003; Baker *et al.*, 2012). There are reports suggesting very high doses in rats (daily doses of 2,000-5,000 mg/kg) over a period of 90 days showed no adverse or toxicological effects (Kelly *et al.*, 2003).

In vitro release profile showed a burst effect with 58% of protein released within the first 4 h followed by a more sustained release until the end of the study (Figure 1). This burst effect could be a typical phenomenon of surface bound protein or the protein in the exterior layers of the particles releasing at much quicker rate than that is in the inner cores. PEGylated particles exhibit a greater burst effect than non PEGylated owing to their increased hydrophilicity of the polymer. Once the superficial layers of the protein are depleted a more sustained release of protein is observed which is controlled by the diffusion as well as the amount of the protein remaining in the particles. *In vivo* release is expected to be slower due, in part, to the presence of tissue surrounding the particles, instead of an aqueous medium.

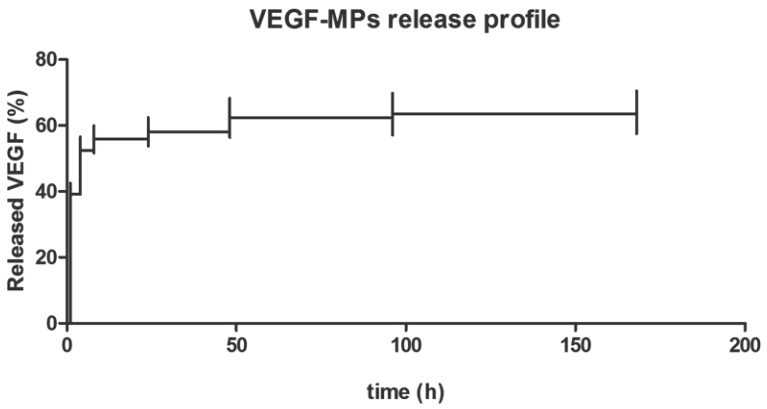

Figure 1. *In vitro* release profile of VEGF-MPs in PBS-pH7.4 at 37 °C.

4.3. Bioactivity of encapsulated VEGF

HUVEC cell assay demonstrates the proliferative activity of VEGF-MPs and is as effective as respective free protein (Figure 2). This study further confirms the integrity of the protein during the encapsulation process by TROMS®.

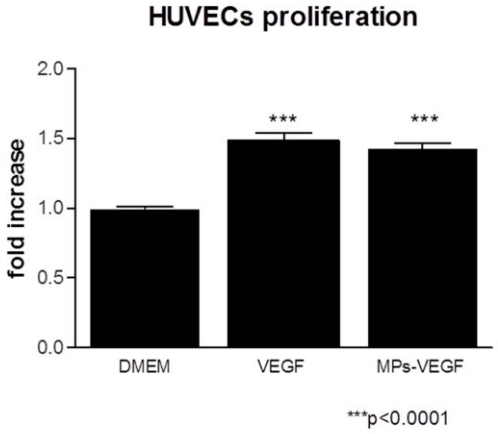

Figure 2. Cell proliferation assay to assess the stability of encapsulated VEGF. Free VEGF and VEGF-MPs are equally good in their cell proliferation ability and are significant when compared to untreated control (***p<0.0001)

4.4. *In vivo* experiments

Animals were divided into eight groups (n=5) and followed up to three months. Echocardiographic functional results have been analyzed grouped in three different efficacy studies to facilitate interpretation of obtained data.

4.4.1. CoQ10 loaded nanoparticle efficacy study

The CoQ-NPs improved the heart function significantly as indicated by increased EF rates when compared to the commercial CoQ10 which showed results comparable to that of the untreated group (Figure 3A). The improved performance of CoQ-NPs can be attributed to their ability to improve their peroral bioavailability and sustain the release of the encapsulated CoQ10 over a period of time allowing dose reduction while maintaining the activity. Even though the commercial formulation used in this study is reported to exhibit improved peroral bioavailability (Chopra, 2001), it is just not enough in the system under the current dosage regimen to elicit any positive response, maybe a daily dosing would have been better with commercial form. Even though a precise mechanism of action of CoQ10 in MI is not known, in general it is attributed to its antioxidant nature, ability to minimize inflammation, improve endothelium function (Dai *et al*., 2011; Lee *et al*., 2012). We have previously reported the efficacy of CoQ-NPs in other experimental models (Ankola *et al*., 2007; Ratnam *et al*., 2009) however a more systematic understanding is required on their therapeutic potential before much efforts are put in to prove the performance of these compounds that are in general high dose molecules and currently in use as supplements.

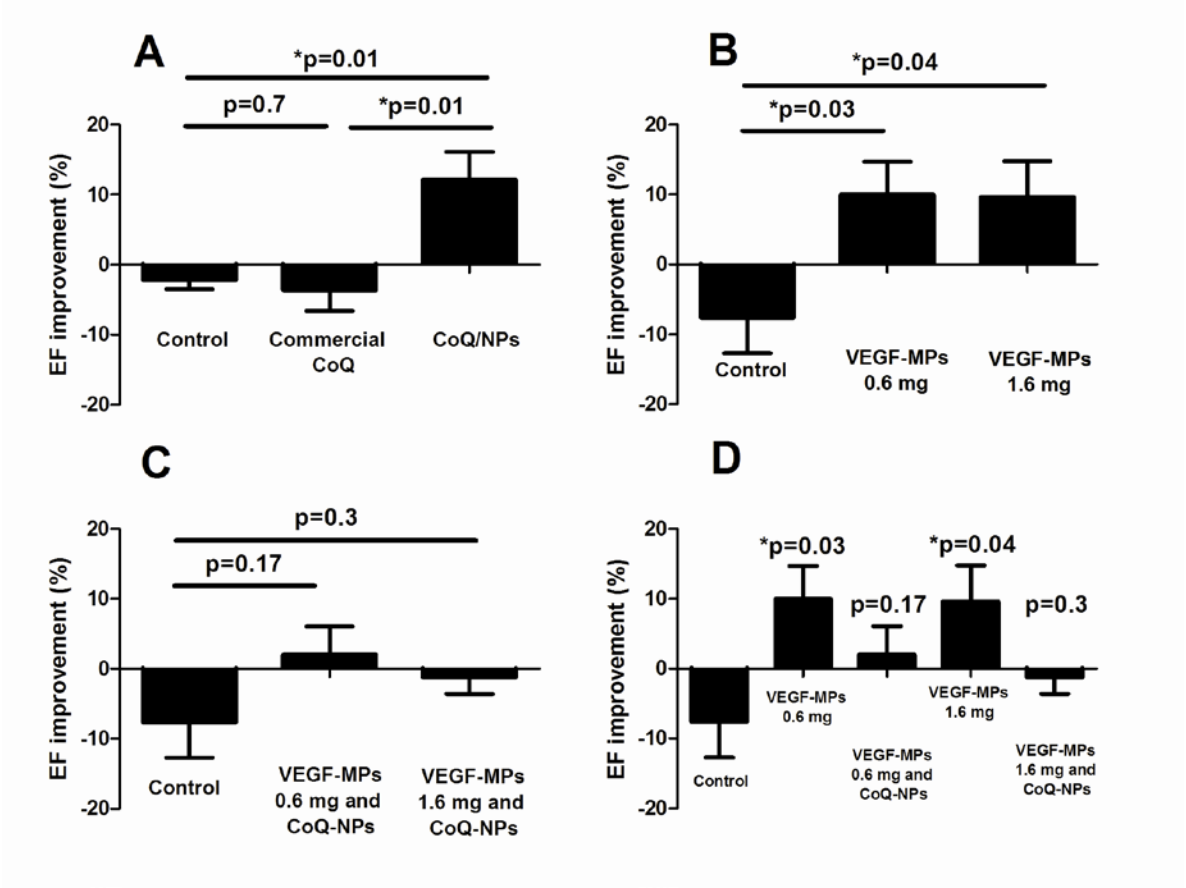

Figure 3. Ejection fraction (cardiac function) observed in different groups: (A) CoQ-NPs (B) VEGF-MPs (C) VEGF-MPs and CoQ-NPs combination and (D) Summary of individual and combination VEGF treatments. EF was assessed by echocardiography before and 3 months after treatment and mean improvement±SD is represented.

4.4.2. VEGF loaded microparticle efficacy study

We have studied two doses of VEGF to establish a dose response and the doses selected were based on the success from our previous study in a rat model of ischemic heart disease (Formiga *et al*., 2010). The animals receiving VEGF-MPs improved the heart function as indicated by the increase in EF however both low and high dose show same response, while the control group which received NL-MPs offered no protection (Figure 3B).

4.4.3. Combined therapy

Though the individual treatments of CoQ10-NPs and VEGF-MPs resulted in significant improvement in the EF (Figure 3C) these two failed to offer synergism when administered together. Expected synergy between proangiogenic and antioxidant therapies mediated by VEGF and CoQ10 was not observed. Moreover, if we put together these results with those obtained with the VEGF-MPs alone it appears that CoQ10-NPs counteract benefits of VEGF therapy (Figure 3D).

There is compelling evidence that CoQ10 is an independent predictor of mortality in chronic heart failure (Molyneux *et al*., 2008), while a recent study suggests the plasma concentrations of CoQ10 is not associated with lower risk of AMI (Naidoo *et al*., 2012). Unfortunately, molecules such as CoQ10 are used as supplements with no clear doses or plasma levels established to prevent/manage/treat any pathology. It is important at this juncture that the inherent biopharmaceutical/physicochemical problems associated with such molecules are addressed by formulating them appropriately before we make substantial claims on their effectiveness or ineffectiveness (Kumar 2012).

CoQ10 role in the angiogenic process has not been studied in depth. There are some published works that explore CoQ10 in the tumor angiogenesis. It has been suggested an antiangiogenic effect of this molecule in the new vessel formation mediated by a mechanism in which endogenous VEGF serum levels are reduced (Premkumar *et al*., 2007, 2008). In our approach VEGF released from the particles in the damaged heart acts in cooperation with endogenous VEGF that is secreted by the cells after MI. An explanation to the results of this study will be that, on one side VEGF-MPs and CoQ-NPs will be exerting its proangiogenic and cardioprotective action respectively, and on the other CoQ-NPs will be also acting reducing VEGF endogenous levels, thus limiting angiogenesis. This result emphasizes how important the co-administration of non-regulated actives can be in the effectiveness of the treatment of

the patients. There is, therefore, a need to go into depth in the interactions of these micronutrients that need to be controlled when patients undergo treatment.

4.4.4. Histological studies

Animals receiving VEGF-MPs showed a statistically highly significant increase ($p<0.0001$) in the number of capillaries in the infarct and peri-infarct area when compared with the control group (Figure 4A). This difference was reduced in the group receiving VEGF-MPs and CoQ-NPs ($p=0.015$). This data are in accordance with the functional results that present the same standard, suggesting that CoQ-NPs are reducing the VEGF-MPs angiogenic effect. In relation with the vessel mean area we have proved that is significantly smaller in the VEGF-MPs & CoQ-NPs animal group ($p=0.02$), indicating that vessel development is more immature in this group (Figure 4B). Finally, differences in the total caveoline positive area were found between control group and VEGF-MPs group, but there were no differences in the VEGF-MPs & CoQ-NPs group in which it was reduced (Figure 4C).

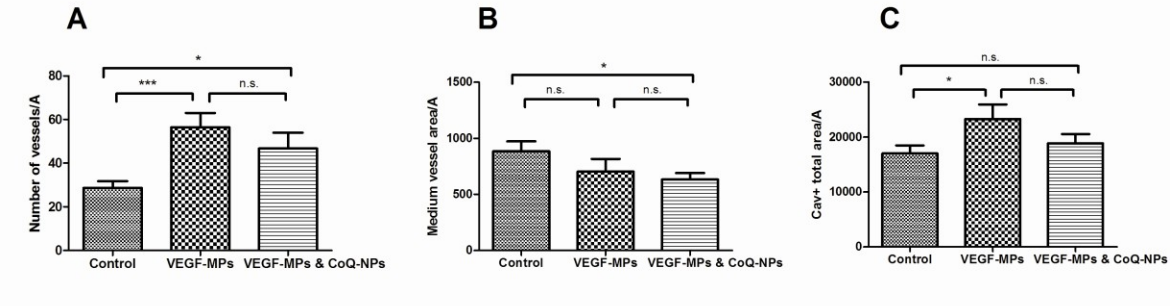

Figure 4. The histological section were imaged and quantified for the analysis of (A) Vessel density (B) Average vessel diameter and (c) Total caveoline positive area. Small vessels were stained using anti-caveoline-1α antibody.

Taking all these data into account we can conclude that VEGF-MPs promotes vessel development, but its angiogenic effect is reduced when it is concurrently administered with CoQ-NPs, resulting in less functional benefit.

5. Conclusion

In this work polymeric nanoparticles containing antioxidant molecule ubiquinone and stealth microparticles containing VEGF were prepared by two different methods, both of them leading to high encapsulation efficiency. These drug delivery systems were applied in an animal model of MI by different administration routes. Treatments resulted in an improvement of the EF of the animals when administered separately, suggesting that they can be effective systems to treat this disease. In the case of VEGF-MPs an angiogenic process underlies this benefit, confirmed by the quantification of larger number of capillaries in the animal hearts. Treatment with the CoQ-NPs present several advantages based on its oral administration, more feasible and non-invasive for the patient, also with lower cost.

However, when both particles were administered concurrently no significant benefit was observed, suggesting that in the dose regime employed in this study, there is a counteraction between them. Histological analysis confirms that small vessel density and size is reduced in those animals included in this group. Finally, results in this study emphasize the need for extra care when using supplements with standard therapeutic interventions.

6. Acknowledgements

This work was partially supported by Royal Society (JP100124, MNVRK & MJB), ISCIII-RETIC RD12/0019/0031, MINECO PLE2009-0116 CARDIOBIO, Program INNPACTO (PROCARDIO), European Union FPVII (INELPY), FUN (University of Navarra) Caja de Ahorros de Navarra (Programa Tu Eliges: Tu Decides). T. Simón-Yarza thanks the Spanish Ministerio de Educación (programa FPU) for the fellowship grant.

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CHAPTER 3

POLYMERIC ELECTROSPUN SCAFFOLDS: NRG ENCAPSULATION AND BIODEGRADATION IN A MODEL OF MYOCARDIAL ISCHEMIA

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POLYMERIC ELECTROSPUN SCAFFOLDS: NRG ENCAPSULATION AND BIODEGRADATION IN A MODEL OF MYOCARDIAL ISCHEMIA

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Keywords: Nrg, scaffolds, protein delivery, electrospinning, tissue engineering, myocardial ischemia.

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ABSTRACT

The latest statistics from the World Health Organization highlight cardiovascular disease as one of the major health challenges in the present century. Numerous clinical trials are under way to identify efficient therapies using growth factors or cells. Tissue engineering takes advantage of biomaterials for the delivery of both cells and proteins, improving cell engraftment and survival in the damaged tissue, and prolonging growth factor delivery, which are the main challenges of cell and protein therapy. In the present study. electrospinning was employed to prepare smooth homogenous polymeric fibers. Parameters were also adjusted to efficiently encapsulate Neuregulin-1, a cardioactive factor. Within the polymer blend PLGA/sP(EO-stat-PO) polymer was incorporated to reduce protein adsorption and to promote specific cell adhesion. A rat myocardial ischemia model was used to characterize the biocompatibility of the system and its degradation at different times: 24h, 1 week, 1 month and 3 months. Histological studies reveal an initial acute inflammatory process followed by a chronic inflammatory stage characterized by the presence of giant cells and fibrotic tissue next to the implant after 1 and 3 months. The scaffold showed progressive degradation signs but it was still present in the heart after 3 months, suggesting that Neuregulin-1 is still being released. Also, the M2:M1 ratio increased from the first 24 hours to at least 1 month after scaffold implantation. This macrophage phenotype is presumably accompanied by constructive tissue remodelling.

The combination of this protein containing scaffold with cells implicated in cardiac regeneration is an ambitious and promising strategy to tackle myocardial ischemia damage.

1. INTRODUCTION

Tissue engineering (TE) is an emerging discipline whose objective is to repair damaged tissues in the organism with a sophisticated approach that includes growth factors (GFs), cells and an appropriate matrix (Simón-Yarza *et al*., 2012a). An interesting field of application is the damaged heart, since cardiovascular disease (CVD) is the leading cause of death worldwide, considered by the World Health Organization(WHO) to be one of the major health challenges of the 21st century (WHO, 2012).

After the occlusion of a coronary artery, cells die due to lack of oxygen, and are then removed from the tissue by macrophages. Fibroblasts and endothelial cells migrate to the zone and within a period of weeks a non-functional fibrotic scar supplies the lost tissue (Martin *et al.*, 2013). Remodeling of the ventricles occurs and at the end the patient develops cardiac insufficiency with a high risk of heart failure (Kurrelmeyer *et al*., 1998). In this context the possibility of building a device that incorporates cells with an active role in the regeneration of the heart (such as those already tested in clinical cell trials) and also incorporating GFs that promote cell recruitment, vasculogenesis or cardiomyocyte replication and/or are able to cooperate with the cells present in the engineered construct, is without any doubt a very attractive approach. (Lakshmanan *et al.*, 2012; Venugopal *et al.*, 2012)

Within the GFs currently under study, Neuregulin-1 (Nrg) has emerged as a promising cytokine that is now being tested in several clinical trials (CT) (Clinicaltrials.gov identifier NCT01131637, NCT01214096, NCT01251406, NCT01258387, NCT01541202) owing to the benefits observed in preclinical studies. This cardioactive GF is a member of the ErbB family indispensable for cardiac development, and for the structural maintenance and functional integrity of the heart (Mendes-Ferreira *et al*., 2013). In an earlier study by our group, polymeric microparticles encapsulating Nrg were demonstrated to improve cardiac function in a rat model of myocardial ischemia (MI) (Formiga *et al*., 2013). Interestingly, in

the animals treated, bone marrow progenitor cells were found near the damaged tissue, and cardiac markers were identified by immunofluorescence on the surface of some of them, indicating differentiation towards cardiomyocytes.

Within the TE matrix, fibrous scaffolds (SCs) made up of biocompatible and biodegradable materials offer the possibility of incorporating proteins in the fiber and at the same time attaching cells on its surface (Ji *et al.*, 2011). A recently published study by Grafahrend and colleagues describes the construction of this kind of fibers by electrospinning (ES) (Grafahrend *et al.*, 2011). ES is a manufacturing process that consists of the application of an electrical charge to a liquid to draw out thin fibers (Fig. 1). Parameters of the process can be controlled to optimize fiber features, such as electric potential, flow rate, distance between the capillary and collection screen, motion and size of target screen (collector) and needle gauge (Rim *et al.*, 2013). In the abovementioned study a novel polymer with appealing features was employed: PLGA/sP(EO-stat-PO). Due to the presence of hydrophilic chains, this polymer reduces protein adsorption, while at the same time it can be easily operationalized to promote specific cell adhesion on the surface of the fibers (Grafahrend *et al.*, 2011).

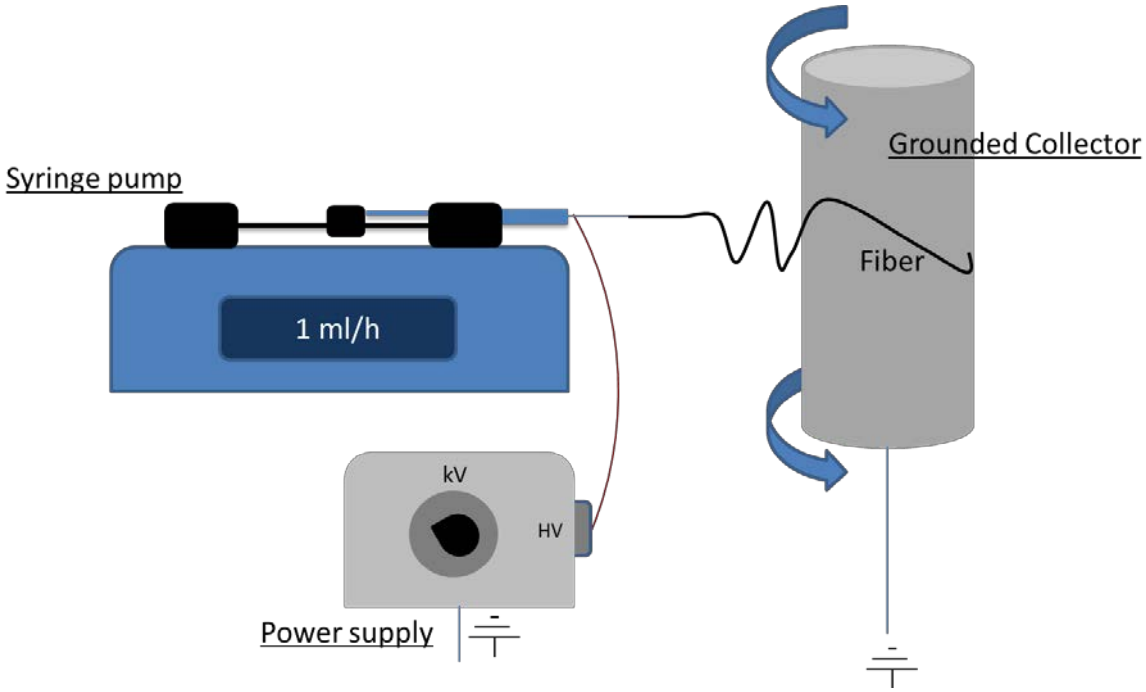

Fig. 1. Schematic representation of the ES components.

The objective of this study was to design, build and characterize a fibrous SC incorporating Nrg which could be used in the future for the treatment of myocardial ischemia in patients. An *in vivo* study with a rat model of MI was done to study the interaction between the SC and the damaged cardiac tissue and to test its biocompatibility.

2. MATERIALS AND METHODS

2.1. aterials

Recombinant human Neuregulin-1b-iso was provided by EuroBio-138 Sciences (Friesoythe, Germany). Chemicals were provided by Sigma-Aldrich (Barcelona, Spain), organic solvents by Panreac Quimica S. A. (Barcelona, Spain), polymers by Boehringer-Ingelheim (Ingelheim, Germany) and western blot reagents by BioRad, unless specified in the text. Clonal anti-human NRG-1 antibody (sc-1793) and horseradish-peroxidase-conjugated donkey anti-goat IgG (sc-2020) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-CCR7 was from Epitomics (Epitomics Inc., 2059-1) and mouse anti-CD163 was from Serotec (AbD Serotec, MCA342R). Isofluorane employed for animal anesthesia was IsoFlo® from Abbot Laboratories S.A. and sutures were from Ethicon (Johnson & Johnson, Brussels).

2.2. Electrospinning conditions

Microfibers were produced at room temperature (RT) (20 °C). Electrostatic field was 10 kV using a high voltage power supply. Distance between the spinneret and the collector was 14 cm. A pump with a flow rate of 1 ml/h was used to spin the melt (World precision instrument 941-371-1003, Sarasota, FL). The collector consisted of a rotating drum with a diameter of 7 cm. A 19 gauge blunt-ended needle was used.

2.3. Fiber composition

Polymers under study were poly (lactic-co-glycolic) acid (PLGA) Resomer® 504, poly [(d,l-lactide-co-glycolide)-co-PEG] diblock Resomer® RGP d 50105 (PEG-PLGA) and PLGA/sP(EO-stat-PO). To prepare the polymers to be electrospun they were first dissolved in acetone 450 µl by stirring for 5 minutes. For the protein encapsulation Nrg was incorporated in an aqueous solution including polyethyleneglycol 200 (PEG200) and tri-fluoro-acetic-acid (TFA). Then this aqueous solution was included in the organic phase by stirring until homogenization (1 minute), to obtain an emulsion ready to be electrospun.

Components of the formulation were evaluated to prepare the fibers. The objective was to obtain smooth fibers suitable for cell adhesion and with a good cytokine encapsulation. Also, we must take into account that in order to allow vascularization to supply oxygen and nutrients to the cells incorporated in the SC, it ought to have a porous size around 50 µm (Venugopal *et al.*, 2012). The variables evaluated included polymer composition, amount of PEG200 included in the aqueous solution, composition of this aqueous solution, amount of protein loaded in the fibers and addition of TFA at different concentrations (Table 1). In all cases, fibers were collected on the surface of a metallic rotating drum forming a film.

2.4. Scanning electron microscopy imaging

To visualize the surface morphology of the fibers, a piece of the SC was cut (0.4 mm x 0.4 mm). Sample preparation was performed using a sputter coater (Emitek K550) and SCs were observed by scanning electron microscope (Zeiss DSM 940A).

Table1 Evaluated fiber composition parameters.

PLGA 504 (mg)	***PEG-PLGA (mg)***	***sP(EO-stat-PO) (mg)***	***PEG (µl)***	***TFA (µl/ml)***	***H_2O (µl)***	***PBS (µg)***	***BSA (µg)***
0	150	0	0	0	0	15	0
75	75	5	10	0.5	15	0	10
150	0	10	20	2	-	-	25
-	-	15	30	10	-	-	50
-	-	-	40	20	-	-	100
-	-	-	50	-	-	-	150

2.5. Neuregulin encapsulation efficiency

To determine the protein loaded into the fibers, western blot was performed. Sample preparation consisted of 2 mg of fibers dissolved in dicholoromethane 40 µl. After electrophoresis (200 V, 50 minutes) and blotting, (350 mA, 1 hour, RT) nitrocellulose membrane was incubated with clonal antihuman NRG-antibody (sc-1793) (1:50, 4 °C, overnight) and horseradish-peroxidase-conjugated donkey anti-goat IgG (sc-2020) (1:2000, RT, 2 hours). Chemiluminescence was detected with LumiLight Plus Western Blot substrate (Roche Diagnosis, Manheim, Germany). A standard curve with Nrg was also included in the western blot to quantify protein content in the samples. To obtain chemiluminiscence images ImageQuant RT ECL was used. ImageQuant TL software was employed to quantify protein bands.

2.6. *In vivo* animal model and scaffold adhesion

Experiments were performed according the ‘‘Principles of Laboratory Animal Care’’ formulated by the National Society for Medical Research and the ‘‘Guide for the Care and Use of Laboratory Animals’’ prepared by the Institute of Laboratory Animal Resources,

Commission on Life Science, National Research Council, and published by the National Academy Press, revised in 1996. Animal procedures were also approved by the University of Navarra Institutional Committee on Care and Use of Laboratory Animals.

For the *in vivo* studies a rat model of cardiac permanent ischemia was employed. Female Sprague Dawley rats (250-300 g) were first anesthetized with isoflurane 4% in an induction chamber. Then they were intubated and ventilation was artificially maintained at 80-90 cycles/min under continuous anesthesia (isoflurane 2%). Surgery consisted of a left thoracotomy through the fourth intercostal space and ligation of the left anterior descendent coronary artery with a non resorbable suture (PROLENE 7.0) 2-3 mm distal from its origin. Finally, the chest was closed in layers. Prior to surgery fentanyl 0.15 mg/kg drug was administered by intraperitoneal route.

One week after artery ligation, the chest was reopened and SC (1.2 cm x 1.2 cm) was applied to the heart covering the infarcted area.

After surgery, ketoprofen (5 mg/kg) was subcutaneously administered for three days. Antibiotic enrofloxacin (Alsir lechones, Esteve Veterinaria) (25 mg/kg) was supplied in the drinking water for 7 days.

Animals were sacrificed at 24 hours, 1 week, 1 month and 3 months. After being anesthetized they were perfusion-fixed and killed. Hearts were harvested and fixed in 4% formalgehyde at 4 °C overnight for its inclusion in paraffin and ulterior histological studies.

2.7. Histological analysis

Hearts were cut into three pieces (apical, mid-ventricular and basal) and embedded in paraffin. They were sectioned at a 5 μm thickness. Hematoxylin-Eosin staining was done by submersion in Harris hematoxylin followed by differentiation through 37% HCl and Li2CO3 solution. Then samples were immersed in 0.5% eosin, dehydrated and mounted in DPX. To

determine macrophage phenotype, immunolabeling was performed. Tissue slides were deparaffined and antigen retrieval was done. For the M1 phenotype, antigen retrieval was performed using the microwave-citrate method (citrate 10mM, pH 6, microwave heating 20 minutes). For the M2 phenotype, trypsin antigen retrieval was employed (trypsin 0.2% in calcium chloride 0.1%, 5 minutes, 37 °C). After washing, sections were blocked with 5%BSA in tris buffered saline. Then primary antibody incubation was performed with rabbit anti-CCR7 (Epitomics, 2059-1) for M1, and mouse anti-CD163 (Serotec, MCA342R) for M2, both diluted 1:100 in blocking solution. Following overnight incubation at 4 °C, samples were washed and secondary incubation for 30 minutes at RT was done using anti-rabbit FITC and anti-mouse Alexa 594. DAPI was also employed for nuclei contrasting.

M1 and M2 macrophages present in the cardiac tissue at different times were visualized and quantified using Zeiss Axioplan 2ie microscope equipped with epifluorescence optics. 6 images per section were analyzed (8 sections per animal, 40X magnification) and digital images were processed using ImageJ software. Data are expressed as mean ± SEM. To analyze statistical significance Student's t-test was used. *P*-values corresponded to a two-tailed unpaired *t*-test for the group comparison, $p < 0.05$ was considered statistically significant. All analyses were conducted using Prism software (GraphPadsoftware, San Diego, CA, USA).

3. RESULTS AND DISCUSSION

3.1. Fiber preparation

Regarding polymer composition, fibers containing PLGA , PEG-PLGA and a combination of both in a ratio 1:1 were prepared. sP(EO-stat-PO) polymer was included in the solution containing the mixture of polymers. During this process it was observed that the

presence of this polymer altered fiber integrity in a dose-dependent manner (Fig. 2). The higher the amount of sP(EO-stat-PO), the less uniform the fibers were.

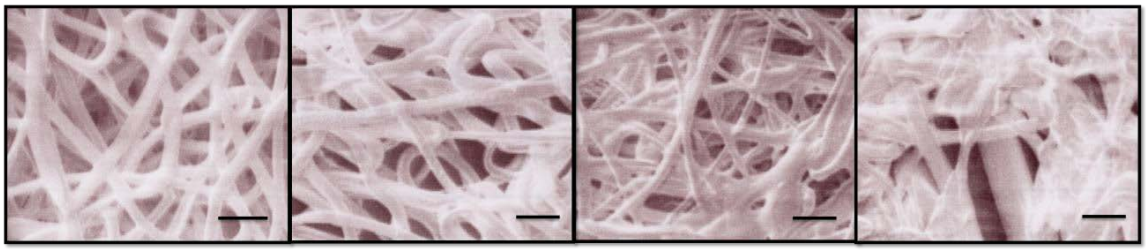

Fig. 2. Fibers prepared with different sP(EO-stat-PO) amounts (from left to right: 5 mg; 10 mg; 15 mg and 20 mg/150 mg total polymer amount). Scale bars: 10 μm.

To overcome this problem TFA was added to improve the conductivity of the blend. It is well known that the conductivity of the solution greatly influences the ES process, which is in fact based on the application of a voltage to the polymer solution. It is necessary that the fluid reaches a critical amount of charge and then a fluid jet erupts from the droplet at the tip of the needle resulting in a Taylor cone (Fig. 3). At that moment the polymer mixture moves towards the grounded collector that presents lower potential, forming the fibers (Ramakrishna *et al*., 2005). When TFA was included in the solution it improved the fibers. However, it was observed that an excessive amount of TFA in the composition had a negative effect (Fig. 4), resulting in the formation of more heterogenous fibers and, in some cases, in their fusion. Also, TFA is an organic solvent that acts by denaturing proteins, so its presence in the formulation must be minimal and direct contact with protein has to be avoided.

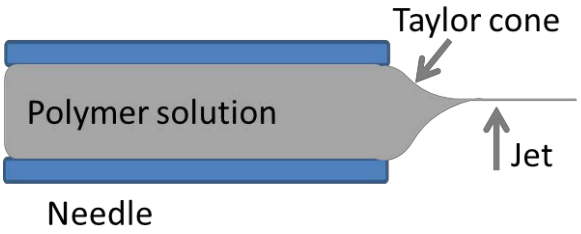

Fig. 3. Schematic representation of Taylor cone formation.

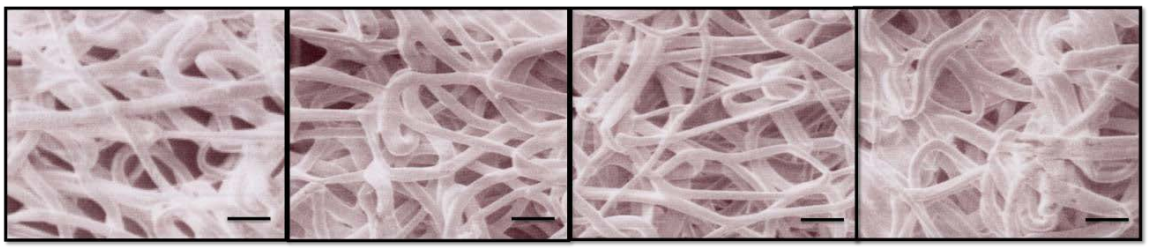

Fig. 4. Fibers prepared with different TFA concentration (from left to right: 0; 0.5; 2 and 10 µl/ml). Scale bars: 10 µm.

PEG200 was added to the aqueous solution, where the protein was included, to protect it from degradation due to the direct contact with the organic solvents. Interestingly, it was observed that, using the same polymer composition, a minimum 30 µl amount of PEG200 was essential to form the fibers. With 20 µl the mixture could be electrospun but in the collector fibers fused (Fig. 5). Below 20 µl PEG200 the mixture could not be electrospun. This effect is probably due to the high viscosity of the blend. Solution viscosity is a crucial parameter in the ES process. It is important to ensure a minimum viscosity so that the polymer jet is not broken when it travels from the injector to the collector. On the other hand, if the viscosity is too high the solution will dry in the needle tip preventing the ES process.

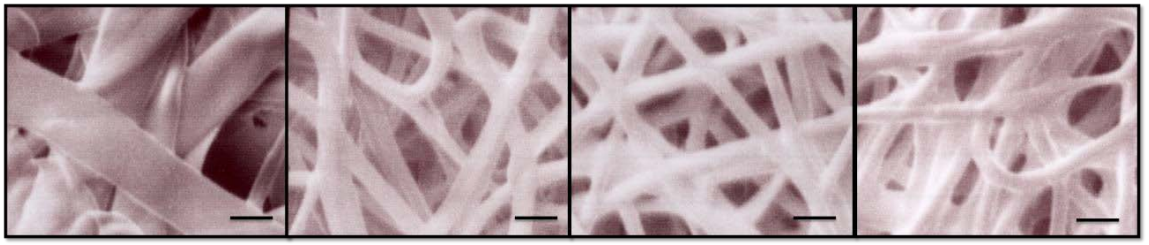

Fig. 5. Fibers prepared with different PEG200 amounts (from left to right: 20; 30; 40; 50 µl/150 total polymer amount). Scale bars: 5 µm.

Proteins are labile molecules that are usually dissolved in buffered solutions containing salts and carrier proteins that interact with them giving protection against degradation. In this study it was observed that both a high amount of carrier protein bovine serum albumin (BSA) (Fig. 6) and the presence of salts in the protein solution altered fiber

formation (Fig. 7). Thus, for protein encapsulation PBS should be avoided and carrier free proteins are desirable.

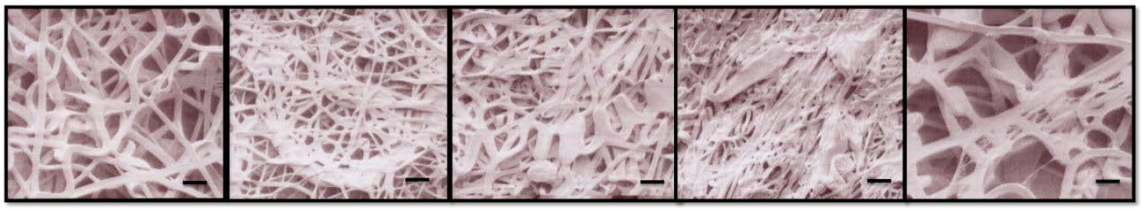

Fig. 6. Fibers prepared with different BSA amount (from left to right: 10; 25; 50; 100; 150 µg/150 total polymer amount). Scale bars: 20 µm.

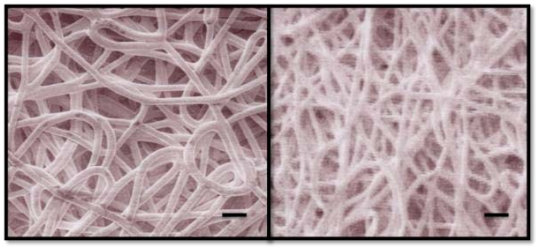

Fig. 7. Fibers prepared including water (left) and PBS (right) in the aqueous solution. Scale bars: 10 µm.

All things considered, SCs with six different compositions were chosen for Nrg encapsulation (Table 2).

Table 2 Composition of the 6 SC formulation prepared, all of them containing Nrg-1 10 µg.

N	*PLGA 504*	*PEG-PLGA*	*sP(EO-stat-PO)*	*TFA*
1	150 mg	0 mg	0 mg	0 µl
2	75 mg	75 mg	0 mg	0 µl
3	0 mg	150 mg	0 mg	0 µl
4	75 mg	75 mg	0 mg	10 µl/ml
5	75 mg	75 mg	5 mg	10 µl/ml
6	75 mg	75 mg	5 mg	2 µl/ml

3.2. Encapsulation efficiency

To evaluate the efficiency of the process to encapsulate proteins in the fibers the amount of protein retained was compared with the protein initially included in the solution and expressed as a percentage. Results indicate that all six SCs were suitable for protein encapsulation, with encapsulation efficiency (EE) values over 85% in all cases. This is a high rate compared with the results of other groups that have also encapsulated GFs in PLGA fibers by ES. For example, Sahoo *et al*. prepared PLGA nanofibers containing bFGF with 54% EE (Sahoo *et al*., 2009). In a more recent study, another group encapsulated BMP-2 in three different PLGA based SCs. EE values were in all cases within 44% and 66% (Nie *et al*., 2012). On the other hand, the EE results demonstrate that the presence of PLGA/sP(EO-stat-PO) polymer in the SC does not affect the protein encapsulation.

3.3. *In vivo* studies and histological analysis

Based on the EE and on the *in vitro* release profile (data not shown), SC composed of PLGA and PLGA/sP(EO-stat-PO) polymer with low TFA concentration (N 6 in Table 1) was chosen for the biocompatibility studies. Since it includes PLGA/sP(EO-stat-PO) and the lowest quantity of TFA it appears to be the most suitable for cell adhesion and therefore for its use in the future *in vivo* studies.

First of all, adhesion of the SC to the cardiac tissue was assessed at different times. It must be noted that the SC was directly applied on the tissue without any suture or natural glue. Adhesion and integration in the tissue were observed from 24 hours after its implantation to the end of the study, after 3 months (Fig. 8). Whereas other patches that have been tested on the heart are not able to integrate due to their rigidity (Araña *et al.*, 2013), this polymeric SC is easily attached and adapted to the cardiac tissue. Moreover, during the whole

study the SC remains in the tissue without holes. It is elastic enough to resist stress and strain due to the contractile activity of the heart muscle.

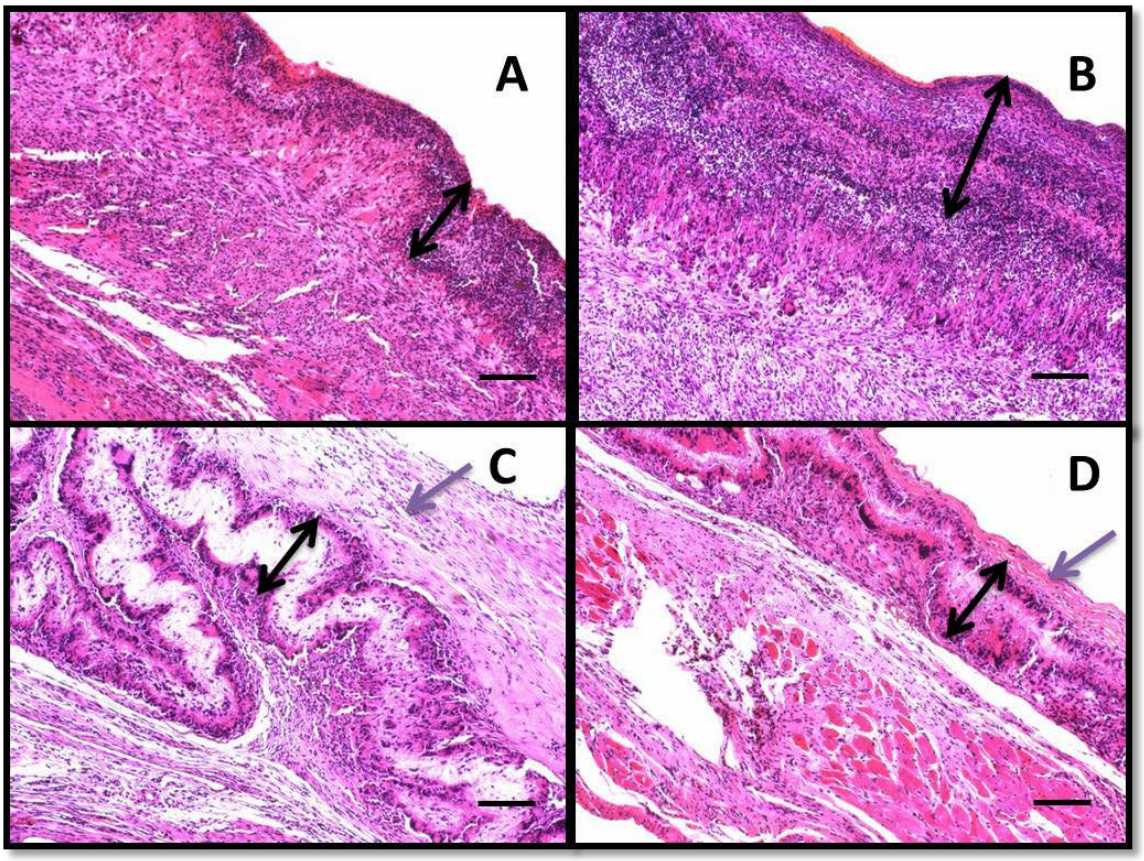

Fig. 8. SCs in the tissue (double arrow) and fibrotic tissue (blue arrow) after 24 h (A), 1 week (B), 1 month (C) and 3 months (D). Scale bar = 50 µm. (Hematoxilin-eosin staining; 10X magnification).

During all the time of the study inflammatory infiltrate appears in the area of the implant, and cells can be seen to have penetrated and diffused through the SC. After the acute inflammation observed at 24 hours, chronic inflammation is established, which can be detected from one week until three months post-implant. One month after its implantation fibrotic tissue appears next to the biomaterial, and the inflammatory infiltrate in the SC is visibly reduced, compared with week 1 (Fig. 8).

After 1 and 3 months, foreign body giant cells can also be observed near the implant area. Macrophages and foreign body giant cells are degradation modulators, which adhere to the surface of the biomaterial and release mediators of degradation such as reactive oxygen species, degradative enzymes, and acid between the cell membrane and the biomaterial

surface. When this degradation occurs in a short period of time it causes device failure, limiting the beneficial effect of the delivery system. All these immunologic events are common in a wound healing process after myocardial ischemia (Freytes *et al.*, 2012) or following the implantation of a biomaterial in the organism (Anderson *et al.*, 2008).

It is interesting to mention that in the study the SC was not completely degraded after three months, meaning that it is suitable for prolonged cytokine release. Nevertheless, long-term studies are necessary to establish the precise *in vivo* degradation time.

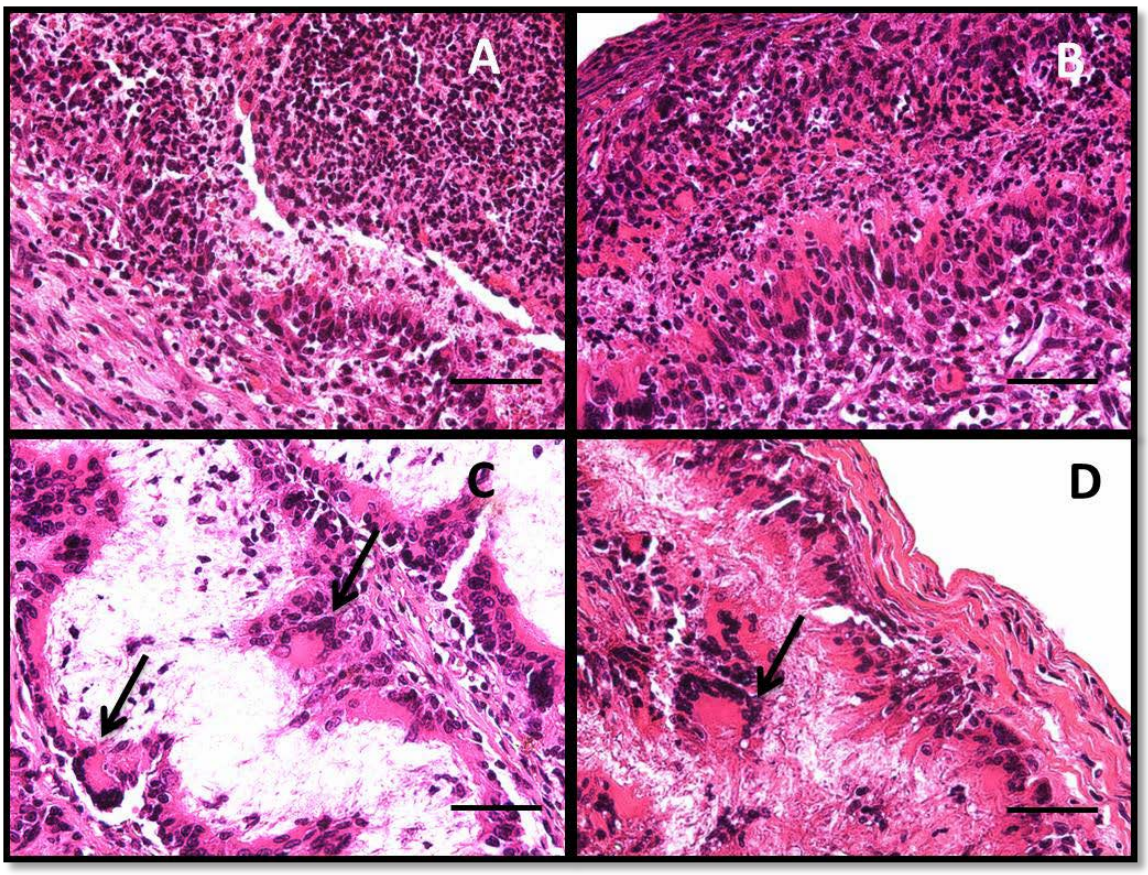

Fig. 9. Implantation site after 24 h (A), 1 week (B), 1 month (C) and 3 months (D). Arrows indicate the presence of foreign body giant cells. Scale bar = 20 μm. (Hematoxilin-eosin staining; 40X magnification).

3.4. M2:M1 macrophage phenotype

Macrophage polarization towards M1 or M2 phenotype has recently been demonstrated to be involved in constructive tissue remodeling. Macrophage response to biomaterials is nowadays being characterized by the M2:M1 ratio (Brown *et al.,* 2012). Using surface markers these two types of cells can be identified and quantified. Whereas M1 phenotype corresponds to classically activated and pro-inflammatory macrophages, M2 is alternative activated and is associated with regulatory and homeostatic functions, and therefore, with tissue regeneration (Badylak *et al.,* 2008). Moreover, it has been demonstrated that the switch from M1 to M2 phenotype promotes progenitor cell differentiation and adequate tissue remodeling (Keane *et al.,* 2012). Based on this, Bryan and colleagues (Brown *et al.,* 2012) implanted 14 different surgical mesh biomaterials in the rat abdominal wall and studied *in situ* macrophage polarization towards M1 and M2 phenotype, as well as the tissue remodeling response. Correlation between M2:M1 ratio and better tissue remodeling was observed, reinforcing the role of M2 in tissue regeneration

In the present study, the macrophage phenotype was quantified at several times in the area where the SCs were implanted. CCR7 and CD163 markers were used to identify M1 and M2 phenotypes respectively (Fig 10). M2:M1 ratio was calculated and a statistically significant increase over time was found (Fig. 11).

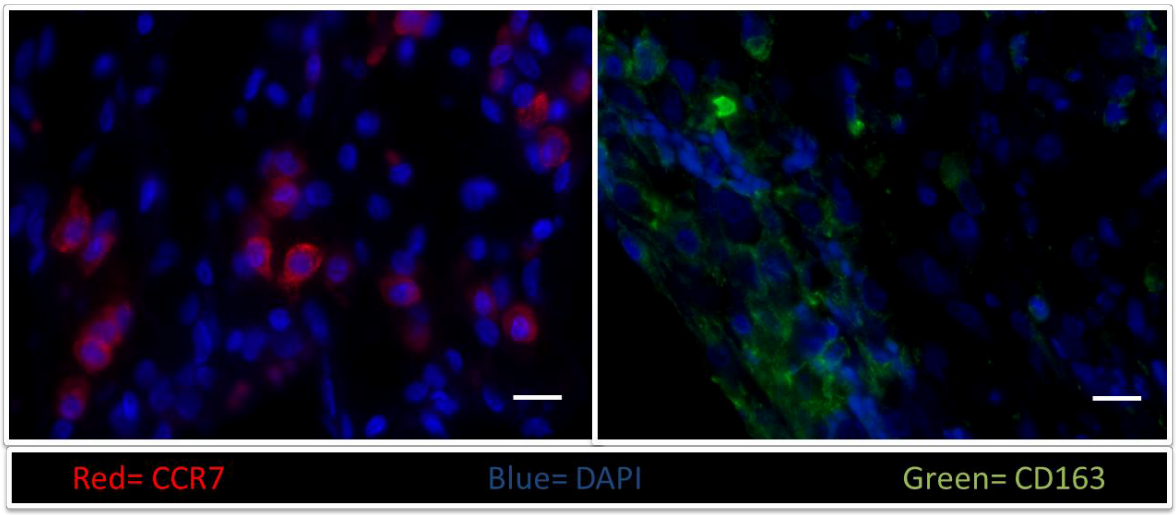

Fig. 10. Immunofluorescent images showing examples of the host macrophage response to scaffolds. Scale bar = 10 μm. CCR7 (M1) = red, CD163 (M2) = green, DAPI (nuclei) = blue.

The results showed how 24 hours after scaffold implantation a predominant pro-inflammatory response is triggered via classically activated M1 macrophages. This response is rapidly attenuated as time goes by, producing a statistically significant M2:M1 ratio increase as soon as one week after implantation. This increment remains at least after one month meaning that the initial pro-inflammatory response moves towards a more constructive response that will produce positive tissue regeneration.

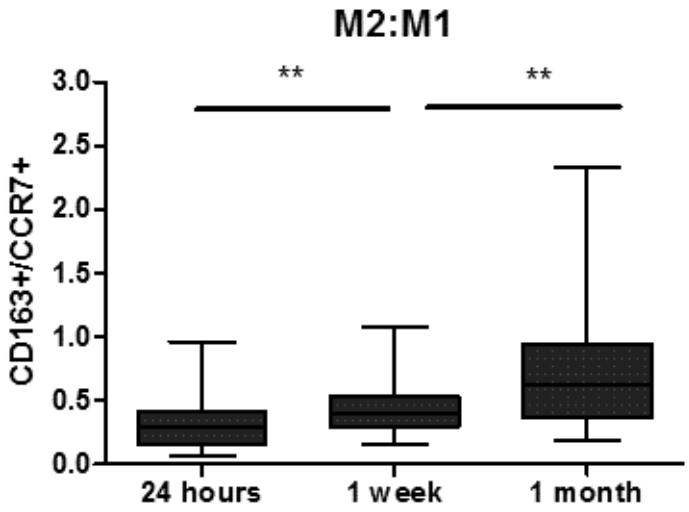

Fig. 11. Ratio of M2:M1 marker expressing cells at 24 hours, 1 week and 1 month.

4. CONCLUSIONS

This paper shows that Nrg was successfully encapsulated within the fibers of polymeric SCs. Prepared by ES, both preparation method parameters and composition of the polymeric solution were established to obtain smooth and homogenous fibers with a diameter suitable for cell adhesion and with high protein EE. A novel polymer with surface modifications to reduce protein absorption and to promote specific cell adhesion was used, with the purpose of combining these GF containing fibers with cells involved in cardiac repair in future experiments. SCs were adhered to cardiac tissue in an animal model of myocardial ischemia and interaction between the polymeric device and the heart tissue was described at different times, from 24 hours to three months, showing prolonged presence of the SC and the normal immunologic response against an implanted biomaterial. Finally, immunohistochemistry revealed that after SC implantation a pro-inflammatory response mediated by M1 macrophages takes place. After one week and one month this response is progressively attenuated, as reflected by the increase of M2:M1 ratio, suggesting constructive tissue remodeling.

Therefore, the polymeric SCs prepared in the present study are suitable for protein encapsulation and cardiac implantation in an animal model of myocardial ischemia, and could be, in the future, a good strategy to promote cardiac tissue regeneration, alone or in combination with cells.

ACKNOWLEDGEMENTS

This work was partially supported by ISCIII-RETIC RD12/0019/0031, MINECO PLE2009-0116 CARDIOBIO, Program INNPACTO (PROCARDIO), European Union FPVII (INELPY), FUN (University of Navarra) Caja de Ahorros de Navarra (Programa Tu Eliges: Tu Decides). T. Simón-Yarza thanks the Spanish Ministerio de Educación (programa FPU) for the fellowship grant.

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GENERAL DISCUSSION

GENERAL DISCUSSION

In this study, poly(lactic co-glycolic acid) (PLGA) microparticles (MPs) containing vascular endothelial growth factor (VEGF) to treat cardiovascular disease (CVD) were optimized to reduce the macrophage clearance of the MPs. As shown in Chapter 1, PEGylation of the MPs succeeded in diminishing macrophage particle clearance, without affecting VEGF high encapsulation efficiency and bioactivity. To our knowledge, this is the first time that the strategy of PEGylation has been applied to VEGF polymeric MPs, which means that this study takes a truly novel approach. Moreover, in Chapter 2, these PEGylated particles were proven to be efficient to improve cardiac function at two different doses in a rat model of myocardial ischemia (MI). Coenzyme Q_{10} (CoQ) orally administered in nanoparticles (NPs) was also found to be beneficial after MI. This result is especially encouraging since no preclinical studies of CoQ-NPs in MI have been reported to date. However, concomitant administration of VEGF particles with orally administered NPs did not improve the treatment. In fact, no beneficial effects were observed in comparison with the control group. Histological analysis revealed a reduced pro-angiogenic effect in those animals receiving combined treatment, compared to those animals receiving VEGF-MPs alone. This result suggests a potential anti-angiogenic effect due to CoQ that should be studied in depth.

In Chapter 3 a new approach to tackling MI is explored. In this case the GF studied was Neuregulin-1 (Nrg). This is a promising factor that has gained relevance in the last three years and is currently being applied in several clinical trials (Clinicaltrials.gov identifier NCT01131637, NCT01214096, NCT01251406, NCT01258387, NCT01541202). In our innovative proposal, Nrg was efficiently incorporated into polymeric fibrous scaffolds. In this preliminary study, protein-containing fibers were manufactured, characterized and applied in an animal model of MI to study their biocompatibility and biodegradation.

Actives involved in heart repair

Cytokines

One of the key points in tissue engineering (TE) is to choose the appropriate cytokine(s) to be incorporated in the drug delivery systems (DDS). This is a complex controversy. Numerous cytokines are under study, some of which have been briefly reviewed in Annex IV. However, the question is not only which one should be used, but also at what dose? At which stage of the disease? Alone or in combination? And if in combination, with which other factor?

In this study we employed VEGF and Nrg. VEGF is probably the most widely studied cytokine, as reviewed in Annex II. After years of research it can be assumed that although VEGF promotes neovascularization, it is not effective enough to promote heart regeneration probably to the immaturity of the newly formed vessels (Reginato *et al.*, 2011). Combining VEGF with other growth factors (GFs) that cooperate with vessel maturation could be more effective. In this context, sonic hedgehog (Shh) is a GF essential during embryonic development. It is known to be involved in vessel maturation, within other mechanisms (Dohle *et al.,* 2011). Some studies have appeared in recent years highlighting its role not only in cardiac development but also in correcting various cardiac dysfunctions, via angiogenic-related processes (Johnson and Wang, 2013; Mackie *et al.*, 2012). Combination of VEGF with Shh could be of interest in this context.

Another candidate to be combined with VEGF is stromal derived factor-1α (SDF-1α). This cytokine has been more extensively explored than Shh. SDF-1α acts at different levels in the damaged heart. It protects cardiomyocytes from apoptosis, it promotes migration of endothelial cells and it acts on bone marrow derived cells promoting recruitment of vascular progenitor cells and angiogenic factor production (Takahasi, 2010). VEGF and SDF-1α secretion after MI are intimately correlated. SDF-1α promotes VEGF secretion that acts, in

turn, promoting SDF-1α secretion and the expression of SDF-1α receptor CXCR4, which is essential for SDF-1α activity. Interestingly, it has been reported how after ischemia there is a lapse between SDF-1α early and brief secretion and CXCR4 late expression (Penn, 2009). This time lapse limits the beneficial effect of SDF-1α. Prolonged release of VEGF concomitant with SDF-1α will help to solve this problem. In that case, VEGF, apart from its inherent angiogenic activity, will contribute to early expression of CXCR4. On the other hand, SDF-1α will be present in the tissue for a longer period of time, thus prolonging the cardioprotective and angiogenic activity.

The other GF under study, Nrg, can also be administered alone or in combination with other factors. In an original piece of research our group combined Nrg-MPs with fibroblast growth factor-1 (FGF-1) MPs (Formiga *et al.*, 2013). It is known that FGF-1 regulates cardiac remodeling by exerting a protective and proliferative effect after MI (Engel *et al.*, 2006). Surprisingly, when it was administered in a model of heart ischemia, no consistent synergistic effect was observed either *in vitro* or *in vivo.* Moreover, the effect of both cytokines combined was lower than either alone. Our hypothesis is that Nrg-1 and FGF-1 use similar pathways, so they may compete by limiting their combined effect (Kuhn *et al.*, 2007; Bersell *et al.,* 2009). In any case, two important lessons can be learned from this study. Firstly, when we combine therapies we should pay attention to the *in vivo* negative interactions between them. Secondly, complex and sophisticated approaches may be conceptually more attractive, but they are not always more effective.

To establish the dose of the cytokines for the efficacy *in vivo* assays, the results of the *in vitro* test were considered. In the case of the VEGF MPs we included two different doses. The highest one is the same as was previously tested in the work in Annex I, and was calculated on the basis of *in vitro* studies. The lowest dose, approximately half of the high dose, allowed us to study whether a dose response occurred. Strikingly, the same effect was observed for both doses, suggesting that calculating the dose for the *in vivo* studies based on the *in vitro* assays is not the optimal way. An alternative way to calculate *in vivo* doses could

be based on the physiological levels of the factors. In the case of VEGF it is well known that in cancer patients it is up-regulated just to promote neoangiogenesis. For example, in a study performed by Kayiannakis and colleagues, the serum levels of VEGF in healthy and gastric tumor patients were compared. Healthy patients' VEGF concentration was around 190 pg/mL, and in cancer patients it was 440 pg/mL (Karayiannakis et al., 2002). This kind of data regarding the physiological active levels of cytokines to trigger a specific process could help in the future to calculate adequate doses of the treatment. If clinical trials with the GF under study have already been performed, a third way to calculate the dose is to extrapolate the assayed doses with non-encapsulated GFs in humans. In that case the total weight of the animals and the volume and weight of their hearts should be considered compared to human values, for dose adjustment.

Finally, in relation to the right moment to administer the treatment, the natural development of the disease must be considered. After MI some areas of the damaged heart constitute the "hibernating" cardiac tissue and can be partially or completely restored to normal function after neovascularization (Elsässer *et al.,* 1997). It seems that early cytokine administration might favor the rescue of this tissue.

Coenzyme Q_{10}

Treatment with cytokines can also be combined with other molecules. For the work described in Chapter 2 we had the opportunity to collaborate with a research group in the university of Strathclyde (Glasgow) to test the efficacy of a CoQ improved DDS. CoQ is a coenzyme with an active role preventing cells from damage due to oxidative stress. This molecule was chosen based on the benefits reported in clinical trials with ischemic heart patients. Nevertheless, we were also conscious of the risk, considering the limited data about mechanisms of action that has already been mentioned in the Introduction and in Chapter 2.

On the one hand, the results of the study are positive because CoQ NPs showed benefits compared to the commercial formulation. On the other hand, the observed antiangiogenic effect of CoQ interfering with VEGF angiogenic effect was disappointing. A deeper study of the CoQ mechanisms mediating heart function improvement is now needed. Also a different administration regimen of CoQ could be interesting, including different doses. However, before doing other *in vivo* studies, more *in vitro* assays need to be done to clarify CoQ activity and its interaction with VEGF. For this purpose, endothelial cells, like human umbilical vein endothelial cells, could be used to study the effect of CoQ in migration, proliferation and tubule formation assays.

Polymeric devices to protect proteins and prolong their delivery

Results included in this study revealed that DDS are suitable for protein encapsulation and therefore able to overcome the limitation of protein therapy related to the short half-life of these macromolecules.

In Chapter 1 VEGF was encapsulated in polymeric MPs made of PLGA and PEG-PLGA. While carrying out this work, we also incorporated other GFs acting in heart repair into polymeric MPs, namely: Shh, SDF-1α, FGF-1 and Nrg (data not included in this report). In all cases, the double emulsion solvent evaporation method was employed to prepare the particles. Total Recirculation One Machine System (TROMS), based on the formation of a multiple emulsion by the injection of the phases under a turbulent regime, was used to avoid stressful conditions affecting protein integrity. High encapsulation efficiency values and *in vitro* bioactivity assays demonstrated protein integrity after the encapsulation process, proving the efficacy of TROMS to efficiently encapsulate cytokines.

In Chapter 3 Nrg was incorporated for the first time into polymeric fibers composed of PLGA and a novel polymer specially designed to reduce the immunological response: PLGA/sP(EO-stat-PO). In this case scaffolds were prepared by electrospinning. To protect

protein from degradation, modifications in the solution to be electrospun were performed. High encapsulation efficiency values were also obtained.

Therefore, manufacturing polymeric systems that protect proteins from degradation is nowadays a real and accessible strategy.

Regarding the capacity of these systems to prolong the release of GFs, it has been demonstrated in Annex III how MPs were detected in the damaged heart tissue at least three months after implantation. However, this study did not demonstrate that those MPs were still releasing the encapsulated protein at that time. Thus, currently we are performing an *in vivo* release study in which Nrg has been biotinylated, encapsulated into MPs (PLGA and PEG-PLGA MPs) and administered in the same animal model used in Chapter 2. Animals were sacrificed at different times and immunohistochemistry has been performed to co-localize the MPs with the biotinylated protein in the site of injection. To date we have analyzed animal hearts from 24 hours to two months after intramyocardial injection and data show that protein continues to be released after two months. Results after three months are still being processed. In most studies with DDS and GFs *in vitro* release profiles are routinely made. However, the conditions of these assays greatly differ from the real conditions in which the DDS are injected in the ischemic heart. Therefore, it is not appropriate to extrapolate the results from the *in vitro* studies to the *in vivo* situation. This work is pioneering in trying to determine the *in vivo* release of proteins from polymeric microparticles after MI.

Polymeric devices in heart repair: particles and scaffolds

One of the advantages of the DDS under study is that they are easily handled, especially in the case of MPs. After being lyophilized we obtain a powder that can be stored under adequate conditions (temperature and humidity) until its use. This powder is then suspended in a solution containing surfactants, which prevent particle precipitation, and this

suspension is locally administered, ensuring dose control and avoiding harmful effects due to the dissemination of the protein through the organism.

In the animal model employed in this study, rats need to be opened to intramyocardially administer the particles. This is an important limitation of these small animal models that is not present when larger animals are used. In pigs, as well as in humans, MPs can be administered using catheter-based delivery systems that make open chest surgery unnecessary (Smits *et al.,* 2003; Dib *et al.,* 2009).

In the case of the scaffolds, what we obtain after electrospinning is a thin sheet, flexible and strong enough to be manipulated under ordinary conditions. To apply the polymeric sheets onto the infarcted area it is mandatory, to date, to perform open chest surgery. This is a negative feature of these devices. On the other hand, they present one important advantage, which is the capability to sustain cells and to improve cell engraftment in the tissue. Also, when the scaffold is applied onto the heart it remains there and it is ensured that no protein is lost during the implantation process. In the case of the MPs, since they are administered in suspension and the heart remains beating during intramyocardial injection, it is frequent to observe how part of the solution flows out, meaning a loss of part of the treatment. Moreover, when injecting particle suspension some volume is also lost due to the dead space volume of syringes or catheters.

In order to administer different cytokines at different doses at the same time, MPs are preferred. It is possible to prepare the particles separately, calculate the necessary dose of one of each and then prepare the cocktail to be administered. Moreover, if different release profiles of each factor are needed, particles can be prepared with different particle size or polymer composition. Possibilities with MPs are numerous in terms of multiple cytokine delivery, different patterns of release, and degradation times. With the scaffolds this is more limited. It is possible to incorporate different macromolecules when the fibers are prepared, but then it is not possible to modify protein content. Also the fibers of this scaffold containing several actives will be homogenous, and therefore, all the factors will present similar release

profiles. Again, these limitations need to be contrasted with the advantage of the scaffolds, which allow cell administration and engraftment.

Throughout the present study, special attention has been paid to the inflammatory response caused by polymeric devices. In the case of MPs, polymer composition containing PEG has reduced the immunogenic response, as demonstrated in Chapter 1. In the scaffold study (Chapter 3), a special polymer that had demonstrated lower immunogenicity in previous studies was also included. However, the inflammatory response was much greater than in the case of the particles, even in the non-pegylated ones (Annex III). This greater immunogenicity has to be attributed to the scaffold itself, irrespective of its composition. Its larger surface and its thickness, together with the long degradation time (more than three months) result in a more aggressive system for the organism than the particles. The scaffold study is a very preliminary one. Modifications of the scaffold are now needed to reduce the inflammatory response. The thickness of the scaffold must be controlled. Polymer composition should also be optimized to reduce scaffold degradation time, providing at the same time the adequate prolonged protein release. However, due to the extent of the scaffolds covering the whole infarct area it can be supposed that they will always trigger a greater inflammatory reaction than MPs. Incorporating anti-inflammatory agents in the fibers could be a strategy to overcome this immunogenicity.

Some of the advantages and disadvantages of the different devices under study have been mentioned, demonstrating the complexity of determining which one is superior. The net profit of scaffolds and particles must be evaluated taking pros and cons into account.

The approach based on MPs encapsulating GFs appears, so far, to be more realistic considering quality control requirements for use in patients. Many preclinical studies have already been conducted. Clinical trials are now necessary to corroborate the efficacy of DDS for cardiac repair. Nevertheless the lack of interest that these systems arouse in the cardiologists' environment is worrying. Most of the preclinical studies including TE

approaches in heart repair are found in journals of drug delivery or TE areas. It is difficult to find articles published in those journals that specialize in cardiac pathology.

Regarding scaffolds combined with cells, these still need to be optimized. For instance, protocols to seed and harvest cells must be reproducible enough to be validated, homogeneity of cell products has to be ensured, safety must be demonstrated and the economic cost of these therapies needs to be reduced to make them accessible to health care systems and to patients. More time and more research efforts are needed to fulfill these requirements. For these reasons, it will probably take more time to find this kind of product at the patients' bedside.

Preclinical animal models of myocardial ischemia

A big handicap when testing the efficacy of treatments in cardiac repair is associated with the limitations of preclinical animal models. This issue becomes even more important when we are dealing with small animal models since, as has already been mentioned, open chest surgery is necessary to administer the MPs.

Several animal models have been used to date to study ischemic heart disease. In general, they can be divided into two kinds: those in which hypercholesterolemia, which is the most important risk factor for atherosclerosis, is induced, and those models in which MI is surgically induced. The first class is not appropriate to evaluate new therapies, due to the lack of control of arterial occlusion and, as a consequence, of the magnitude of the damage. Hence it is important to make use of models in which ischemia is surgically induced, with a better control over time, localization and extent of the lesion provoked (Klocke *et al*., 2007). Regarding the animal to be used, in the first stages of the efficacy studies, it is preferable, from an ethical point of view and also for economic reasons, to use small animals from less evolved species. In the case of animal models of MI the use of rats is frequent since they are

easily handled. Surgical models in rats are performed by the occlusion of the left descendent coronary artery. In the ischemia-reperfusion model, the ligature is maintained for a period of time and then the artery is reopened allowing tissue reperfusion. This model has the advantage of being more similar to what occurs in clinical situations. When a patient suffers acute infarction, the artery is frequently spontaneously reopened, due to the dissolution of the thrombus or the vascular musculature relaxation. If the obstruction does not revert, unblocking of the artery is performed, in most cases by a percutaneous coronary intervention. In both situations, after arterial occlusion, a reperfusion stage takes place. This reperfusion is beneficial as it allows cell oxygen supply to be restored. However, it has been well described that it is also responsible for inflammatory damage mediated by oxidative stress, an increase in the intracellular calcium levels, aggressive pH modifications and an increase in inflammation (Hausenloy and Yellon, 2013). This damage, the result of cardiac reperfusion, makes it difficult to evaluate the efficacy of therapies whose objective is the regeneration of the ischemic tissue mediated by the mechanisms described above: neovascularization, antiapoptotic effect, cardiomyocyte replication or bone marrow cell mobilization. On account of this, for the *in vivo* study performed in Chapter 2 and Chapter 3, the permanent ligation model was chosen, even though we were conscious of its limitations.

Finally, it must be mentioned that, in all cases, cardiovascular rat physiology differs greatly from its human counterpart, so in subsequent studies this therapy has to be evaluated in a suitable large animal model, like the pig.

As an overall conclusion, protein therapy with our polymeric devices shows promising results to treat CVDs. PEGylated microparticles containing VEGF are able to improve cardiac function in an animal model of MI. To boost this effect, these MPs can in the future be combined with other factors that will contribute to vessel maturation. In the case of the polymeric scaffolds, although we are still at a preliminary stage, they have been effective at encapsulating Nrg. The degradation time of the scaffold suggests prolonged release of the

protein, which should be demonstrated by performing an *in vivo* release study. *In vivo* biocompatibility was satisfactory, but can still be improved by modifying the polymer composition and the thickness of the scaffold, for instance. Once optimized, PLGA/sP(EO-stat-PO) polymer included in these scaffolds will allow selective cell attachment and good cell engraftment in the heart. This device will be a combination of GF, cells and DDS, a paradigm of the TE triad.

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CONCLUSIONS

CONCLUSIONS

Studies included in this work allow concluding:

1. PLGA microparticles previously prepared in our laboratory were optimized by including a more hydrophilic polymer in the initial formulation: poly(ethylene glycol). This polymer reduced the immunogenicity of the microspheres, demonstrated by a reduction in the particle macrophage clearance.

2. VEGF was successfully encapsulated into PEGylated microparticles. During the elaboration process VEGF bioactivity was not affected, as demonstrated in a HUVEC proliferative assay.

3. VEGF PEGylated microparticles were effective to ameliorate cardiac function in an animal model of myocardial ischemia. Treatment with two different doses of VEGF microparticles significantly improved animal ejection fraction, when compared to the control group. No dose-dependent response was observed in infarcted animals after treating them with two different doses of VEGF-microparticles.

4. Antioxidant CoQ in PLGA nanoparticles orally delivery improved cardiac function after myocardial ischemia. Ejection fraction was significantly improved compared to the control group. Moreover, combined VEGF microparticle treatment and CoQ nanoparticle oral therapy did not significantly improve cardiac function, refuting the hypothesis of a synergistic effect.

5. The histological analysis demonstrated increased angiogenesis in those animals treated with VEGF microparticles. This effect was reduced in those animals in which

this treatment was combined with antioxidant CoQ nanoparticles, suggesting the antiangiogenic role of the CoQ.

6. Smooth and homogenous polymeric fibrous scaffolds were prepared by electrospinning process, including in their composition sP(EO-stat-PO), a polymer that reduces protein adsorption and allows specific cell adhesion. Additionally, neuregulin-1 was efficiently encapsulated within polymer fibers.

7. The polymer scaffolds implanted in an animal model of myocardial ischemia unleashed the immunological response typical of wound healing.

8. The scaffolds implantation caused a pro-inflammatory response mediated by M1 macrophages. After one week and one month this response was progressively attenuated, as reflected by the increase in the M2:M1 ratio, suggesting constructive tissue remodeling. Three months after implantation, the scaffold presented degradation signs but remained in the damaged tissue, confirming its ability to prolong growth factor delivery.

CONCLUSIONES

CONCLUSIONES

Los estudios incluidos en este trabajo permiten concluir:

1. Las micropartículas de PLGA, previamente preparadas en nuestro laboratorio, fueron optimizadas mediante la inclusión de un polímero más hidrofílico en la formulación inicial: poli(etilén glicol). Este polímero redujo la inmunogenicidad de las microesferas, hecho demostrado por la reducción del aclaramiento por macrófagos de las mismas.

2. VEGF fue encapsulado en las micropartículas PEGiladas. Durante el proceso de elaboración su bioactividad no se vio afectada, tal y como se ha demostrado en un ensayo de proliferación de células HUVEC.

3. Las micropartículas PEGiladas con VEGF fueron efectivas en la mejora de la función cardiaca en un modelo animal de isquemia de miocardio. El tratamiento con dos dosis diferentes de micropartículas de VEGF aumentó de manera significativa la fracción de eyección, comparado con el grupo control. No se observó respuesta dosis-dependiente en los animales infartados tras ser tratados con dos dosis de VEGF encapsulado en las micropatículas.

4. Las nanopartículas de PLGA con el antioxidante CoQ administradas por vía oral mejoraron la función cardiaca tras un infarto de miocardio. La fracción de eyección aumentó de manera significativa comparado con el grupo control. El tratamiento combinado de micropartículas de VEGF y nanopartículas de CoQ administradas por la vía oral no mejoró de forma significativa la función cardiaca, refutando la hipótesis de un efecto sinérgico.

5. Los análisis histológicos demonstraron mayor angiogénesis en aquellos animales tratados con las micropartículas de VEGF. Este efecto se vio reducido en los animales en los que el tratamiento se combinó con nanopartículas conteniendo CoQ, sugiriendo la actividad antiangiogénico de la CoQ.

6. *Scaffolds* de fibras poliméricas lisas y uniformes fueron preparados a través de un proceso de *electrospinning*, incluyendo en su composición sP(EO-stat-PO), un polímero que reduce la adsorción proteica y permite la adhesión celular específica. Además, la neuregulina-1 fue encapsulada con eficacia en las fibras poliméricas.

7. Los *scaffolds* poliméricos implantados en un modelo animal de isquemia de miocardio desencadenaron la respuesta inmunológica típica de un proceso de cicatrización.

8. La implantación de los *scaffolds* provocó una respuesta proinflamatoria mediada por macrófagos M1. Tras una semana y un mes, esta respuesta se vio atenuada, como se desprende del aumento de la proporción M2:M1. Este incremento sugiere un remodelado constructivo del tejido. Tres meses después de ser implantados, los *scaffolds* presentaron signos de degradación pero seguían presentes en el tejido dañado, confirmando su capacidad para prolongar la liberación de factores de crecimiento incluidos en los mismos.

ANNEXES

ANNEX I

SUSTAINED RELEASE OF VEGF THROUGH PLGA MICROPARTICLES IMPROVES VASCULOGENESIS AND TISSUE REMODELING IN AN ACUTE MYOCARDIAL ISCHEMIA–REPERFUSION MODEL

Formiga FR, Pelacho B, Garbayo E, Abizanda G, et al. Sustained release of VEGF through PLGA microparticles improves vasculogenesis and tissue remodeling in an acute myocardial ischemia–reperfusion model. J Control Release 2010;147(1):30–37

http://hdl.handle.net/10171/18289

ANNEX II

VASCULAR ENDOTHELIAL GROWTH FACTOR-DELIVERY SYSTEMS FOR CARDIAC REPAIR: AN OVERVIEW

Theranostics 2012, 2(6) 541

IVYSPRING INTERNATIONAL PUBLISHER

Theranostics

2012; 2(6):541-552. doi: 10.7150/thno.3682

Review

Vascular Endothelial Growth Factor-Delivery Systems for Cardiac Repair: An Overview

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Received: 2011.10.21; Accepted: 2011.12.23; Published: 2012.06.04

Abstract

Since the discovery of the Vascular Endothelial Growth Factor (VEGF) and its leading role in the angiogenic process, this has been seen as a promising molecule for promoting neovascularization in the infarcted heart. However, even though several clinical trials were initiated, no therapeutic effects were observed, due in part to the short half life of this factor when administered directly to the tissue. In this context, drug delivery systems appear to offer a promising strategy to overcome limitations in clinical trials of VEGF.

The aim of this paper is to review the principal drug delivery systems that have been developed to administer VEGF in cardiovascular disease. Studies published in the last 5 years are reviewed and the main features of these systems are explained. The tissue engineering concept is introduced as a therapeutic alternative that holds promise for the near future.

Key words: VEGF, protein delivery, cardiovascular disease, angiogenesis, tissue engineering.

VEGF

Discovery

Vascular Endothelial Growth Factor (VEGF) was first isolated and purified from the medium conditioned by bovine pituitary folliculo-stellate cells by Henzel and Ferrara [1]. The newly discovered heparin-binding factor induced proliferation of vascular endothelial cells (ECs), showing no effects on corneal ECs. This apparent target cell selectivity induced the authors to name it "at least provisionally" VEGF [1, 2].

Some months later Ferrara *et al* [2] published a study in which they demonstrated that VEGF was a secreted protein. Interestingly, they screened cDNA and isolated clones encoding mature monomers of 121, 165 and 189 amino acids. At the same time Keck *et al* published the purification of the Vascular Permeability Factor [3], identified 5 years before by Senger *et al* [4]. Surprisingly, this protein was identical to VEGF 189.

Since that moment a considerable volume of research has been carried out, and it is now known that the VEGF 121 isoform is the most diffusible [5]. Two mechanisms by which this protein is released into a soluble form, namely alternative splicing and proteolytic cleavage, have been described, *in vivo* pro-angiogenic activity has been tested, several tyrosine kinase VEGF receptors are now known, and an

increasing number of molecular studies on VEGF is being published [6].

Biological aspects

Research focusing on the VEGF family has led to the discovery of several members encoded by different genes: VEGF-A or Vascular Permeability Factor (firstly discovered), B, C, D, E, F and PlGF (Platelet Growth Factor)[6, 7]. The most widely studied member of the family as far as angiogenesis is concerned has been VEGF-A, which we will refer to as VEGF from now on. Different VEGF isoforms from alternative splicing have been described: $VEGF_{121, 145, 165, 189 \text{ and } 206}$ (number indicates amino acid residues). $VEGF_{121}$ is the freely diffusible form, $VEGF_{145}$ remains bound to cell surface and extracellular matrix (ECM) and $VEGF_{189}$ and $VEGF_{206}$ are sequestered in the cell surface and ECM [5, 7]. Regarding its different role in neovascularization, briefly, $VEGF_{121}$ is a mitogenic agent and a chemo-attractant for endothelial cells (ECs) during angiogenesis and vasculogenesis. $VEGF_{165}$ is a soluble heparin binding protein, and is thus less diffusible than $VEGF_{121}$, but it exhibits higher mitogenic activity. On the other hand $VEGF_{145}$ induces EC proliferation and angiogenesis *in vivo* [5].

From the beginning, researchers realized the clinical relevance of those factors implicated in the angiogenic process. In fact, the role of anti-angiogenic strategies to treat human cancer was proposed by Folkman in 1971, 18 years before VEGF was discovered [8]. On the other hand, pro-angiogenic activity also appeared promising as an innovative therapeutic approach in ischemic disorders such as myocardial ischemia. As VEGF is a secreted protein with EC selective activity it was proposed from the first moment as a promising means of regulating angiogenesis.

VEGF in anti-angiogenic therapy

Only 4 years were necessary to develop a successful strategy against human cancer based on VEGF's known involvement in vasculogenesis. The proof of this concept was carried out by Ferrara's group [9]. They administered an anti-VEGF monoclonal antibody into nude mice that had been previously injected with different human tumor cell lines. The treatment was successful in inhibiting tumor growth. This effect was associated with a diminished vessel density in the treated tumors. Studies continued and in 1997 the first humanized anti-VEGF monoclonal antibody was developed. Approved in 2004 by the Food and Drug Administration (FDA) for its use in patients with previously untreated metastatic colorectal carcinoma, this antibody, named Bevacizumab [10], is nowadays used in several diseases, namely breast cancer and macular degeneration, among others.

Another strategy that has been explored is the use of small molecule VEGF receptor tyrosine kinase inhibitors. In 2005 Sorafenib received the FDA approval for its use in patients with advanced renal cell carcinoma [11]. In 2007 approval was extended for the treatment of patients with unresectable hepatocellular carcinoma. In the meantime, in 2006 another molecule with the same mechanism (Sunitinib malate) was approved for the treatment of advanced (metastatic) renal cell carcinoma and for the treatment of gastrointestinal stromal tumor after disease progression, or intolerance to the treatment of choice [12].

VEGF in pro-angiogenic therapy

To study the role of VEGF in the formation of new vasculature it is important to identify different processes in vessel formation [13]. Vasculogenesis takes place during mammalian embryo development. It consists of the formation of *de novo* vessels by differentiation of angioblasts into ECs. Sprouting during angiogenesis is the subsequent process, which ensures the expansion of the vessel network. Arteriogenesis involves the covering of EC channels by pericytes or vascular smooth muscle cells. Besides these steps, other mechanisms can occur, such as intussusception of pre-existing vessels or recruitment of bone marrow derived cells and endothelial progenitor cells that are incorporated into the endothelial lining in a process known as postnatal vasculogenesis [14]. In all these processes, VEGF is present and plays a critical role [15].

In the adult organism, quiescent vessels are constituted by quiescent ECs and pericytes. These suppress EC proliferation and release cell survival signals like VEGF. When a hypoxic stimulus activates quiescent vessels, pericytes are detached from the vessel wall. Matrix metalloproteinases (MMPs) start proteolytic degradation and pericytes are released from the basement membrane; ECs lose their junctions, allowing vessels to dilate. VEGF acts at this point by increasing the permeability of the EC layer, and plasma protein flows out establishing an ECM scaffold. Following integrin signaling, ECs migrate onto this ECM surface. Angiogenic mediators of the ECM such as VEGF and fibroblast growth factor (FGF) are released by proteinases. These factors are implicated in the constitution of the ECM as an appropriate angiogenic environment. After these steps, a cell is selected to lead vessel enlargement. VEGF gradient, regulated by soluble and matrix bound isoforms, makes tip cells upregulate delta-like ligand 4 (DLL4) expression, activating NOTCH in stalk cells, then downregulating

VEGF receptors. As a consequence, stalk cells are less responsive to VEGF, helping the tip cells to take the lead. Tip cells respond to stimuli and move towards the angiogenic signal. Stalk cells, on the other hand, elongate the stalk by division and establish the vessel lumen [14].

The complexity of this vasculogenic process has not always been as clear as it is now, but the importance of VEGF in the neovascularization process has been evident since it was first discovered. In the last two decades, research to find a VEGF-based therapy to treat tissues damaged due to hypoxia has concluded in various clinical trials. In the next section, an overview of the clinical trials performed in the context of cardiovascular repair will be discussed.

Clinical trials with VEGF: results and conclusions

The results of small phase I trials using intracoronary and intravenous infusions of VEGF in patients with coronary artery disease were encouraging [16-18]. For example, Hendel *et al.*[17] conducted a study with 14 patients who underwent exercise and myocardial perfusion measurements before as well as 30 and 60 days after VEGF administration. Although it was not designed to demonstrate VEGF efficacy, the study showed a significant improvement in exercise capacity without any safety issues. Also, the resting nuclear myocardial perfusion scans indicated a VEGF treatment effect. In another small study, a dose escalation trial was designed to determine the safety and tolerability of intracoronary VEGF infusions in 15 patients with underperfused myocardium. As a result, myocardial perfusion imaging was improved in 7 out of 14 patients at 60 days and all 7 patients with follow-up angiograms had improvements in the collateral density score. This study also established that VEGF can safely and tolerably be administered to humans by intracoronary infusion for up to 20 minutes at 0.050 μg/kg/min [19]. In a study employing intravenous administration of VEGF in 28 patients, the authors reported evidence of improvement in rest myocardial perfusion and in collateral density [16]. In spite of the promising results of these small phase I trials, a randomized, double-blind, place- bocontrolled phase II trial failed to show differences between the VEGF and placebo groups. The VIVA (Vascular endothelial growth factor in Ischemia for Vascular Angiogenesis) study compared two doses of VEGF to placebo in 178 patients with coronary artery disease. A single intracoronary infusion followed by three separate intravenous infusions was given. Despite the safety and tolerability, the administration regimes revealed that VEGF offered no improvement beyond placebo by day 60, although high-dose VEGF resulted in better improvement in angina and favorable trends in exercise treadmill test time and angina frequency, by day 120 [20].

The results of myocardial clinical trials using VEGF delivery have generally been disappointing and the studies have failed to consistently demonstrate improvements in treated patients as compared with placebo. Many of these trials relied on an intravenous infusion or intracoronary delivery of the recombinant protein. It is noteworthy that VEGF is not effective when delivered intravenously [21]. Therefore, these negative results have been attributed, at least partially, to the short-lived effect and high instability of the protein when injected as a bolus. Intravenous administration of VEGF is limited by its short *in vivo* half life (~30 min) and overall dose is limited by off-target site toxicity issues [18]. In the case of myocardial ischemia, the amount of VEGF localized in the ischemic region after systemic administration is minimal and does not persist for more than 1 day [22]. Based on these issues, some unusual characteristics of the VIVA trial make interpretation of therapeutic efficacy of VEGF somewhat difficult; in particular, suboptimal dose or route of administration and uncontrolled delivery method of VEGF. Perhaps the most striking contribution of the VIVA trial was to consider that more preclinical data were needed with regard to the time course of angiogenesis and the optimal dose and route of administration to induce effective VEGF therapy in the myocardium. Also, given that the low recovery in the myocardium of the administered VEGF might be another important cause of the missing clinical effect, local and sustained VEGF delivery by controlled release approaches in the heart tissue might be a better strategy to achieve higher efficacy in VEGF-based therapy for myocardial ischemia. Table 1 summarizes the main clinical trials using VEGF recombinant protein for cardiac repair.

DELIVERY SYSTEMS

Protein delivery systems

When administering drugs to an organism, the goal is to reach the appropriate dose at the site of action for the necessary period of time, so that the drug acts in its optimal condition, with the minimum adverse effects. Drug delivery systems (DDS) are designed taking into account the specificities of the drug to be administered, the organism in which it is administered and the disease being treated. Depending on these conditions, release profiles are designed and materials and device architecture are chosen. Even though the concept of drug delivery is relatively old,

its application to biomolecules, such as proteins, hormones, antibodies or genes, has been explored only in the last 15-20 years.

Table 1. Clinical trials using VEGF recombinant proteins for cardiac repair.

Route	Trial	n	Outcomes	Reference
Intracoronary	Phase I	14	Some improvement in perfusion in patients treated with low-dose VEGF; five of six patients had perfusion improvement at rest and stress at higher doses	[17]
Intracoronary	Phase I	15	Dose screening study; well tolerated up to 0.05 mg/kg/min; myocardial perfusion imaging was improved in 7 out of 14 patients at 60 days	[18]
Intravenous	Phase I	28	An increase in exercise capacity was reported without any safety issues	[16]
Intracoronary/ intravenous	Phase II	178	VIVA study; safe and well tolerated; no improvement beyond placebo in all measurements by day 60. By day 120, high-dose VEGF resulted in significant improvement in angina; no improvements in myocardial perfusion	[20]

As scientists make progress in their understanding of the mechanisms of disease, there is increasing awareness that protein delivery is a very attractive approach in many diseases. In our context, as clinical trials have highlighted, promoting angiogenesis via growth factors (GFs) or cytokines demands the use of a DDS that preserves their properties and prolongs their short half life [23]. Proteins are labile systems with primary, secondary, tertiary and quaternary structures. Primary structure relates to the amino acid sequence, whereas higher structures refer to protein fold. Chemical instability is due to modification at the first level, such as deamidation, oxidation, beta elimination, incorrect disulfide formation or racemization. Physical instability refers to secondary, tertiary and quaternary structure, with phenomena like denaturation, aggregation, precipitation and surface adsorption. All these instability issues result in the short half life of VEGF when administered directly in the tissue. Incorporating VEGF in an adequate carrier serves to protect it until it is released from the device. However, incorporating VEGF, and any protein, in an adequate carrier poses unique difficulties due to physical and chemical instability. Temperature, pH, pressure, the presence of metal ions and denaturing agents like surfactants need to be tightly controlled during the manufacturing process to avoid chemical instability.

The first marketed protein included in a DDS was the luteinizing hormone releasing hormone (LHRH), which was commercialized by Astra Zeneca as Zoladex, with FDA approval in 1989 for the treatment of prostate cancer. It was a 1.5 mm sized subcutaneous implant prepared with the copolymer polylactide-co-glycolide (PLGA). Nowadays other protein formulations have come on the market: long release octreotide (Octreotide® LAR®, Novartis Pharmaceuticals), human growth hormone (Nutropin de-

To achieve sustained presence of VEGF in the damaged tissue, both gene therapy and DDS have been designed. However, the aim of this review is focused on the second option. Updated information about gene therapy in this field can be found in the bibliography [24-27].

Studies published in the last five years related to VEGF delivery systems and cardiovascular diseases have been reviewed. Those corresponding to the last two years are summarized in Table 2. During this period of time, two systems have been the most widely employed: scaffolds and particulated constructs, represented in Figure 1.

Scaffolds

Most authors propose the use of scaffolds as delivery platforms. In general, the requirements that scaffolds must fulfill are several [28]: adequate 3D architecture with the desired shape, volume and mechanical strength; highly porous structure to allow tissue growth and diffusion of biomolecules; biocompability; appropriate degradation rate; and good interface adherence so that proteins attach in a desired way to the material. Particularly, basic physical requirements for myocardial engineered constructs are robust yet flexible mechanical properties, contractile ability, and electrophysiological stability [28, 29]. The physical and biochemical effects of scaffolds in cardiovascular differentiation have been reviewed elsewhere [30]. Moreover, a scaffold designed for protein delivery needs to show high loading capacity, homogenous protein distribution, a protein binding affinity that allows adequate protein release and, importantly, ability to maintain protein stability and bioactivity.

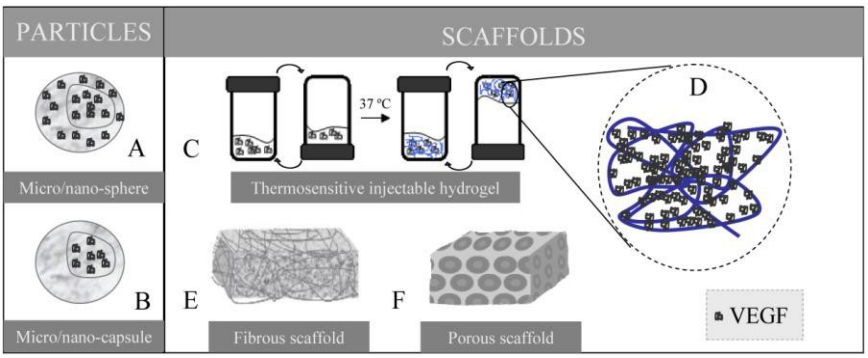

Figure 1. The main DDSs employed in recent years in the field of VEGF and cardiac repair are represented. In the particles (nano or microsized) VEGF can be included homogenously through the entire particle (A) or only in the core of the system (B), which affects the release profile. A wide variety of scaffolds has been designed. In a thermosensitive injectable hydrogel (C) at temperatures under 37 °C the blend of the vehicle and the VEGF remains liquid, but when it is injected and reaches the body temperature the mixture solidifies and VEGF is entrapped within the gel matrix (D). By electrospinning fibrous scaffolds are built up (E) whereas porous scaffolds (F) can be formed by solvent casting and particulate leaching method.

Table 2. Summary of VEGF delivery and cardiac repair in the last 2 years.

	DDS	COMPOSITION	ANGIOGENIC FACTOR/S	PREPARATION METHOD	ANGIOGNENIC EFFICACY ASSAY	AUTHOR'S CONCLUSION	REF.
SCAFFOLD	surface cross-linked Heparin	polycaprolactone	VEGF	Solvent casting and particulate leaching method	Subcutaneous implant model in mice	Modification of the scaffold with heparin improves VEGF efficacy	[37]
	hollow-fiber membrane	cellulose acetate	VEGF & S1P	Double injection extrusion/ precipitation method	Subcutaneous implant model in mice	System capable of exploring sequential delivery of angiogenic factors. Sequential delivery of VEGF followed by S1P resulted in recruitment of more ECs and higher maturation index	[43]
	biomimetic hydrogel (adhesion peptide sequence RGDS)	PEG diacrylate	VEGF	Photopolymerization	*In vitro (HUVECs and hMECs)*	The system promotes EC proliferation, migration and viability maintenance	[44]
	patch with covalently immobilized VEGF	collagen	VEGF	Commercial scaffold	Right ventricular free wall resection and replacement with the scaffold	Collagen scaffold with covalently immobilized VEGF improved tissue formation	[32]
	hydrogel with surface cross-linked Heparin	star-PEG	VEGF & FGF-2	Cross linking	*In vitro* (HUVECs) and Chicken chorioallantoin membrane angiogénesis assay	Angiogenic activity superior to the administration of single factors	[45, 46]
	hydrogel	alginate	VEGF	Cross linking	Hindlimb ischemia in mice		[47]
	covalently immobilized factors	collagen	VEGF & Ang-1	Commercial scaffold	Chicken chorioallantoin membrane angiogenesis assay	Scaffolds with co-immobilized VEGF and Ang-1 further improved angiogenesis as compared to independently immobilized VEGF or Ang-1	[31]
	composite scaffold	Poly(ether)urethane-polydimethylsi	VEGF & bFGF	Spray-phase inversion method	Subcutaneous implant and uni-	Incorporation of VEGF, bFGF and heparin in the composite scaffold	[38]

		loxane -fibrin			lateral hind limb ischemia model in rat	enhances angiogenesis	
	temperature sensitive injectable hydrogel	PVL-b-PEG-b-PVL	VEGF	Metal-free cationic method	Myocardial infarction rat model (coronary artery ligation)	The system preserved ventricular function by stabilizing the infarct and reducing angiogenesis	[41]
	fibrous membranes	Dextran/PLGA	VEGF	Coaxial electrospinning	*In vitro*	The system positively promotes cell proliferation	[48]
	dual layered scaffold combined with an osmotic release mechanism	Poly(trimethylene carbonate)	VEGF & HGF	Cross linking	*In vitro (HAECs)*	System able to release combined GFs at similar rates, and at controllable sequences	[49]
PARTICLES	NPs	Hyaluronic acid/chitosan	VEGF & PDGF-BB	Ionic gelification technique	-	NPs entrap efficiently both factors. PDGF-BB is released in a sustained manner over 1 week and VEGF within the first 24 hours.	[50]
		PLGA/heparin/fibrin	VEGF	Spontaneous emulsion solvent diffusion method	Rabbit ischemic hind limb	The system strongly increases the *in vivo* therapeutic angiogenic effects of VEGF	[51]
		PLGA	VEGF	Modification of the double emulsion method	Hindlimb ischemia in mice	Feasibility of the system to produce a more viogorous revascularization when compared with free VEGF administration	[52]
	MPs	PLGA	VEGF	Double emulsion/solvent evaporation method	*In vitro (HUVECs)*	The system allows VEGF encapsulation and bioactive protein release up to 21 days	[53]
		PLGA	VEGF	Double emulsion/solvent evaporation method	Myocardial infarction rat model (coronary artery ligation)		[54]
		Collagen	VEGF	Cross linking	*In vitro (HUVECs)*	The system allows VEGF encapsulation and bioactive protein release up to 4 weeks	[55]
PARTICLES-SCAFFOLD	MPs MPs-Scaffold	PLGA (MPs)PLGA-N-methyl pyrrolidone (scaffold)	VEGF	MPs: spray dry Scaffold: gelification	Murine model of peripheral angiogenesis	Both formulations provide a method to incite neovascularization from a single injection	[56]
	NPs Scaffolds (hydrogel or polymeric) NPs-Scaffold (hydrogel or polymeric)	NPs: Dextran-sulfate/chitosan Scaffold: Matrigel® or PLGA	VEGF	NPs: complex formation and coacervation Scaffold: gas foaming/particulate leaching method (Matrigel®: commercially obtained)	Subcutaneous injection (NPs-Matrigel®) NPs-PLGA scaffolds implanted into the intraperioneal fat pad of mice	Angiogenesis was clearly improved by VEGF encapsulation and further incorporation into implants, compared to direct VEGF incorporation into implants	[57]
	MPs-Scaffold co-administered with ECs	Alginate MPs collagen/fibronectin gel	VEGF & MCP-1	Cross linking	Subcutaneous implant model in mice	Delivery of multiple therapeutic proteins to enhance the efficacy of cell-based vascularization	[58]
OTHER	Polymeric injectable carrier	Poly(trimethylene carbonate)	VEGF	-	Subcutaneous injection in rat	The approach has potential for providing effective, local, bioactive growth factor delivery.	[59]
	Collagen patches	Collagen	VEGF	Collagen binding domain is fused to VEGF	Myocardial implantation	Patches improve left ventricular cardiac function and increase the vascular density	[60]

These devices can be constructed with natural or synthetic biomaterials. In the past the use of non-degradable constructs involved a second intervention to extract these. Nowadays the use of biodegradable devices is almost presupposed. Components found in the ECM are preferably from within the range of natural materials: collagen, fibrinogen, hyaluronic acid (HA), chitosan, alginate, etc. The use of these natural scaffolds has increased during recent years and companies have already put them on the market. In the last two years, Radisic *et al* have published two papers in which they immobilize VEGF in a commercial ultrafoam [31, 32], demonstrating that the system has suitable mechanical properties for potential use in repairing heart defects and that it improves angiogenesis both *in vitro* [31] and *in vivo*, in a rat heart injury model [32]. Because of their natural origin, most of these components are biocompatible, bioactive and tend to show similar mechanical properties to the native tissue. However, synthetic materials have been developed with optimal qualities for tissue regeneration. Elastomeric scaffolds such as poly (glycolic acid) (PGA) and poly (lactid acid) (PLA), as well as their copolymer PLGA, have been successfully applied [33-35]. Like natural materials, polyesters are biocompatible and biodegradable, and by controlling polymerization it is possible to modulate their mechanical properties. In fact, PLGA has already received FDA approval for its use in drug delivery [36] Also polyanhydrides and other polyesters, such as polycaprolactone (PCL) [37], have been shown to be possible alternatives as VEGF carriers. In recent years, different materials have been combined to modulate the mechanical and biological properties of the scaffold. To give an example, fibrin-based biomaterials have been found to stimulate and support the growth of new blood vessels, but their poor mechanical properties have encouraged researchers to combine them with synthetic materials. Losi *et al* [38] have obtained composite scaffolds made of a synthetic layer of poly(ether)urethane-polydimethylsiloxane, contributing to mechanical resistance, and a fibrin layer that acts as a GF delivery carrier.

Numerous preparation methods have been reported for manufacturing 3D porous scaffolds, namely fiber bonding, emulsion freeze drying, solvent casting/particulate leaching [37], high pressure processing, gas foaming/particulate leaching, thermally induced phase separation, electrospinning [39] and rapid prototyping (reviewed in [40]). Recently, injectable biomaterials that form scaffold *in situ* have been used. These can be administered parenterally, but also locally, with the advantage that the scaffold acquires the shape of the tissue defect and surgery is avoided. Recently, Wu *et al* [41], prepared a VEGF-conjugated injectable hydrogel that was intramyocardially injected into Sprague Dawley rat infarcted hearts, preserving ventricular function after myocardial ischemia by stabilizing the infarct and inducing angiogenesis. The gel was composed of a temperature sensitive aliphatic polyester (poly (δ-valerolactone)-block-ply (ethylene glycol)-block-poly (δ-valerolactone)). This polymer is called thermosensitive since it dissolves in water at room temperature, but gels at 37 ^{o}C (see Fig. 1C). This property makes this kind of material especially interesting.

The route of administration to reach the infarcted heart still remains challenging (see Figure 2) [42]. When implanting a scaffold in the heart, surgery is necessary, with the consequent inconvenience and risks for the patient. The scaffold in that case needs to be placed covering the infarcted zone, or surrounding it, so that GFs are released towards the cells acting in the repair process and are responsive to VEGF (Fig. 1A). If an injectable gel is used, surgery may be avoided, since it can be directly injected into the heart without surgical intervention (Fig. 1C).

To optimize scaffold effectiveness, it can be surface modified. Collagen and gelatin are known to modulate adhesive properties. The attachment of a cell adhesive peptide on the surface improves ligand-receptor interaction, as well as cell adhesion. In a recent article, Porter *et al* [44] propose the preparation of a biomimetic hydrogel by including an adhesion peptide sequence derived from fibronectin (RGDS), in a VEGF covalently incorporated PEG hydrogel, to enhance cell adhesion. However, in this study only *in vitro* assays were performed. Hyaluronic acid (HA) is a substance present in the ECM that interacts with CD4 receptor, promoting wound healing, making its use attractive. Heparin modification has also been extensively studied for the release of GFs. Heparin is a highly sulfated glycosaminoglycan in the ECM, and is known for its specific interactions with various angiogenic GFs [61, 62]. A wide variety of scaffolds including nanofibers, prepared from collagen, fibrin, chitosan, alginate, PLA and PLGA, have been immobilized with heparin to achieve sustained release of GFs [40, 63]. Based on this, Zieris *et al* [37, 45] prepared a biohybrid hydrogel with star-shaped PEG and carbodiimide/N-hydroxysulfosuccinimiede-activated heparin, obtaining appropriate VEGF binding and release, with good *in vitro* results (included in Table 2). On the other hand, Singh *et al* [37] demonstrated an enhanced VEGF angiogenic effect, in a subcutaneous implant model, when a PCL scaffold was surface

modified with heparin.

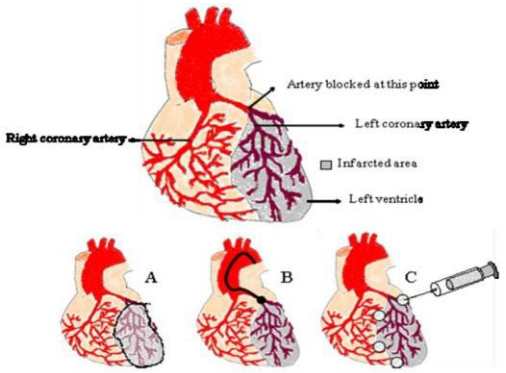

Figure 2. Depending on the VEGF carrier different routes of administration can be chosen. Solid scaffolds need to be attached to the heart, frequently covering the infarcted area (A). Particles can be injected in the desired zone intracoronarily using a catheter (B) or by direct injection (C). Injectable scaffolds can also be implanted using this route.

Nano/Microparticles

Among the particulated delivery systems MPs (>1 μm and < 1,000 μm sized) and NPs (< 1 μm sized) have been used for VEGF delivery. Microsized particles are not readily internalized by the cells, but they have the potential to provide sustained release kinetics after implantation. In addition, MP diffusion from the implantation site is infrequent, which avoids undesired effects in other tissues. Thus, MPs provide control over the release rate and dose, yielding desirable concentrations for a period of time [64]. On the other hand, NPs can penetrate through capillaries into the cell machinery. Therefore, the pro-angiogenic potential of GFs in NPs or MPs in the tissue is not necessary equivalent. In relation to drug distribution within the particle, we can distinguish between nano/microcapsules and nano/microspheres (see Fig. 1A and 1B). A clear advantage of these DDS, when compared with scaffolds, is the route of administration. As it has been mentioned before, most of the scaffolds need to be implanted through a surgical intervention. In the case of the particles, they can be injected directly into the myocardium without surgery. Frequently, several injections are performed around the infarcted area, in the confluence of the healthy and the damaged tissue (see Fig. 2C). Another non-invasive route for these DDS is the intracoronary route via a catheter (see Fig. 2B).

To prepare these DDSs, numerous strategies have been employed, among others, solvent extraction/evaporation, spray drying and phase separation method [65]. Spray drying is associated with low process efficacy and protein denaturing due to dehydration [66]. The phase separation method is mediated by a solvent and coacervating agent that can react with the protein and disrupt the secondary structure. Moreover, this is a process that is not well suited to producing particles in the low micrometer size range [67]. The solvent extraction/evaporation method is thus thought to be, to date, the most appropriate. Two different modifications of this procedure are the single emulsion and the double emulsion solvent evaporation method. In the first one, since proteins are hydrophobic molecules, low encapsulation efficiency (EE) values are obtained. EE is an important feature and can be defined as the capability of immobilizing most of the protein added during the process. In the single emulsion case, protein is incorporated in the external phase of the emulsion (o/w), whereas in the double emulsion the protein is localized in the internal aqueous phase (w/o/w), diminishing the loss of the molecule. Nevertheless, this method presents some drawbacks, such as the need to reach high temperatures or to employ mechanical forces that can damage the protein. Systems to avoid elevated temperatures and stirring during the emulsion preparation have now been designed. An example is the Total Recirculation One Machine System® (TROMS) [68]. In the TROMS, the inner aqueous phase of the emulsion is injected into the organic phase. This blend is forced to circulate through a closed circuit with a specified inner diameter for a period of time. Once the first emulsion is formed, it is injected into the external aqueous phase leading, after a period of time in circulation, to the constitution of the double emulsion without inflicting too much stress on the protein so that bioactivity is preserved. Employing TROMS we have been able to encapsulate VEGF and other proteins in PLGA microparticles with EE values over 80% while maintaining protein bioactivity during the formulation process [54, 69]. On the other hand, the formation of aqueous/organic solvent interfaces is the main disadvantage of this method. Also, when using PLGA copolymer, hydrophobic interactions between the protein and PLGA are responsible for protein aggregation and denaturing. To minimize these effects, addition of human serum albumin (HSA) or surfactants such as poly ethylene glycol (PEG), helps to protect the protein by preventing hydrophobic contacts [70]. Another important factor to take into account is pH modification. When PLGA degrades, pH decreases due to the presence of

glycolic and lactic acid [71], and this is associated with a loss of bioactivity as a consequence of protein aggregation and chemical degradation. It may also be possible that PLGA degradation products and proteins react, leading, for example, to protein acetylation [71]. Therefore approaches to reduce acid-induced damage have been discussed, such as incorporating Mg(OH)2 in the formulation [72].

PLGA particles with VEGF have been studied extensively for ischemia tissue repair [52-54] and they appear to be a promising strategy. Guldberg *et al* [52] demonstrated a vigorous revascularization response when treating a hindlimb ischemia model in mice with VEGF-NPs, which was greater than the response obtained after 5 µg VEGF, 2.5 µg VEGF, and saline treatment. VEGF-MPs were also prepared by Baysal *et al* [53] to face therapeutic angiogenesis. In this study, MPs succeeded in promoting HUVEC proliferation and migration. Our group developed VEGF loaded PLGA MPs which, when intramyocardially injected in an infarcted heart rat model, improved vasculogenesis and tissue remodeling [54].

Particles included in scaffolds

More sophisticated systems can be prepared by incorporating protein loaded particles into scaffolds. These kinds of system present some features that can be of interest in some situations. Firstly, the release profile is modified (Fig. 3). In short, the burst effect is maintained while the sustained release of the protein is prolonged [64]. However, possibilities are unlimited, since the GF can be included both in the particles and in the scaffold, or just inside the particles. Also, GFs can be covalently immobilized in the carriers or non-covalently, leading to different delivery. Moreover, the scaffold architecture can modulate different release profiles, depending on whether it is a multilayered system or a core-shell system. All these alternatives, and others, have been recently reviewed by Chen *et al* [64].

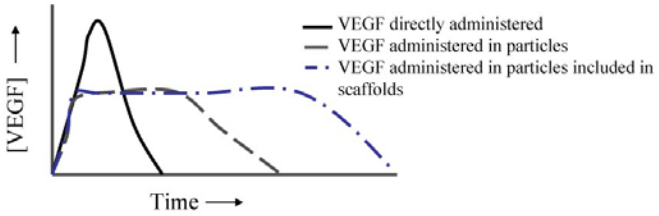

Figure 3. Simplification of how VEGF release profile can be modified when included in particles or in particles embedded in scaffolds.

An elegant construct has been described by Chung *et al* [51]. They compare the angiogenesis potential of VEGF when included in a fibrin gel or when included in a heparin-functionalized nanoparticle incorporated in the same fibrin gel. In the first case, almost 100% of VEGF was released within the first 3 days, whereas sustained release of the protein was observed for more than 30 days in the second approach, resulting in an enhanced angiogenic effect. Recently, des Rieux *et al* [57] investigated whether angiogenesis is enhanced when administering VEGF that is freely incorporated in two types of matrices (Matrigel® or PLGA) or when VEGF is previously encapsulated in dextran-chitosan nanoparticles. The results lead these authors to conclude that a more interesting approach could be to fill the PLGA scaffold pores with the hydrogel, both systems being loaded with VEGF nanoparticles, and thus combine three carrier systems.

Combination of VEGF with other biomolecules

When trying to regenerate heart tissue, the combination of several factors (pro-angiogenic, cardioprotective and chemoattracting) could be a good strategy [73]. As described above, a hypoxic stimulus in the heart activates not only VEGF and neovascularization, but complex and tightly spa- tiotemporally coordinated pathways, involving different cells and inter-regulated factors. DDSs appear in this context not only as the way to prolong the short half life of all these biomolecules, but also as a suitable platform to mimic the optimum environment for the tissue to regenerate, by delivering various GFs in a different but controlled manner. Work in this field has increased in recent years. VEGF has been incorporated into carriers together with other factors; the next step is to prepare systems that allow a dual or sequential delivery of factors with a tight dose control [46]. Recently Chapanian and Amsden [49] have been

able to create a system for the combined and sequential delivery of VEGF and hepatocyte growth factor. The construct is composed of trimethylene carbonate based elastomers and is combined with an osmotic mechanism to release acid-sensitive GFs, preserving its bioavility. Another approach is the combination of VEGF with Angiopoietin-1, another GF implicated in vascular stabilization and remodeling [31]. These were covalently immobilized in collagen scaffolds and a chicken chorioallantoic membrane angiogenesis assay was performed to test the system. The results led these authors to conclude that scaffolds with co-immobilized VEGF and Ang1 further improved angiogenesis, as compared to independently immobilized VEGF or Ang1. Also, covalent immobilization of growth factors on the scaffolds yielded better results compared to simple addition of soluble growth factors to scaffolds (Table 2). Other factors combined with VEGF have been FGF-2 in PEG based hydrogels [45, 46], PDGF-BB in alginate hydrogels [74] and in HA/chitosan NPs [50], bFGF in a composite scaffold [38] and sphingosine 1-phosphate [43]. The work of Hao *et al* [74] is particularly worth mentioning. While the majority of the studies have just proved the ability of the system to incorporate GFs and to deliver them, in this case the system was applied to a myocardial infarction rat model, with the formation of more mature vessels and improvement of cardiac function when compared to delivery of single factors [17, 74]. Despite these results, it is still necessary to answer the question as to which the appropriate VEGF co-factor/s are and what the ideal release profile for each one is.

Application of Drug delivery systems in the ischemic heart

To sum up, it is possible to conclude that each delivery system has its own advantages and limitations. As mentioned before, the administration route is a key point to take into account. Several methods of delivery are used (Figure 2.) depending on the pathology of the patient. In the case of chronic myocardial ischemia, direct intramyocardial injection is preferred, since it allows delivery in the ischemic tissue with an occluded artery [75]**.** Direct intramyocardial injection can be trans-epicardial, trans-endocardial, and less frequently, trans-venous. The first is usually performed during open heart surgery, and due to the high risk, is not performed as a standalone procedure. Another context is an acute myocardial infarction, in which intracoronary injection is the most frequent method [76].

On balance, solid scaffolds present the disadvantage of needing to be implanted in the heart, making surgery mandatory. Solid scaffolds would therefore not be an adequate DDS to treat acute myocardial ischemia, or for chronic myocardial ischemia when the patient requires open heart surgery.

The administration route is not the only feature to be taken into account when choosing a DDS. For instance, scaffolds have the drawback of the administration route but they show the benefit of covering the total area of the infarct. In consequence, if they are built with materials able to reproduce the extracellular matrix, they can help the heart to overcome the consequences of the negative remodeling [77]. Furthermore, if the aim is to administer more than one factor with different release profiles, the scaffold elaboration process becomes more complicated, as it is necessary to include in it, for instance, particles that modify the release rate of one or more of the active molecules.

On the other hand, use of particles makes all the administration routes possible. It is also easier to combine different particles containing several factors, theoretically providing a more orchestrated signaling environment to the damage tissue.

In relation to the possibility of preparing an engineered construct including factors, DDS and cells, both scaffold and MPs are potentially useful, whereas the reduced area of NPs does not provide enough space to attach cells to their surface. Sustained release of the factors is also difficult to achieve when administering NPs, not to mention their greater likelihood of being removed from the tissue by phagocytic uptake.

Most of the published studies using VEGF loaded in DDSs carried out limited *in vivo* studies, and just a few of them have applied VEGF-DDS in the ischemic heart. Thus, it is still too soon to conclude which DDS is the most suitable to apply in cardiac repair.

Therefore, as all DDS present advantages and disadvantages, combining them to prepare more sophisticated constructs could make it possible to exploit the benefits of each one in order to give the optimal signals to the cardiac tissue that needs to regenerate.

FUTURE DIRECTIONS: THE TISSUE ENGINEERING TRIAD

As the same time as the number of papers in which GFs and DDSs are combined has increased, numerous researchers have focused on cell therapy to treat cardiac injury[42]. Just as DDSs appear in the first case as a suitable approach for prolonging the permanence of GFs in the tissue, the cell engraftment approach may well be extended by including cells in scaffolds. So far, these scaffolds have progressed to

mimic the cell's natural environment as far as possible, aiding in its survival and development. In this sense, the incorporation of factors in the scaffold helps not only to modulate cell life, but also acts in the tissue and improves its recovery. The combination of engineered platforms, cells and GFs has been named the tissue engineering triad, and seems to be a promising tool in the field of cardiac repair. A considerable research effort is now needed to establish the "winning combination".

Acknowledgments

We gratefully acknowledge support from the Spanish Ministry of Science and Innovation PSE SINBAD (PSS 0100000-2008-1), Caja Navarra Foundation (Programa Tu Eliges: Tu Decides), Spanish Ministry of Sciences and Education and Agencia Española de Cooperación Internacional para el Desarrollo (AECID).

Competing Interests

The authors have declared that no competing interest exists.

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ANNEX III

BIODEGRADATION AND HEART RETENTION OF POLYMERIC MICROPARTICLES IN A RAT MODEL OF MYOCARDIAL ISCHEMIA

ANNEX IV

ANGIOGENIC THERAPY FOR CARDIAC REPAIR BASED ON PROTEIN DELIVERY SYSTEMS

Formiga FR, Tamayo E, Simon-Yarza T, Pelacho B, Prosper F, Blanco-Prieto MJ. Angiogenic therapy for cardiac repair based on protein delivery systems. Heart Fail Rev 2012 May;17(3):449-473

http://dx.doi.org/10.1007/s10741-011-9285-8

http://hdl.handle.net/10171/29331