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NEW STRATEGIES FOR CARDIAC REPAIR USING POLYMER-BASED DRUG DELIVERY SYSTEMS LOADED WITH GROWTH FACTORS

Trabajo presentado por Simón Pascual Gil de Gómez

para obtener el grado de Doctor

Fdo. Simón Pascual Gil de Gómez

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Certifican:

Que el presente trabajo, titulado "New strategies for cardiac repair using polymer-based drug delivery systems loaded with growth factors", presentado por **D. SIMÓN PASCUAL GIL DE GÓMEZ** para obtener el grado de Doctor, ha sido realizado bajo su dirección en el Departamento de Farmacia y Tecnología Farmacéutica y en el Área de Terapia Celular del Centro de Investigación Médica Aplicada (CIMA). Revisado finalmente el trabajo no encuentran objeciones para que sea presentado a su lectura y defensa.

Y para que así conste, firman la presente:

Fdo.: Dra. María Blanco Prieto Fdo.: Dr. Felipe Prósper Cardoso

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Paso a paso

se asciende hasta el Parnaso,

Yo no quiero las alas de Pegaso.

Gerardo Diego

TABLE OF CONTENTS

ABBREVIATIONS

AMI	acute myocardial infarction
ADSC	adipose derived stem cell
aiMac	embryonic derived anti-inflammatory macrophages
Akt1	serine–threonine protein kinase
BMSC	bone marrow derived stem cell
bNRG-1	biotinylated neuregulin-1
BSA	bovine serum albumin
CMC	cardiomyocyte
CPC	cardiac progenitor cell
CQ10	coenzyme Q10
cTnT	cardiac troponin
DDS	drug delivery system
ECM	extracellular matrix
EF	ejection fraction
EPO	erythropoietin
EPC	endothelial progenitor cell
ErbB4	biological receptor for neuregulin 1
ESC	embryonic stem cell
FGF1	fibroblast growth factor-1
FS	fractional shortening
G-CSF	granulocyte-colony stimulating factor
GF	growth factor
GlcNAc	N-acetyl-D-glucosamine
HGF	hepatocyte growth factor
HMGB1	high-mobility group box 1

HRP	horseradish peroxidase
iCM	induced cardiomyocyte
IGF-1	insulin like growth factor 1
IL	immunoliposome
I/R	ischemia/reperfusion
iPS	induced pluripotent stem cell
LV	left ventricle
LVEF	left ventricular ejection fraction
Mac	macrophage
MI	myocardial infarction
MMP9	matrix metalloproteinase 9
MP	microparticle
MSC	mesenchymal stem cell
NF	nanofiber
NIPAM	thermo-responsive poly(N-isopropylacrylamide)
NP	nanoparticle
NRG1	neuregulin-1
PDGF	platelet-derived growth factor
PEG	poly(ethylene-glycol)
pErbB4	phosphorylated biological receptor for neuregulin 1
PGCL	poly-glycolide-co-caprolactone
PIGF	placental growth factor
piMac	bone-marrow derived pro-inflammatory macrophages
PLGA	poly (lactic-co-glycolic acid)
PS	phosphatidylserine
PU	polyurethane
PVA	poly(vinyl alcohol)

SC	stem cell
SDF-1	stromal cell-derived factor-1
SEM	scanning electron microscopy
SLX	Sialyl Lewis X molecule
SM	skeletal myoblast
SOD1	superoxide dismutase 1
TΒ4	thymosin β4
TROMS®	Total Recirculation One-Machine System®
UPy	2-ureido-4-pyrimidone
VEGF	vascular endothelial growth factor
WB	western blot

FOREWORD

- Acute myocardial infarction history

Heart problems have been associated with human beings since as long ago as the birth of writing in ancient Egypt, as reported in scroll dating from 1550 B.C. discovered by Georg Ebers in 1873. The papyrus contains information on how to detect heart failure, with references to cardiac dysfunction, fluid retention due to heart failure, patients with weak hearts and so on [1,2]. Surprisingly, there is a gap in history and no more references regarding heart diseases can be found until 400 years ago when Friedrich Hoffmann (1660–1742), chief professor of cardiology at the University of Halle (Germany), noted that coronary heart disease started with the "reduced passage of the blood within the coronary arteries" [3]. Although some interesting research concerning cardiovascular diseases was conducted later [4], it was not until 1912 that the American cardiologist James B. Herrick (1861–1954) gave the first definition of myocardial infarction (MI) and described it properly [5]. He proposed that thrombosis in the coronary artery leads to the symptoms and abnormalities of MI, mainly anemic necrosis and fibrosis of the myocardium, ultimately causing the rupture or dilatation of the heart [6].

It is generally accepted that the identification and characterization of MI was the keystone and turning point in our knowledge of heart diseases. For instance, it led to the formation of the well-known American Heart Association in 1924. During the next 100 years, great efforts were invested in the characterization and understanding of MI. The definition of MI underwent substantial revisions during this time. The latest version is the "Third Universal Definition of Myocardial Infarction", published in 2012 by the European Society of Cardiology, American College of Cardiology Foundation, American Heart Association and World Heart Federation [7]. Importantly, this document establishes that the term MI makes reference to myocardial necrosis in a clinical setting consistent with acute myocardial ischemia. When pathological findings indicate the presence of MI but there is no evidence of ischemic causes, the disorder must be named "prior MI", possibly indicating the beginning of a process that will end up as MI. Thus, the basic term MI normally refers to acute MI (AMI).

At the same time, our increasing knowledge about MI led to the evolution of treatment for this pathology. James B. Herrick originally established the importance of rest in post-infarction recovery. In fact, rest was the only therapeutic orientation existing in the first half of the 20th century [8]. Regarding surgical interventions, the first reported coronary artery bypass graft in humans using an internal mammary artery was performed by Robert Goetz in 1960 [9]. The first heart transplant in humans took place in Cape Town, South Africa, by the group led by the South African surgeon Christiaan Barnard in 1967 [10]. Some years later, in 1977, Gruntzig and Myler carried out the first coronary angioplasty during coronary artery bypass graft surgery

[11]. The 1980s marked the beginning of the "Pharmacologic Era", which was heralded by the first Vasodilator Heart Failure Trial [12]. During this time, digitalis, diuretics, vasodilators and inotropes played a prominent role [13]. Angiotensin-converting enzyme inhibitors, beta blockers, and spironolactone (for the treatment of advanced heart failure) were introduced in the 1990s, and significantly improved patient outcomes, altering the natural history of the disease progression [14,15].

As a result of these advances, a diagnosis of heart disease today is no longer necessarily a death sentence, and patients' quality of life has improved notably. Nevertheless, we are still far from completely erasing this disease from human history. In fact, MI represents the main cause of death and disability worldwide [16], causing 7.4 million deaths in 2015 [17]. Unfortunately, risk factors associated with MI such as alcohol, insufficient physical activity, obesity, and diabetes are increasing considerably in modern society. Consequently, it has been estimated that the number of MI events will rise in the next few years [16].

- Protein therapy and delivery systems

Current interventions for AMI (bypass, stents and pharmacological treatments) are palliative, which means that they stabilize the infarcted area already formed, preventing its expansion [18]. However, loss of heart functionality remains an issue, and patients who have had a MI normally have to face important functional limitations for the rest of their lives. In the end, the only real solution to MI is heart transplant, but there is an obvious lack of healthy donors, not to mention immune rejection considerations. Therefore, taking into account the current clinical relevance of MI, it is imperative to develop new and more efficient treatments with the aim of recovering heart contractility and function after MI.

One such treatment is protein therapy, which is based on the administration of therapeutic proteins (generally referred to as growth factors (GFs) or cytokines) [19]. GFs trigger and control a variety of biological processes responsible for the final cardioreparative action (Figure 1). Nowadays, a long list of GFs have been tested as cardioreparative and/or cardioprotective drugs [18], but particular attention must be paid to neuregulin-1 (NRG1). NRG1 induces heart regeneration in zebrafish, and positive heart remodeling in mammalian hearts [20]. These effects are due to the fact that NRG1 improves embryonic stem cell differentiation into the cardiac lineage, stem cell survival [21], cardiomyocyte (CMC) proliferation, growth of new coronary vessels [22] and cardiac myofiber trabeculation [23]. In fact, NRG1 and its biological receptors ErbB2 and ErbB4 have proved to be essential in heart development during the neonatal stage [24,25]. Another cytokine of interest is acidic fibroblast growth factor (FGF1). FGF1 regulates cardiac remodeling

by exerting a protective and proliferative effect after MI via induction of cardiomyocyte mitosis, angiogenesis, scarring reduction and decrease of cardiomyocyte apoptosis [26,27].

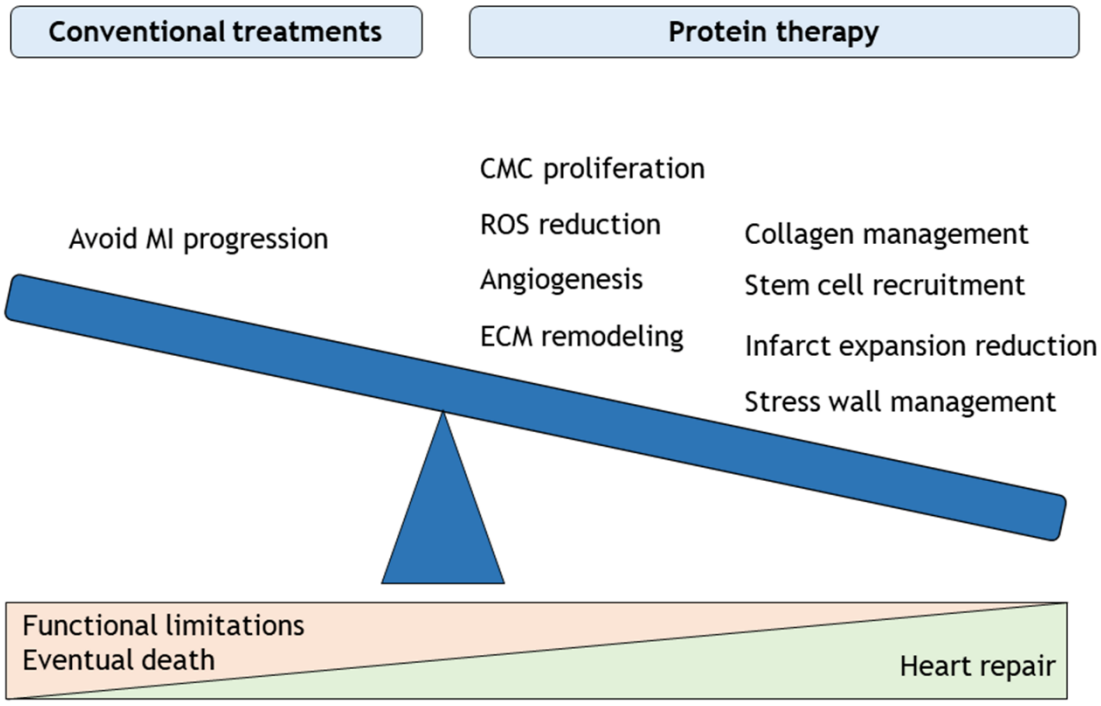

Figure 1: Contributions and advantages of conventional treatments and protein therapy to heart repair after MI.

However, proteins present one major limitation: they have a very short half-life *in vivo*. For instance, NRG1 is almost totally degraded within thirty minutes of administration in the blood or in tissues [28,29]. FGF1 follows a similar degradation profile, with a half-life of only one hour [30]. This explains the lack of efficacy and controversial results of protein therapy in clinical trials so far [18,31,32]. Consequently, during the last few years, there has been worldwide investment and interest in the development of vehicles to deliver therapeutic GFs in a safer manner. In this sense, biomaterials acting as drug delivery systems (DDSs) have attracted all the attention. Several types of DDSs have been developed so far, as is the case with nanofibers, hydrogels, nanoparticles (NPs) and microparticles (MPs) [33–35] (Figure 2A). On the one hand, biomaterials confer DDSs biodegradability and biocompatibility properties [36,37]. On the other hand, DDSs act as carriers oriented to 1) control the release of drugs over time, 2) deliver drugs in a specific body compartment [38] and 3) protect therapeutic agents against *in vivo* degradation [18] (Figure 2B). All this together leads to an increase of the

drug's bioavailability [39–43], and protein therapy in combination with DDSs is attracting renewed interest.

One of the DDSs more broadly used is MPs, specifically polymeric MPs made of poly (lactic-co-glycolic acid) (PLGA MPs). PLGA obtained the Food and Drug Administration approval in the 1970s [44], and since then its clinical applications have grown exponentially. Moreover, PLGA MPs have been tested by several international recognized groups for treating MI in preclinical models, showing promising results (reviewed elsewhere [27,45]), and nowadays it is generally accepted that PLGA MPs are one of the most promising vehicles to deliver GFs in the ischemic heart region.

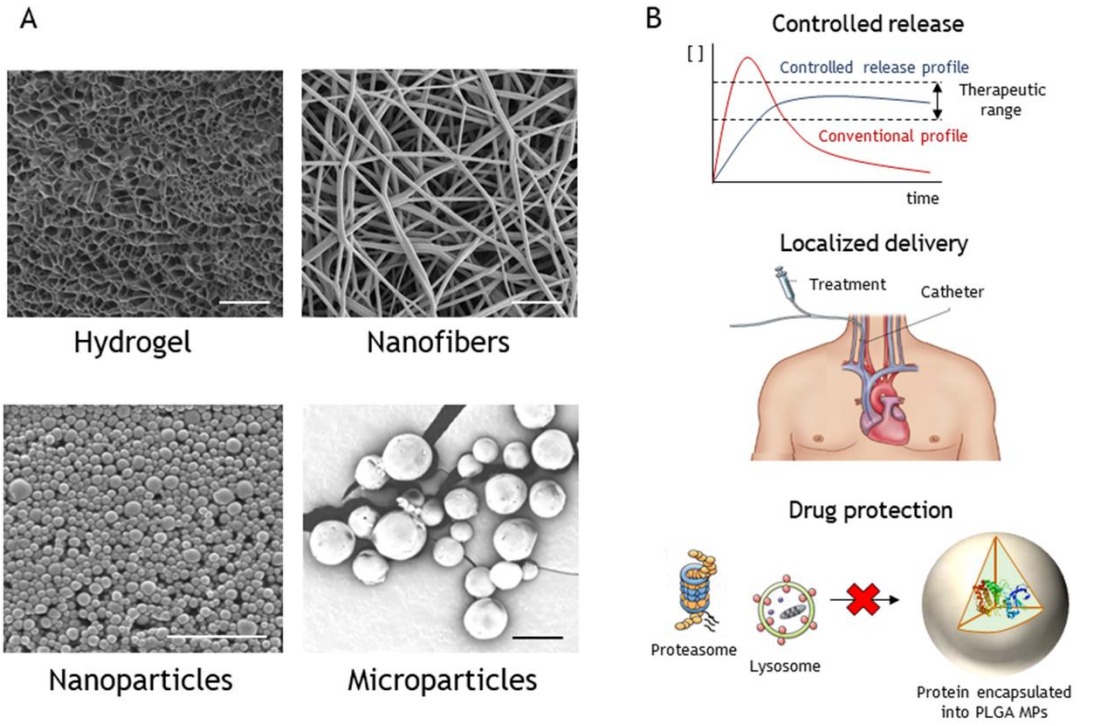

Figure 2: Principal types of DDSs used for cardiac repair. A) Scanning Electron Microscopy showing external structure of hydrogels, nanofibers, nanoparticles and microparticles. Scale bar: 10 μm. B) Advantages of using DDSs for the administration of therapeutic proteins into the heart.

- Inflammation modulates MI progression and resolution

Very recently several mechanisms have been suggested to rule and determine MI evolution, such as inflammation, extra cellular matrix remodeling and myofibroblast behavior [47–50]. In particular, the inflammatory response of the heart after a MI event may play a central role in left ventricle (LV) remodeling [49,51,52]. In the steady state, the heart is inhabited by embryonic derived anti-inflammatory macrophages with reparative properties

(aiMac) [53]. However, after MI, the macrophage (Mac) population in the heart changes dramatically due to the inflammatory response. In adult mammals, this inflammatory process can be classified into three major phases: inflammatory, reparative, and maturation [54]. The inflammatory phase begins with a rapid influx of neutrophils and bone-marrow derived monocytes within hours of the ischemic event, particularly in the setting of reperfusion. These monocytes differentiate into bone-marrow pro-inflammatory macrophages (piMac), displacing resident aiMac [53]. By day 3, piMac totally dominate the inflammatory phase, clearing debris and secreting inflammatory cytokines. The reparative phase starts at day 4, and is characterized by piMac reduction whereas myofibroblasts, endothelial cells and remaining aiMac secrete several anti-inflammatory cytokines and ECM components. Although this phase is focused on replacing lost ventricular tissue, the previous and excessive inflammatory environment leads to fibrin deposition and the creation of a collagen scar. Finally, the maturation phase is marked by apoptosis of the majority of the inflammatory and reparative cells and scar maturation [55,56]. Although scar formation serves initially to prevent LV wall rupture, in the long term it causes LV negative remodeling that may lead to heart failure.

A coordinated cellular response and timely progression and resolution of the three phases of inflammation are necessary for proper infarct healing [57–59]. Importantly, very recently the role of aiMac in cardiac repair has been further identified in different animal models such as teleost fish (zebrafish), urodeles (salamander) and neonatal mice (reviewed in [60]). Such challenging studies have demonstrated that following fibrin deposition, the zebrafish heart does not go through the intense collagen deposition and scarring seen in mammalian hearts after injury. Instead, lost cardiomyocyte tissue is replaced and contractile function of hearts appearing grossly normal in a couple of months. Similar reparative capacity was also found in urodeles. This regenerative capacity is maintained throughout adult life. On the other hand, mammals lose this ability in adulthood. While neonatal mice are able to fully repair the organ, the adult mammalian heart lacks regenerative capacity [61]. The main difference between zebrafish, urodeles and neonatal rodents on the on hand, and adult rodents on the other hand, is that adult hearts contain different cardiac macrophage subsets, with diverse functions, developmental origins, and homeostasis mechanisms. In particular, reparative capacity is correlated with aiMac, which are missing after MI in adult mammals. This strongly suggest that therapies that modulate and induce aiMac proliferation, maintenance and shift may be candidates for clinical use [62] (Figure 3).

In this sense, special interest must be focused on the relationships among inflammation, protein therapy and biomaterials. For instance, NRG1 is known to have effects on inflammation [63–65], and PLGA may trigger an

inflammatory response as well, mainly when the polymer is degraded in the tissue [66,67]. In any case, how NRG1 and PLGA affect inflammation in the heart after MI has yet to be elucidated, and still remains an issue.

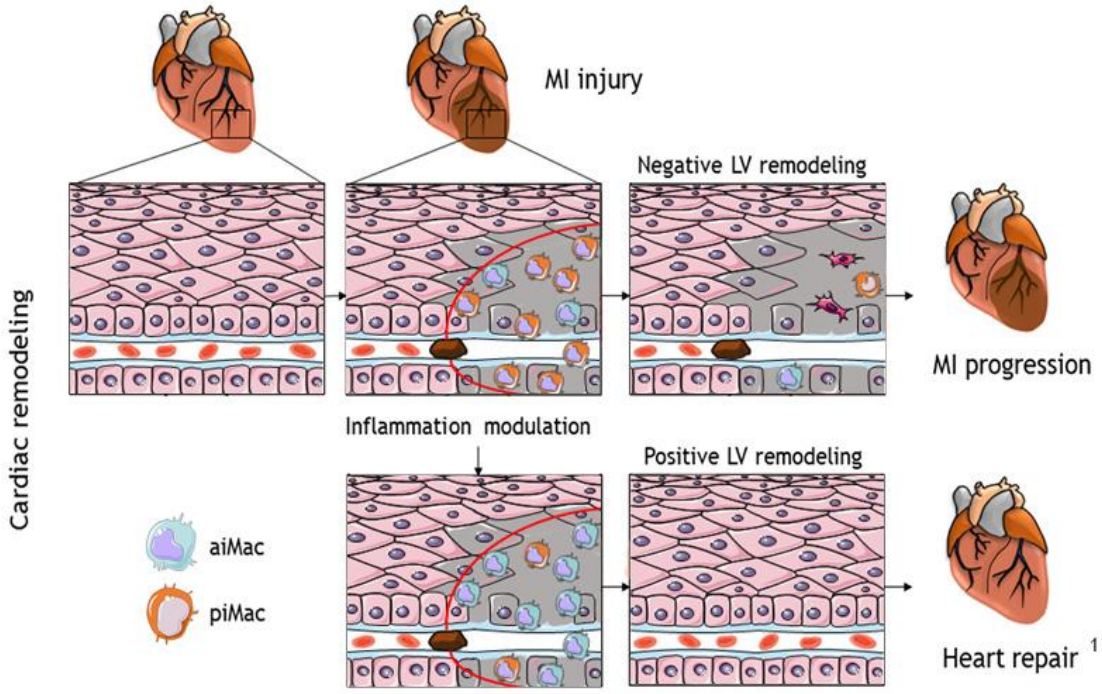

Figure 3: Cardiac remodeling after MI. Inflammation plays a key role in tissue restructuring. In particular, aiMac are associated with positive LV remodeling. Therefore, modulation of immune response in order to enhance aiMac proliferation and maintenance may be essential for proper heart repair after MI. aiMac: embryonic anti-inflammatory macrophages. piMac: bone-marrow derived pro-inflammatory macrophages.

In short, although MI patients' outcomes have improved significantly in the last few decades, current treatments are not able to restore the functionality of the organ or to repair the infarcted tissue. This unmet clinical need has triggered the development of novel approaches for treating MI, such as protein therapy in combination with MPs. Protein therapy is focused on tissue repair, thus favoring positive LV remodeling to recover organ functionality. MPs play a key role, protecting GFs against degradation and delivering them in a controlled manner, thus enhancing GF's bioavailability. Importantly, the recent discovery of inflammation as a key mechanism underlying MI progression and resolution, make it mandatory for these new treatments to be optimized and fully characterized in order to combat the inflammatory response. In this work, we focus on NRG1 PLGA MP synthesis, characterization, efficacy, and mechanisms of action, and their impact on inflammation after MI.

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INTRODUCTION

HEART REGENERATION AFTER MYOCARDIAL INFARCTION USING SYNTHETIC BIOMATERIALS

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Abstract:

Myocardial infarction causes almost 7.3 million deaths each year worldwide. However, current treatments are more palliative than curative. Presently, cell and protein therapies are considered the most promising alternative treatments. Clinical trials performed until now have demonstrated that these therapies are limited by protein short half-life and by low transplanted cell survival rate, prompting the development of novel cell and protein delivery systems able to overcome such limitations. In this review we discuss the advances made in the last 10 years in the emerging field of cardiac repair using biomaterial-based delivery systems with focus on the progress made on preclinical *in vivo* studies. Then, we focus in cardiac tissue engineering approaches, and how the incorporation of both cells and proteins together into biomaterials has opened new horizons in the myocardial infarction treatment. Finally, the ongoing challenges and the perspectives for future work in cardiac tissue engineering will also be discussed.

Key words:

Myocardial infarction, Cell therapy, Protein therapy, Clinical trials, Synthetic biomaterials, Delivery systems, Tissue engineering.

1. Introduction

1.1. Myocardial infarction and current treatments

Myocardial infarction (MI) remains a leading cause of morbidity and mortality worldwide, being responsible for nearly 7.3 million deaths each year. Moreover, as the World Health Organization highlighted in the last "Global Atlas on cardiovascular disease prevention and control" report [1], the number of deaths is expected to increase within the next decades due to the rising prevalence of the key risk factors for this pathology, such as behavioral and metabolic factors.

MI is principally caused by the occlusion of a coronary artery due to atherosclerotic and thrombotic processes, with the consequent reduction of the blood flow to the heart muscle. That loss of blood supply to the myocardium induces functional and morphological consequences. First, ischemic conditions lead to cardiomyocyte (CMC) death by necrotic or apoptotic processes, generating an infarcted area and causing a defect in contractile function. As a consequence, progressive and negative left ventricle (LV) remodeling and scar tissue formation take place [2]. These changes affect the ventricular chamber geometry, leading to the emergence of a larger, thinner and more spherical heart shape. Although the collagen-rich scar provides a rapid solution that avoids total LV wall disintegration, progression of the MI event often culminates in total heart failure and death [2]. A schematic representation of MI development with the principal steps is shown in Fig. 1.

Death from MI could be prevented by accurate early-stage diagnosis and proper subsequent proper treatment [3]. The more quickly the blood flow is restored, the better the outlook. Current therapies include surgical procedures such as coronary bypass, balloon angioplasty, stents and heart transplant as a last option [4]. Surgical interventions are generally combined/complemented with pharmacological treatments in order to improve patient outcomes [5]. However, although

conventional interventions are useful in mitigating MI symptoms [6,7], they cannot repair the infarcted tissue, and so cardiac dysfunction remains an issue [8]. In view of the fact that current treatments are not able to regenerate the cardiac tissue and that the heart has shown limited post-natal cardiomyogenesis [9], patients who survive a MI might face serious functional limitations for the rest of their lives, which leads to secondary complications that impair their quality of life and place a major annual economic burden on the country [10].

1.2. New therapeutic strategies under investigation for myocardial infarction

As already stated above, conventional treatments are not enough to deal with functional and economic complications derived from MI and many aspects of the treatment for this pathology remain challenging. Therefore, in recent decades there has been a great research effort aimed at finding new alternative therapies for MI, focusing on myocardial regeneration. In these investigations, angiogenesis, CMC proliferation and recruitment of stem cells (SCs) to enhance endogenous healing of the heart have played an essential role, since they are considered to be key factors for adequate post-ischemic repair [11,12]. The advent of new molecular and cellular targets together with advances in genomics, proteomics and other biotechnologies have led to the discovery of novel pharmaceutical compounds with the potential to definitively change MI treatment. This emerging class of substances includes biological agents, genes, siRNAs, small molecules such as growth factors (GFs) and other therapeutics [13]. Among them, the ones that have shown the best results so far are cells and proteins [14, 15]. In fact, exciting preclinical studies carried out to evaluate regenerative therapies for MI have prompted the initiation of clinical trials based on administration of SCs or GFs to the heart, as shown in the sections below.

1.2.1. Cell therapies for MI in clinical trials

Cell therapy relies on the administration of living cells for therapeutic purposes. Focusing on myocardial regeneration, it requires the administration of multipotent cells able to differentiate into the main cardiac cell lineages myocytes, vascular smooth muscle cells and endothelial cells [16] and to develop both CMCs and coronary vessels [17]. To date, the most popular cell candidates used to regenerate the damaged tissue include adipose derived stem cells (ADSCs), mesenchymal stem cells (MSCs), embryonic stem cells (ESCs), endothelial progenitor cells (EPCs), bone marrow derived stem cells (BMSCs), induced pluripotent stem cells (iPS), cardiac progenitor cells (CPCs) and induced cardiomyocytes (iCMs) [18]. However, although several SCs have been tested in *in vitro* and *in vivo* preclinical studies with promising results [19], not too many SCs have reached clinical trials. In fact, only BMSCs, myoblasts, CPCs and ADSCs have been employed in clinical trials yielding both encouraging and disappointing results [20]. The discrepancies in the results of different clinical trials using the same cell source have led researches to investigate the key aspects that determine the success of cell therapy.

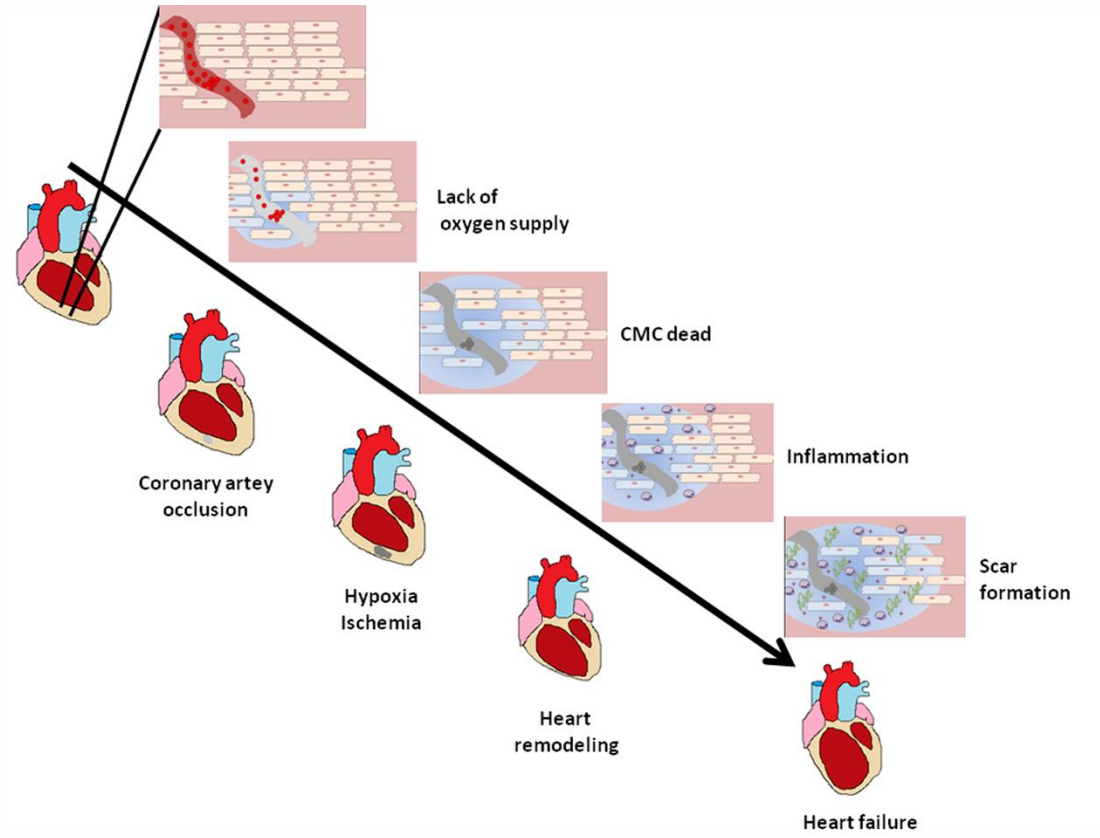

Fig. 1. Schematic representation of MI development including the principal steps. After a coronary artery occlusion, the heart gradually loses its function and suffers a negative remodeling that ends in total heart failure. The black arrow indicates the progression of the pathology.

A careful analysis of the available data shows that negative results may be fundamentally due to the poor permanence of the injected cells inside the tissue [21,22], since SCs' therapeutic efficacy depend on their ability to survive in the hostile milieu of the damaged heart and to engraft within the myocardium [23]. Another critical point is the complexity of obtaining a reliable source of functional CMCs. Moreover, there are other aspects such as ideal cell type, source and dosing, route and time of delivery [24] and clinical trial design which should undergo further analysis to validate the safety and efficacy of cell therapy for MI [25]. In addition, cardiovascular regeneration may not be identical among individuals, and there should be an optimal cardiac regeneration therapy for each patient [26]. In summary, although the outcomes of clinical trials performed so far have displayed promising results, the overall beneficial effects of SCs therapies are still relatively modest. Moreover, the fundamental mechanisms of SC-mediated repair are largely unknown and controversial. Interestingly, the slight improvement observed after cell administration is frequently due to the paracrine effect of the cells rather than to their differentiation [19,27]. Thus, bioactive factor secretion may mediate the improvement in cardiac remodeling, function and

metabolism. The latest research trend in SC therapy for cardiac healing identified exosomes secreted by SCs as crucial mediators of cell therapy-induced regeneration [28].

1.2.2. Protein therapies for MI in clinical trials

In addition to cell therapy, the administration of GFs able to promote cardiac repair holds great promise as a therapy capable of contributing to myocardial regeneration. GFs are administered next to the damaged tissue with the aim of favoring angiogenesis, chemotaxis, SC differentiation, CMC survival and proliferation, reduction of apoptosis and remodeling [5]. First, it was reported that during MI evolution the administration of therapeutic GFs could help to enhance the endogenous angiogenic process [29], thereby improving cardiac function and recovery. Interestingly, more recently it has been demonstrated that GF administration also has effects on stimulating progenitor cell recruitment to the heart and on inducing differentiation of SCs and existing CMCs [15]. In fact, the combination of all the three processes is mandatory for achieving the best possible heart regeneration. Therefore, optimism about how protein-based approaches can be effective for cardiac regeneration and can avoid the fatal consequences of MI disorder has spread considerably in the last few years. Consequently, several GFs have been brought to clinical trials to test their therapeutic potential to regenerate the infarcted heart. This is the case of fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), erythropoietin (EPO), hepatocyte growth factor (HGF), neuregulin (NRG), granulocyte-colony stimulating factor (G-CSF) and stromal cell-derived factor-1 (SDF-1) [5,30]. Again, as reported in clinical trials with cells, important but controversial results have been observed in clinical trials with GFs. By summarizing these results some interesting conclusions can be outlined. Firstly, the study designs vary considerably from one trial to another in terms of population, GF administered, route and dose of administration. Thus, a better definition of clinical trial requirements is needed in order to obtain more comparable results and conclusions. Secondly, a common drawback observed in all these trials is the low half-life of therapeutic proteins in the organism, which are rapidly degraded or removed from the site of injection. Therefore, the low efficacy and variable results reported so far might be attributed to this bioavailability issue. Thus, protein therapy needs to overcome those obstacles before it can attain clinical relevance.

1.2.3. Current challenges

Taking an overview of the aforementioned results, it can be gathered that SC and protein based therapies are potentially powerful strategies for treating MI. However, only partial improvements have been achieved, and more research is needed to optimize such therapies. The advantages and challenges of cell and protein therapies are illustrated in Fig. 2.

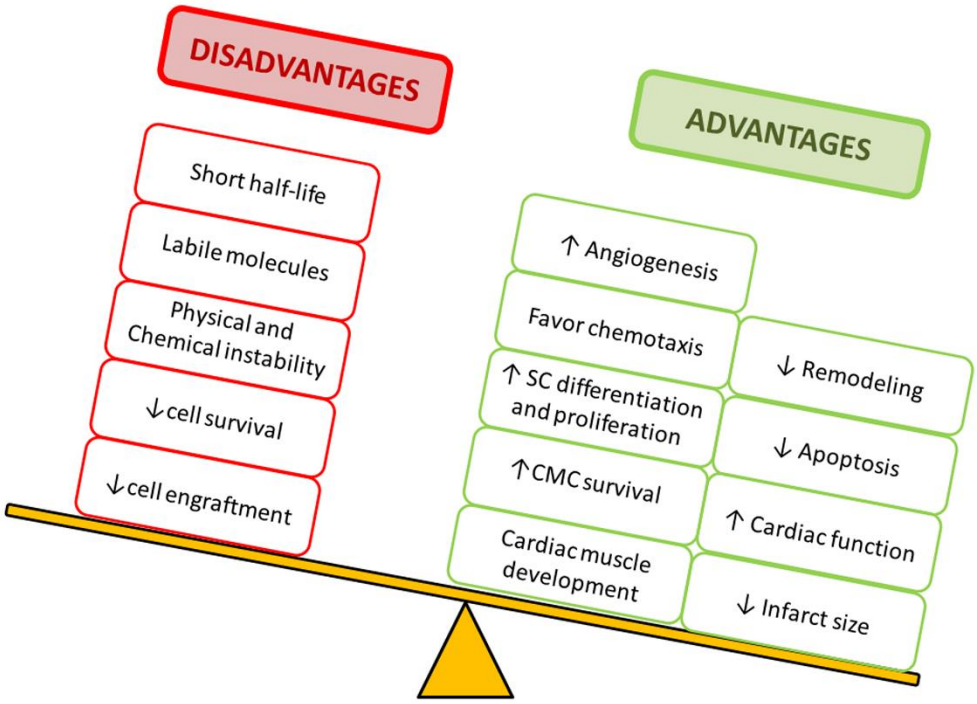

Fig. 2. Advantages and challenges of cell and protein therapies.

Principally, enhancement of cell engraftment, integration and coupling in the tissue must be at the center of efforts in cell therapy, whereas in protein therapy, improvements in bioactivity half-life and stability of therapeutic proteins are the principal points in where deeper investigation is mandatory. It is necessary to improve the efficacy of these novel strategies to reach their full potential. Importantly, specialized delivery modalities are highly recommended to achieve these goals.

2. Biomaterials to enhance cell and protein delivery to the heart

2.1. Biomaterials in cardiac repair

The development of new technologies that enable effective cardiac drug delivery would optimize cardiovascular treatment and would address the shortcomings of current and novel therapies. Biomaterials were developed to be used as medical devices for transplantation. However, the term biomaterial has evolved from simple implants to complex multifunctional interfaces with the body. The current definition proposed by the European Society for Biomaterials is a “material intended to interface with biological systems to evaluate, treat, augment or replace any tissue organ or function of the body” highlighting the role and importance of the material in influencing

biological processes. Biomaterials should play a crucial role in the repair of the damaged heart. In a cardiac context, there are 4 ways in which biomaterials have shown to be useful (Fig. 3):

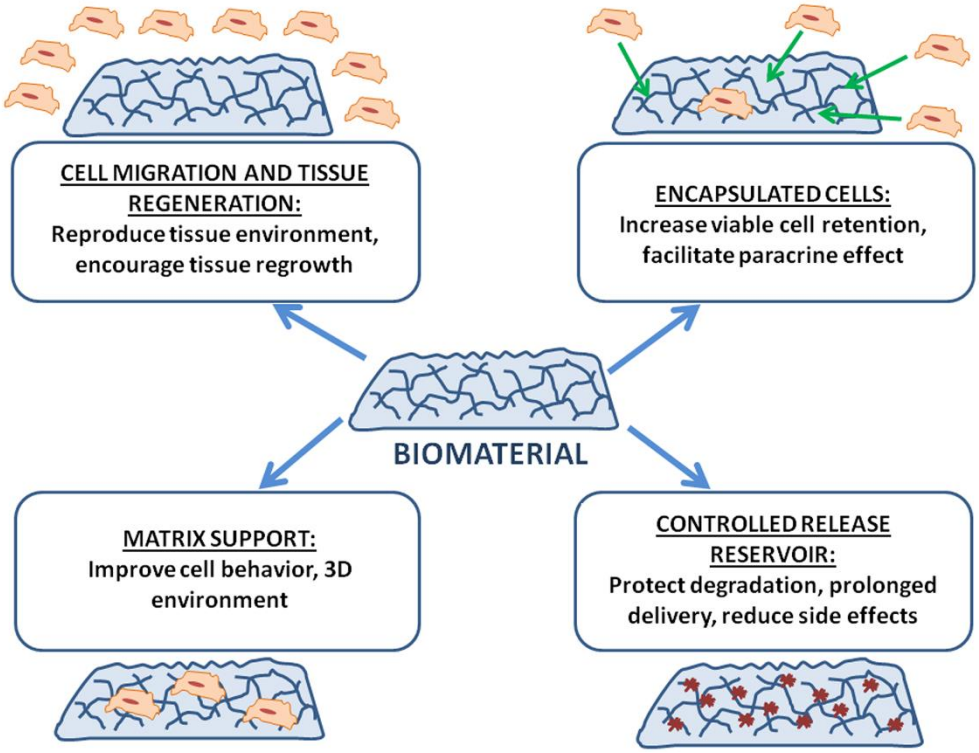

Fig. 3. Principal benefits of biomaterials in cell and protein therapies.

a) The biomaterial by itself promotes cell migration or tissue regeneration. In this case, biomaterials reproduce some aspects of the natural cardiac tissue environment and encourage tissue regrowth. Biomaterials are used for general cardiac reconstruction, vascular grafts, pediatric shunts, etc. Synthetic materials, metals, combinations of both and decellularized materials have been used for many years with significant success [31].

b) The biomaterial is used to encapsulate cells acting as an immunoisolation barrier. Encapsulation is one potential strategy to increase viable cell retention while facilitating paracrine effects. Synthetic biomaterials have evolved from polymers with no cell-recognition moieties to compounds mimicking the extracellular matrix, thus favoring cell–biomaterial interactions [32]. Nevertheless, they have not yet reached the cell viability and proliferation rates observed with natural biomaterials. Consequently, synthetic polymers are used in combination with natural compound or small peptide sequences in order to promote cell–biomaterial interactions for tissue regeneration [33].

c) The biomaterial is used as a matrix to support cell growth and integration. The biomaterial improves cell behavior due to the 3D environment as well as to the mechanical and signaling cues they provide to transplanted cells. These biomaterials are thus used as scaffolds. Several parameters must be taken into consideration in scaffold design to meet heart-specific requirements, such as shape, size and physical and mechanical properties [34].

d) The biomaterial is used as a controlled release reservoir to locally deliver bioactive molecules. Biomaterials can be used to prepare drug delivery systems (DDSs) that might provide protection from degradation to biomolecules (e.g. GFs, transcription factors, soluble paracrine factors) and a prolonged delivery. Thus, DDSs are able to decrease the amount of drug given to the patient, reducing serious side effects besides promoting cardiac repair. Each biomaterial, regarding its physico-chemical properties, provides a particular release profile. Thus, a specific biomaterial must be used for achieving the desired controlled release.

2.2. Current studies using biomaterial-based delivery systems in heart regeneration

Biomaterial-based delivery systems are essential in enhancing the therapeutic outcomes of cells and proteins in cardiac tissue engineering. The number of studies using biomaterials in combination with cell and protein therapies has therefore increased exponentially over the last decade. This section provides an overview of the advances made in the last 10 years in the field of cardiac repair using biomaterial-based delivery systems with a focus on the progress made on preclinical *in vivo* studies done with synthetic biomaterials. We center on the use of synthetic biomaterials because they have been recently favored as cell and protein carriers. Nowadays there is a large list of synthetic biomaterials under investigation, including caprolactone, polyglycolic and polylactic acids, polyurethane and self-assembling peptides among others (Table 1). Each one has its own characteristics, but, in general all present important advantages over their natural counterparts. For instance, their physical, chemical, mechanical and biological properties can be modified, they can be produced uniformly in large quantities offering a multitude of possibilities. They also offer limited batch-to-batch variability. Concerning toxicity, nowadays synthetic biomaterials have reached similar safety levels to natural compounds, and their biocompatibility and biodegradability are well established in almost all cases (Table 1).

A point that must be considered when designing biomaterial-based delivery systems for heart regeneration is the size of the DDS, since it could induce important side effects. On one hand, administration of “big” DDSs might favor tissue necrosis or hamper cardiac muscle contraction. On the other hand, very

small DDSs could be rapidly phagocyted or cleared by the blood flow diffusing to other body organs. For instance, our preclinical studies using small (rat) and large (minipig) animal models of MI demonstrate that particles between 5 and 20 μm have the ideal size for intramyocardial injection. Smaller particles presented poor retention in the injected tissue, whereas injection of bigger particles resulted in damaged cardiac tissue [35–37].

2.2.1. Hydrogels

Injectable hydrogels are three-dimensional polymer networks extensively swollen in water (Fig. 4) [44], and represent a powerful delivery system for cardiac repair, since they are tri-dimensional networks that mimic the extracellular matrix and reproduce the natural environment and, in addition, they can be administered using non-invasive techniques like cardiac catheterization, thanks to their liquid-gel controllable nature [45]. In accordance with this potential, hydrogels have been developed using a long list of biomaterials. Nowadays synthetic materials have achieved high degrees of biodegradation and biocompatibility, like their natural counterparts. In fact, cardiac administration of synthetic hydrogels has proved to be effective in terms of promoting contractile phenotype smooth muscle tissue formation [46,47], preventing LV remodeling and scar expansion and improving cardiac function [48–51]. Interestingly, the timing of administration seems to be important because hydrogels assumed markedly different morphologies that determined heart remodeling. Thus, very early time points may not be beneficial, whereas hydrogel injection one week after the infarct event results in positive remodeling and cardiac function improvements [50].

2.2.1.1. Hydrogels in cell-based therapies.

The use of hydrogels as cell carriers to repair the heart is a relatively new strategy. The resulting network that hydrogels form can reproduce specific biological functionality of the natural cardiac extracellular matrix and thus, seeded cells grow under conditions as similar as possible to those of the natural environment. To examine the effect of cell delivery via hydrogels, Wall S.T. *et al*. developed a semi-interpenetrating hydrogel made of a crosslinked copolymer network of N-isopropylacrylamide and acrylic acid, interpenetrated with linear chains of polyacrylic acid with chemically tethered peptides combined with cell-surface integrin receptors for encouraging cellular attachment. The system was used as an assistive microenvironment for BMSC transplantation (2 × 105 cells per hydrogel) and tested in a mouse MI model. Six weeks after implantation, hydrogels were at the site of injection and GFP-BMSC could be detected. However, BMSC-hydrogel treatment did not show any improvement in ejection fraction (EF) and fractional shortening (FS) compared to non-loaded hydrogels and free BMSC injection [52].

Table 1
Most employed synthetic biomaterials used to prepare cardiac DDSs and their principal advantages and disadvantages.

Synthetic biomaterial	Advantages	Disadvantages
Caprolactone and derivatives	Non-toxic, tissue compatible, mechanical properties, modifiable nature, pH sensitivity	Difficult to synthesize, slow biodegradation [38,39]
Polyglycolic and polylactic acids and derivatives	Well established biodegradation and biocompatibility, extended release rates	Acidic environment during degradation, bulk erosion [39]
Polyurethane	Biocompatible, mechanical properties	Biodegradable only when copolymerized with other polymers, no conductivity [39]
Self-assembling RAD 16 peptides	Self-assembly properties, bioreabsorbable, designed 3D microenvironment	Unknown toxicity and side effects [40,41]
Carbon nanotubes	Excellent mechanical and electrical properties	Strong hydrophobicity, physicochemical Properties related toxicity, expensive [42]
Polyketals	Biodegradable, non-immunogenic, neutral degradation products, acid sensitivity, low cost	Rapid macrophage uptake and biodegradation, complex synthesis [43]

Concurrently, Li X.Y. *et al*. developed a crosslinked polymer hydrogel by mixing dextran-hydrophobic poly(e-caprolactone)-2-hydroxylethyl methacrylate chains with thermo-responsive poly(N-isopropylacrylamide) (NIPAM) chains. The biomimetic network was then combined with BMSC and placed onto the LV of rabbit MI model. A significant increase in cell engraftment 48 h after injection compared to free SC administration was observed. One month after treatment significant LV-EF preservation and attenuated LV dilatation accompanied by enhanced neovascular formation and prevented scar expansion were found in BMSC-hydrogel group compared to the rest of the groups [53].

Limited positive results have been reported with hydrogels prepared using only poly(ethylene-glycol) (PEG) [54]. However, several PEG copolymer hydrogels have shown benefits. For instance, PEG has been combined with synthetic caprolactone by Wang T. *et al*. A triblock polymer was synthesized by mixing methoxy PEG and poly(caprolactone)-(dodecanedioic acid)–poly(caprolactone). BMSCs (2 × 107) were resuspended in α-cyclodextrin solution, which was intramyocardially co-injected with the hydrogel solution in a rabbit MI model. BMSC- hydrogels significantly enhanced cardiac function and increased both cell retention and vessel density around the infarct, preventing scar expansion compared with cells injected alone 4 weeks after treatment [55]. PEG has also been combined with natural materials to formulate hydrogels for cell transplantation. Naturally occurring biomaterials allow for the appropriate cell–matrix interactions, thus favoring cell engraftment [56]. The first example is the study by Habib M. *et al*. who PEGylated bovine fibrinogen to synthesize a biocompatible matrix where neonatal rat ventricular CMCs (3 × 106) were seeded. Irgacure 2959 photoinitiator was added to allow UV-light-activate in situ polymerization of the hydrogels after injection into the myocardium of rats suffering MI. Owing to the PEG additional crosslinkers, hydrogel permanence in the tissue varied from less than one month on the absence of additional PEG, to more than one month when additional PEG up to 2% was added. The combination of CMCs and hydrogels resulted in a favorable effect on cardiac remodeling with a significant increase in FS and functional outcomes 30 days after treatment administration. Higher anterior wall thickness was also detected in the CMC-hydrogel group when compared to controls, and transplanted CMCs were detected one month after administration inside the infarcted region [57]. In another approach Bearzi C. *et al*. synthesized a PEG–fibrinogen hydrogel using PEG–diacrylate as crosslinker and the photoinitiator Irgacure 2959 to control gelation. iPS (5 × 106) overexpressing placental growth factor (PlGF) and/or matrix metalloproteinase 9 (MMP9, protease involved in vascularization and engraftment processes) were combined with the hydrogels and tested in a mouse MI model. Animals that received iPS-hydrogel, regardless of whether cells overexpressed the therapeutic proteins or not,

showed a significant increase in capillary density and cardiac function and a decrease in fibrotic and apoptotic indexes. Administration of SCs overexpressing both proteins resulted in better outcomes [58]. Positive results were also observed by Wang H. *et al.*, who combined PEG with fumarate. In this case, APS/TEMED solution was used for controlling hydrogel gelation. ESCs (1 × 106) were seeded and system efficacy was tested in a rat MI model. 24 h and 4 weeks after treatment a significant higher injected cell population was detected in the infarcted tissue in ESC-hydrogel group compared to injection of cells alone. The heart area covered by transplanted cells was also significantly bigger when the hydrogel was used. Concerning cardiac parameters, echocardiography values, infarct size and arteriole/venule density were significantly improved in ESC-hydrogel group when compared to controls 4 weeks after treatment [59].

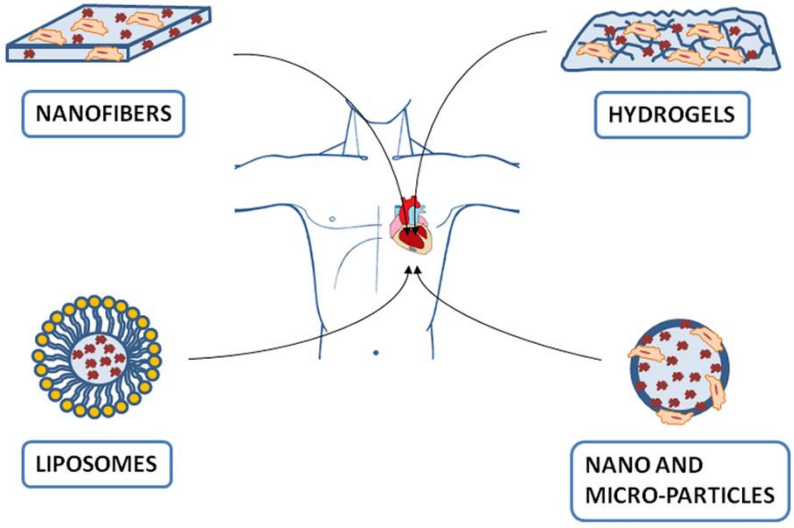

Fig. 4. Main types of drug delivery systems made of biomaterials that are being investigated in combination with proteins and/or cells for treating myocardial infarction.

In addition to cardiac hydrogels based on PEG there is a growing interest on the development of hydrogels made from self-assembling peptides. Self-assembling is a process that is mediated by non-covalent interaction between molecules via ionic bonds, hydrogen bonding, hydrophobic interactions and van der Waal interactions [60]. Self-assembling peptides are normally 8–16 amino acids long and they are composed of alternating hydrophilic and hydrophobic residues that form a stable hydrogel of flexible nanofibers (NFs) upon exposure to physiological salt concentration or pH [61]. This is the case with the self-assembling polypeptide RADA16-II and its derivates. This polypeptide is able to spontaneously assemble into a stable three-dimensional NF scaffold that mimics natural extracellular matrix [62]. Davis M.E. *et al.*

demonstrated that RADA16-II peptides rapidly gel when mixed with sterile sucrose solution. The resulting hydrogel created a microenvironment in the myocardium which promoted vascular cell recruitment and favored injected cell survival [63]. Then, Lin Y.D. *et al*. seeded BMSC (108) in such DDS and tested them in a pig MI model. BMSC-hydrogel injection resulted in significant higher improvement of cardiac function compared to other groups, and was accompanied by a significant increase in transplanted cell retention and capillary density in the peri-infarct area. Similar significant results were observed regarding the scar length fibrosis area, which were reduced in animals treated with seeded hydrogels compared to other groups [64]. More evidence about the use of RADA16 peptides for cardiac implantation was given by Cui X.J. *et al*. Here, MSCs (5 × 105) were seeded on the polypeptide hydrogel and injected in a rat MI model [65]. Injected cells underwent myogenic differentiation in the infarct and peri-infarct regions 4 weeks after administration. Smaller infarct size, higher capillary density and improved global cardiac function were observed, with significant differences between animals treated with MSC-RADA16-hydrogel and the rest of the groups [66]. These encouraging results led Guo H.D. *et al*. to attach an RGDSP cell-adhesion motif to RADA16 peptide to enhance cell survival and differentiation and thereby to improve SC efficacy. 5 × 106 BMSCs were seeded in these systems and final constructs were tested in a rat MI model. The system protected SCs from apoptosis and necrosis processes present in the ischemic myocardium. Moreover, MSC survival rate, cardiac function and collagen deposition were improved in animals treated with RGDSP-MSC-hydrogels with respect to MSC-hydrogels group 4 weeks after treatment [67]. Tokunaga M. *et al*. further demonstrated the efficacy of RADA16 peptide as cell carrier in a mice MI model. In this study, Puramatrix™, a commercial variant of RADA16 peptide, was used to create self-assembling hydrogel that underwent gelation in the presence of salts from the body. Authors seeded 2 × 104 BMSC, SM, ADSC or CPC in such systems and injected them onto the infarcted area of a mouse MI model. 2 weeks after treatment CPC-hydrogel significantly attenuated infarct size expansion, improved echocardiography parameters and favored neoangiogenesis. Cell apoptosis was reduced when hydrogels were employed compared to free cell administration. These results suggest that CPCs are a promising cell source for preventing cardiac remodeling and dysfunction [68].

More recently, carbon nanotubes have been explored for cardiac delivery. They have good electrical conductivity and suitable and adaptable mechanical properties for cardiac application. A novel hydrogel made of carbon-nanotubes mixed with thermo-sensitive NIPAM was developed in order to enhance ADSC therapeutic efficacy. 2 × 106 ADSCs were seeded onto such systems and injected in a rat MI model. One week after treatment significant enhanced engraftment of seeding cells was detected when hydrogels were co-

administered with the SCs in comparison to free ADSC administration. Moreover, LV-EF and FS, infarcted area and LV wall thickness were significantly improved in the ADSC-hydrogel group [69] providing evidence for the myocardial application of carbon nanotubes.

2.2.1.2. Hydrogels in protein-based therapies.

The use of hydrogels for therapeutic protein delivery into the myocardium is quite recent. However, due to the liquid nature of the hydrogels, which facilitates cardiac administration, this DDS has rapidly attracted a great deal of attention.

Thermo-responsive hydrogels have been proved to favor positive remodeling and to improve cardiac function when combined with GF. For instance, a temperature-sensitive aliphatic polyester copolymer hydrogel made of PEG, N-hydroxysuccinimide and poly(δ-valerolactone) was conjugated with VEGF (40 ng) and intramyocardially injected in a rat MI model. No significant differences among groups were observed in any of the echocardiography parameters 7 days after treatment, but 35 days after treatment FS, EF, end-systolic elastance and end-systolic volume were significantly improved in the VEGF-hydrogel group compared to controls. Interestingly, conjugation of VEGF with the biomaterial prevented scar expansion and ventricular dilatation. Vascular density was significantly higher when VEGF was encapsulated inside the hydrogel [70]. Similar results were observed by Garbern J.C. *et al*. that entrapped biotinylated-FGF (5 μg) into a sharply pH–temperature-responsive injectable hydrogel system composed of a random terpolymer of NIPAM, propylacrylic acid and butyl acrylate. Hydrogels increased GF retention in the heart for 0–7 days when tested in a rat MI model. On the other hand, FS, regional myocardial blood flow, LV wall thickness and angiogenesis were significantly improved in the FGF-hydrogel group when compared to the other groups 28 days after treatment [71].

Apart from temperature sensitive hydrogels, other devices have been tested. For instance, Projahn D. *et al*. encapsulated Met-CCL5, a chemokine that inhibits neutrophil infiltration by competitive antagonism of CCL5 receptors, and SDF-1 in a star-shaped poly(ethylene oxide-stat-propylene oxide) and linear poly(glycidol) hydrogel. In this strategy, polymer chemical modification led to different GF release profiles. Thus, Met-CCL5 (0.5 μg) was mixed with fast degradable hydrogel and SDF-1 (3 μg) with slow degradable one in order to optimize GF retention in the myocardium and to adjust the GF release to heart necessities over time. One day after injecting the systems in a mouse MI model, high levels of Met-CCL5 were detected in mouse sera, but this trend was not maintained 4 weeks after hydrogel injection. On the other hand, levels of SDF-1 remained constant due to the slow release. Regarding heart recovery, EF was significantly higher 4 weeks after treatment when both GF-

hydrogels were administered. Regarding neovascularization, apoptotic levels and infarcted area size, they were significantly improved when SDF-1 was administered, alone or in combination with the other GF, suggesting that there was accelerated wound healing in these groups [72].

In another study, PEG-based hydrogels were formulated by mixing this compound with maleimide macromers, and the systems were pre-functionalized with RGD adhesion peptide. HGF and/or VEGF (both at concentration of 1 μg per injected hydrogel) were incorporated into the matrices, which were tested in a rat MI model. The chemical-sensitive precursor hydrogel solution was crosslinked into a hydrogel by addition of a cysteine-flanked protease-degradable peptide sequence. Thus, before administration, crosslinked agent was added and final solution was injected into the ischemic zone. Only when both GF were co-administered a significant cardiac function improvement could be observed 21 days after treatment with respect to the non treated group. Significant increase in both vessel density and fibrosis and in c-kit+ cells were observed after HGF–VEGF-hydrogel treatment [73]. Nevertheless, some controversial results were observed in larger animal models when other PEG based hydrogels were employed. This was the case of the study by Koudstaal S. *et al.*, who developed a pH-switchable supramolecular hydrogel with self-healing properties made of PEG and 2-ureido-4-pyrimidone (UPy). These systems were combined with insulin like growth factor 1 (IGF-1, 2 μg) and HGF (2 μg) and administered in a pig MI model. Although the dual GF-hydrogel combination resulted in improvements in LV end-systolic volumes, EF and formation of new capillaries in the infarct border zone one month after the injection procedure, no other benefits were detected. Interestingly, regarding CMC hypertrophy rate, although GF administration attenuated CMC degeneration, no significant differences were observed between the hydrogel group and the free GF administration group [74].

Another type of chemical-sensitive hydrogels was used by Wang T. *et al*. who employed their biocompatible PEG-based hydrogel solution, previously used as cell carrier (see [55]), as delivery system to administer EPO into rat MI model. In order to favor hydrogel formation, PEG solution and EPO solution dissolved with α-cyclodextrin were co-injected together. One month after treatment, animals that had received EPO dissolved in saline medium or incorporated into the hydrogel showed significant improvements in echocardiography parameters. However, significant infarct size reduction and apoptotic index, as well as increase in CD34+ cell density and neovasculature formation were only detected in the EPO-hydrogel group indicating the benefits of this strategy [75]. Self-assembling peptides are nowadays attracting growing interest for protein delivery. For instance, RADA16-II peptide solution in combination with platelet-derived growth factor (PDGF, 4 and 8 μg) has been

used to synthesize chemical depending hydrogels, which undergo gelation once peptide solution is mixed with sterile sucrose [76]. The highest dose system showed decreased CMC death and preserved systolic function 14 days after being injected in a rat MI model. Previous observations were correlated with a decrease in infarct size and induced PDGF receptor ß expression and Akt phosphorylation in cardiomyocytes *in vivo* that indicated that CMCs were protected by endothelial cells through PDGF-pathway. The same group performed a long-term study in which intramyocardial delivery of PDGF by self-assembling peptide hydrogel led to an improvement in cardiac performance for at least 3 months [77]. In order to prolong and slow angiogenic factors release, Guo H.D. *et al*. constructed a novel self-assembling peptide by attaching the heparin-binding domain sequence LRKKLGKA to the self-assembling peptide RADA16 encapsulating VEGF (100 ng). In a rat MI model EF, FS, scar size, collagen deposition, cell survival and microvessel density were significantly improved in animals treated with the novel hydrogel compared to VEGF-RADA16 hydrogel (without LRKKLGKA sequence) 4 weeks after treatment [78]. Segers V.F. *et al*. also observed positive results in cardiac recovery using RADA16-II peptides. In this case, SDF-1 variant resistant to protease degradation was encapsulated into hydrogels and administered in a rat MI model. Treatment resulted in significant enhancements on SC recruitment, improved cardiac function and capillary density 28 days after administration [79]. Interestingly, when animals were treated with the SDF-1 resistant variant, a better heart recovery was observed compared to the normal SDF-1 treated group, although no significant differences were reported.

Encouraged by RADA16-II peptide result, Kim J.H. *et al*. optimized the therapy by combining PDGF and FGF-2 in the same hydrogels. The systems were injected in a rat MI model and dual GF loaded hydrogels showed the smaller CMC apoptosis rate compared to the other groups. Infarct size and wall thickness followed similar significant trends 4 and 8 weeks after treatment. Interestingly, animals treated with PDGF–FGF-hydrogel showed similar vessel density to non infarcted animals, suggesting an important angiogenic synergy between both GFs [80]. Dual GF delivery strategy for preserving cardiac function was also explored by Webber M.J. *et al*. in a mouse MI model, who loaded VEGF and FGF (10 ng of each GF per hydrogel) in heparin-binding-peptide-amphiphile hydrogels. VEGF–FGF-hydrogel treatment resulted in significantly improved LV contractility 30 days after administering the treatments [81].

Hydrogels made of semi synthetic materials have also been explored as protein carriers for cardiac repair. He Y.Y. *et al*. used dextran in combination with hydrophobic poly (e-caprolactone)-2-hydroxylethyl methacrylate chain and thermo-responsive NIPAM forming thermosensitive hydrogels. 2.5 µg of

high-mobility group box 1 (HMGB1, cytokine that attenuates cardiac remodeling after MI) were added per hydrogel, and then tested in a rat MI model. 24 h after treatment administration, cardiac SC proliferation and differentiation were found to be significantly higher in HMGB1-hydrogel group compared to the other groups. One month later, HMGB1-hydrogel treated animals showed the greatest increase in EF and the lowest collagen deposition, with significant differences from all other groups. Nevertheless, both HMGB1-hydrogel and free HMBG1 significantly increased arterial density in the peri-infarcted area when compared to controls, but no significant differences were observed between these groups [82].

Hyaluronan is a natural polysaccharide which has been mixed with synthetic compounds to prepare hydrogels due to its excellent biocompatibility and biodegradability [83]. For instance, sodium hyaluronate was chemically modified with hydroxyethyl methacrylate to favor hydrolytic degradation, as in the work of MacArthur J.W. Jr. *et al*. A synthetic analog of SDF-1α was encapsulated at a concentration of 25 μg/50 μL and APS/TEMED was used for hydrogel gelation. These systems were injected intramyocardially in a rat MI model, where they proved to have significant benefits in improving echocardiography parameters such as EF, cardiac output and contractility when compared to controls. Loaded hydrogel also augmented capillary density. However, no significant differences were found between SDF-hydrogel and hydrogel groups regarding preservation of ventricular geometry and infarct size region, although both were significantly improved when compared to control groups [84].

2.2.2. Nanofibers

NFs are tridimensional, polymeric matrices with a network structure made of engineered fibers with diameters less than 500 nm (Fig. 4). To date several biomaterials have been tested as potential NFs for inducing cardiac repair after MI. For instance, in the work of Castellano D. *et al*., collagen, poly(3-hydroxybutyrate), poly(e-caprolactone), poly-lactic acid and polyamide NFs were generated by electrospinning, being then transplanted into a rat MI model. Interestingly, poly(3-hydroxybutyrate) was the scaffold with the most beneficial reparative potential and positive remodeling capacity [85]. In another study polyester urethane urea NFs showed suitable mechanical properties and biocompatible characteristics, allowing cellular integration and endocardial endothelialization with minimal inflammation [86]. Thus, the evidences suggested that NFs result in positive outcomes for MI treatment.

2.2.2.1. Nanofibers in cell-based therapies.

Since NFs are solid networks, cells can be entrapped within the polymeric matrix, augmenting their engraftment and survival. With this aim, Jin J. *et al*.

combined MSCs with poly(lactide-co-ε-caprolactone) NFs. The systems, containing 1× 106 MSCs per NF construct, were sutured onto the epicardial surface over the infarcted region of a rat MI model. Four weeks after treatment, echocardiography showed that SC administration, regardless the co-administration of NFs, resulted in LV dilation and improved EF compared with the control groups, and SCs survived and differentiated into cardiomyocytes. Only infarct area was significantly reduced in the

MSC-NFs group compared to other groups [87]. More recently poly(ε-caprolactone) was mixed with gelatin to prepare NFs by electrospinning. 2 × 106 MSCs were seeded onto these hybrid scaffolds and transplanted into a rat MI model. Cells within the NFs were able to migrate towards the scar tissue, promoting new blood vessel formation at the infarct site. Consequently, 4 weeks after transplantation, the seeded NFs restricted the expansion of the LV wall, reduced the scar size and improved cardiac function significantly compared to the other groups [88]. Other similar co-polymers such as poly-glycolide-co-caprolactone (PGCL) and polyglycolic-acid (PGA) have been used as synthetic and biocompatible NFs for myocardial implantation. Piao H. *et al*. seeded 2 × 106 BMSC on PGCL-NFs and injected such systems into the epicardial surface in a rat MI model. Four weeks after implantation, BMSC-NF group showed higher but no statistically significant migration of BMSC into the epicardial region, as well as a greater differentiation rate towards cardiomyocytes. Induction of neovascularization, reduced fibrosis, positive remodeling and ameliorated LV function were detected in BMSC-NF treated group when compared to controls [89]. In the work of Ke Q. *et al*. PGA-NFs were combined with ESC (5 × 104), being then transplanted onto the surface of ischemic myocardium of infarcted mice. ESC-NF treatment not only improved blood pressure and ventricular function, but also had significantly higher survival rates compared to all other groups eight weeks after treatment [90].

More recently, polyurethane (PU) has drawn attention due to its softness, elastic and biodegradation characteristics. In addition, PU allows CMC to grow in organized layers matching physical and mechanical properties of the native tissue [91,92]. Thus, Blumenthal B. *et al*. seeded SM (5 × 106 cells) on such systems but, interestingly, they previously transfected the SM with DNA of VEGF, HGF, SDF-1, or serine–threonine protein kinase (Akt1). Their final constructs resulted in GF-producing myoblast-seeded PU NFs. After being sutured at the epicardial zone of infarcted rats, SM-NFs were found to be accepted by the host with no inflammatory reaction detected after 6 weeks. This was correlated with enhanced angiogenesis when SMs were transfected with VEGF, HGF and Akt1, and with reduced infarction area when SM overexpressed SDF-1 and Akt1 or when SMs were untransfected [93]. Two years later, in 2012, a couple of interesting studies were performed in the

same direction. On one hand, von Wattenwyl R. *et al*. used VEGF-overexpressing myoblasts (5 × 106) seeded on PU-NFs [94]. On the other hand, Poppe A. *et al*. transfected SM (5 × 106) with HGF and then seeded them on the same DDS [95]. Apart from stimulating endothelial cell motility and enhancing angiogenesis, intramyocardial HGF secretion after ischemic injury was associated with less severe ventricular enlargement and with an improved cardiac function [96]. In both cases, the seeded scaffolds were intramyocardially transplanted in infarcted rats, and six weeks later hemodynamic parameters and histological analysis were performed. The administration of HGF-overexpressing SM in PU-NFs resulted in an increased capillary density on the infarcted and peri-infarcted regions. Nevertheless, statistical analysis showed no significant changes in infarct size between groups. Regarding cardiac function, only the HGF overexpressing SM-NF treated group showed a significant improvement from baseline at the end of the study [94,95]. These results are in correlation with the study of Giraud

M.N. *et al*., who studied how myoblast-seeded PU-NFs could prevent cardiac dysfunction. Highly porous NFs with SM (5 × 106) were attached to the outer myocardial scar surface of MI rats. Only SM-NFs significantly prevented progression towards heart failure 9 months after treatment compared to the other groups, but this effect vanished 12 months after treatment. Interestingly, the systems were correctly incorporated into the cardiac tissue as new-formed vessels were formed inside the DDS [97].

2.2.2.2. Nanofibers in protein-based therapies.

The use of NFs as protein delivery systems is a relatively new field and only a limited number of studies have explored their application in cardiac repair. For instance,

Wang Y. *et al*. formulated poly(lactic-co-glycolic acid) (PLGA) NFs loaded with FGF (15 μg). This system significantly enhanced neo-vascular formation, blood flow, FS and the number of proliferating cells 6 weeks after implantation in a mini-swine MI model [98]. In another approach, poly-vinyl-alcohol (PVA) was combined with dextran to form solid injectable NFs for the delivery of FGF (100 μg). These systems were tested in a large ovine MI model. FGF-NFs were sutured to animals' epicardium, showing a sustained release of FGF that strongly stimulated angiogenesis and increased wall thickness index in the infarcted myocardium 2 months after treatment. The NFs also significantly attenuated the increase in LV end-systolic diameter, but did not improve cardiac function [99]. Positive results were reported by Zhang G. *et al*., who reported that PEGylated fibrin NFs loaded with SDF-1 (100 ng), when injected in a mouse MI model, significantly increased myocardial recruitment of c-kit+ cells compared to controls two weeks after treatment. Enhanced stem cell homing was maintained at 28 days, when LV function was significantly

improved in comparison with the controls [100]. More recently, our group prepared smooth polymeric NFs of stat-modified PLGA to deliver NRG to the heart. *In vivo* biocompatibility studies demonstrated that NFs were present in the heart 3 months after administration and a constructive tissue remodeling was observed indicating good incorporation into the organism [101]. In ongoing studies, the efficacy of this system is being evaluated.

2.2.3. Nano and micro-particles

DDSs based on nano and microparticles (NPs and MPs, respectively) have shown great potential to improve the treatment of many diseases, including cardiovascular disorders. They are solid particles in the nanometer or micrometer size range in which the active principle is dissolved, entrapped, encapsulated or adsorbed [102]. There is a long list of materials that can be used to prepare particles of a desired size. Depending on the raw materials employed, drug release profiles, particle degradation and location of the particles can be controlled. Generally, NPs and MPs suffer faster degradation processes than hydrogels or NFs. This higher biodegradability allows their total elimination from the biological tissues avoiding chronic inflammation responses. Together, these characteristics make particles one of the more versatile DDS on the market [103].

2.2.3.1. Nano/microparticles in cell-based therapies.

Regarding strategies based on the use of SCs combined with NPs, covalent coupling, adsorption and internalization of NPs inside cells have been used [104]. It is important to note that NPs were not used for encapsulating or conveying SCs on their surface due to their relatively small size, but for augmenting their circulation time, targeting cells towards specific tissues, improving SC function *in vivo* [104], modifying cell behavior [105], delivering biomolecules and genes and for diagnostics and imaging methods [106].

On the other hand, MPs can be formulated to encapsulate [107,108] or to convey [37,109] cells on their surface. Nevertheless, although alginate and matrigel MPs have reported promising results [107,108], only few studies have been performed with MPs made of synthetic materials. In a recent study human amniotic fluid SCs (1 × 106) were encapsulated inside PLGA porous MPs of about 250 μm. The efficacy of these systems was tested in a rat MI model, showing that animals treated with SCs–MPs had a significantly increased capillary density and positive remodeling, which resulted in improved cardiac function 4 weeks after treatment compared to other groups. SCs were clearly retained at the site of injection and were differentiated towards cardiomyogenic and angiogenic lineages [110]. In another study, Penna C. *et al*. demonstrated that PLGA-MP enhanced MSC survival and regeneration in the hostile environment of post-ischemic tissues [109].

2.2.3.2. Nano/microparticles in protein-based therapies.

Encapsulation of proteins in NPs/MPs is one of the approaches that have been most extensively investigated to protect therapeutic molecules against *in vivo* degradation and to release drugs in a controlled manner [36,102]. Apart from their well-established efficiency as DDS, their surface modification possibilities [111] have given NPs and MPs an interesting therapeutic potential. In fact, active targeting [112] is a very common strategy that has given NPs a particular interest for intravenous administration [113], since they can pass through the microcirculation easily [114] and they are not very vulnerable to immune clearance [115,116], finally reaching heart tissue. Nevertheless, any targeted NP formulation has been clinically approved yet [111]. Regarding MPs, their relative large size makes their intravenous administration impossible without causing undesired side effects. Consequently, in the few *in vivo* studies developed so far, local delivery of NPs and MPs remains the most common way of administration. Along these lines, Sy J.C. *et al*. developed poly(cyclohexane-1,4-diyl acetone dimethylene ketal) MPs (polyketal-MPs) and PLGA-MPs of around 20 μm encapsulating SB239063 (0.5 mg), which were intramyocardially injected in a rat MI model. SB239063 molecule is an inhibitor of apoptotic protein p38, which is related to the progression of cardiac dysfunction. Both types of MPs allowed an *in vivo* sustained release of SB239063 at least for 7 days. Authors observed significant less fibrosis and improvements in cardiac function 21 days after treating MI rats with the SB239063-polyketal-MP group but, interestingly, no significant improvements with respect to controls were detected in the SB239063-PLGA-MP animals [117]. Based on these results, the same group also synthesized superoxide dismutase 1 (SOD1) polyketal-MPs (protein:polymer ratio of 0.05) (10 μm). SOD1 is a protein with antioxidant effects that has proved to favor infarct size reduction after a MI event [118]. When injected intramyocardially in a rat MI model, MPs were detected for up to 10 days in the myocardium. Superoxide levels were decreased in animals treated with SOD1-MPs when compared to controls, and the same significant trend was observed in CMC apoptosis ratios. Three days after treatment, improvements in FS were only observed when SOD1 and SB239063-MPs were co-injected, suggesting the need of multiple therapeutics dosage to combat the different phases of the disease [119]. In order to enhance NP uptake by CMC, SB239063-polyketal-NPs were covered with the sugar N-acetyl-D-glucosamine (GlcNAc) [120] and their efficacy was tested in a rat MI model. The number of apoptotic CMCs was significantly lower in the GlcNAc-SB239063-NP group compared to other groups 24 h after treatment. This result was confirmed by an uptake study, where GlcNAc- polyketal-NPs were clearly more captured by CMCs than non-coated NPs. Three days after treatment echocardiography analyses showed that only rats that received loaded-NPs had a significant reduction in infarct size/area-at-risk and an improved FS [121].

Due to their well-established *in vivo* biocompatibility, safety and FDA approval, polyesters like PLGA are widely used in cardiac tissue engineering. One interesting example is the study by Chang M.Y. *et al.*, where PLGA-NP of 60 nm, 200 nm and 1 μm were synthesized containing different concentrations of IGF-1. When intramyocardially injected in a MI mice model, 24 h after treatment IGF-1 was significantly more in IGF-1-NP treated group compared to free IGF-1 administration. IGF-NP treated animals also showed a significant reduction in infarct size and number of apoptotic CMCs and improved LV-EF 21 days after treatment compared to free IGF administration and non-loaded NPs. Finally, the authors reported that 60 nm NPs were most effective in binding IGF-1 and consequently preventing CMC apoptosis [122]. Our group has also examined the feasibility of using PLGA-MPs encapsulating therapeutic proteins to promote cardiac regeneration. First, Formiga F.R. *et al*. prepared PLGA-MPs (5 μm) containing VEGF (35 μg per 50 mg of MPs) by solvent extraction/evaporation method using TROMS technology. This technology based on double emulsion and solvent evaporation methods allowed them to encapsulate labile proteins without altering their natural properties and bioactivity [123]. The systems were administered via intramyocardial injection in a rat MI model. One month after treatment, PLGA-MPs were present in the myocardium, and significant increments in angiogenesis and arteriogenesis in the infarct and peri-infarct areas of the injured hearts in the VEGF-MP group were detected in comparison to controls. The increased revascularization of the tissue translated into a beneficial effect in the remodeling processes, with a significantly greater thickness of the LV wall in the VEGF-MP treated animals in comparison to the rest of the groups [123]. In addition, Simón-Yarza T. *et al*. combined both angiogenic and antioxidant drugs to establish potential synergistic effects. With this aim, PLGA-MPs of 5 μm containing VEGF (50 μg per 50 mg of MP) and PLGA-NPs (150 nm) encapsulating Coenzyme Q10 (CQ10, 1.5 g per 3 g of NPs) were formulated. CQ10 is known due to its antioxidant and cardioprotective roles [124]. The efficacy of VEGF-MPs and CQ10-NPs was studied in a rat MI model, where MPs were intramyocardialy injected and NPs were administered orally. Separately, both treatments demonstrated significantly increased EF three months after administration when compared to the other groups. That was correlated with a highly significant increase in the number of capillaries in the infarct and periinfarct areas. Interestingly, CQ10-NPs showed better outcomes than commercial CQ10, what was attributed to the ability of NPs to improve oral bioavailability and to the sustained release of the encapsulated CQ10. Unfortunately, combined treatment failed to offer synergy, and no EF improvements could be observed [125]. In another study, we successfully delivered FGF-1 and NRG-1 to the ischemic tissue using PLGA MPs (5 μm), administering a final amount of 1740 ng of FGF and/or 1300 ng of NRG in treated animals. Three months after treatment, global cardiac function,

infarct size, fibrosis, revascularization and cardiac stem cell recruitment were significantly increased in GF-MP treated groups (FGF, NRG or FGF/NRG) when compared to controls [126] (Fig. 5). Our study is providing very useful data regarding the underlying mechanisms contributing to the beneficial effects of this therapy, especially those linked to endogenous regeneration, which might be very useful for the design of novel cardiac repair approaches. As a prerequisite for clinical application, we next determined the long-term therapeutic effectiveness and safety of this therapeutic strategy in a preclinical large animal model of myocardial infarction (minipigs) demonstrating that cytokine delivery MPs are able to restore cardiac function [35]. This technology could soon be translated to humans.

Finally, Oh K.S. *et al*. prepared semi-synthetic NPs made of a lecithin core and a Pluronic F-127 coating with a Capryol 90 hydrogel system in order to achieve a stable localization of VEGF-NPs at the ischemic area. In this case, VEGF (5 µg) was encapsulated inside the natural core of NPs and final systems (270 nm) were epicardially injected in a rat MI model. Although both VEGF-NP and VEGF-NP-hydrogel resulted in significantly improved capillary density, significantly higher cardiac function was observed in VEGF-NP-hydrogel group compared to all other groups [127].

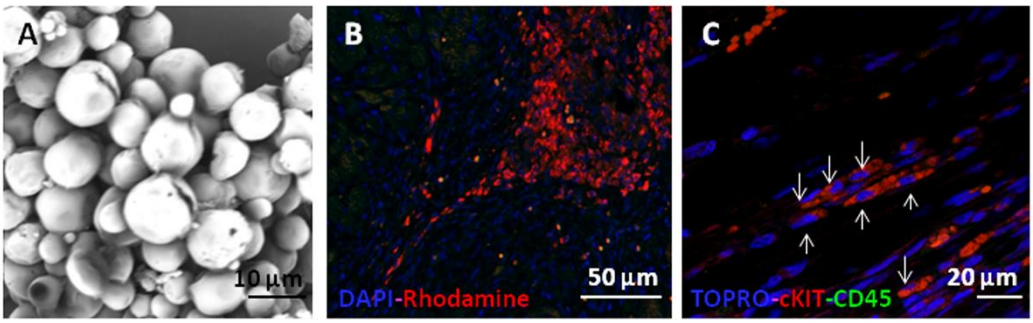

Fig. 5. NRG–PLGA MPs. A) Scanning electron microscopy of NRG–PLGA MPs. B) Tissue retention of fluorescent PLGA MPs 1 month after intramyocardial injection in a rat MI model. C) Cardiac progenitor cell recruitment 1 week after intramyocardial injection of NRG–PLGA MPs.

2.2.4. Liposomes

The latest example of biomaterial-based DDSs reviewed here are liposomes. Liposomes are sphere-shaped vesicles consisting of one or more phospholipid bilayers (Fig. 4) [128]. The liposomal drug Doxil was the first lipid system to be used in clinical practice in 1999. Although nowadays they are used in other diseases [129,130], there are still no liposomal formulations approved for human use for the treatment of cardiovascular disease.

Concerning liposomes, active targeting has been deeply explored, and surface-attached targeting molecules have been employed for preparing liposomes used to target MI, constituting a promising approach for heart therapy [131]. Thus, immunoliposomes (ILs) presenting phosphatidylserine (PS) on their surface can be easily recognized by macrophages, which are relevantly concentrated in the cardiac tissue after MI due to the inflammatory process, providing specific accumulation of targeted ILs in the damaged heart. This strategy has been used by Harel-Adar T. *et al*. who synthesized PS-ILs. Firstly, the systems were intraperitoneally injected in rats, and 3 h later, the peritoneal cells were analyzed. The state of macrophages changed from pro-inflammatory to anti-inflammatory. That was translated in significantly higher levels of anti-inflammatory cytokines on the peritoneal lavage fluids in treated animals compared to controls, which confirmed the previous result. When this was translated to a rat MI model, the same protective trend was observed. Interestingly, PS-ILs induced cardiac macrophages to secrete anti-inflammatory cytokines 3 days after treatment, which is 1 day earlier than under normal conditions. The treatment also promoted angiogenesis, prevented ventricular dilatation and remodeling, and small scars were detected in comparison with control groups [132].

Concerning cell therapy and liposomes, no results have been published yet, so the following section will be focused on liposomes for protein delivery.

2.2.4.1. Liposomes in protein-based therapies.

A large number of the physicochemical properties of liposomes [133] have been explored for active targeting of therapeutic drugs to myocardial ischemic regions [134]. PEGylation strategy is used to improve permanence time and to reduce the opsonization process of systems administered in the blood [135,136], consequently increasing liposome therapeutic efficacy [137]. Regarding heart targeting, one adhesion molecule that is upregulated on endothelium in response to ischemia and inflammation is P-selectin [138]. In the work of Scott R.C. *et al*., PEGylated phosphati-dylcholine/cholesterol liposomes were synthesized and incubated with IgG2a mouse antibody to rat P-selectin. VEGF (0.12 g/kg animal weight) was encapsulated in such systems and administered via tail vein immediately after induction of MI in rats. ILs were selectively accumulated in the myocardial infarct region [139], allowing targeted VEGF delivery to post-MI tissue, which resulted in significant increase of FS and improved systolic function. These functional improvements were associated with an increase in the number of vessels in the MI region of treated animals [140]. Similarly, Wang B. *et al*. developed anti-P-selectin conjugated ILs to target the delivery of VEGF to the heart, which significantly improved vascularization and cardiac function [141]. Using other approach, Yamada Y. *et al*. prepared Sialyl Lewis X molecule (SLX) ILs (100 nm)

encapsulating EPO [142]. SLX is a carbohydrate present in the leucocyte membrane known for interacting with selectin cell-adhesion proteins and to play a vital role in cell-to-cell recognition processes [143]. SLX-EPO-ILs were intravenously administered in a rabbit MI model (2500 IU of EPO/kg body weight). Only ILs but no non-targeted liposomes were selectively accumulated at the border area of the infarcted myocardium, significantly increasing EPO levels in the heart 48 h after treatment. LV remodeling, EF, FS and reduction on MI size were significantly improved in the SLX-EPO-ILs group when compared to controls. Similar results were observed for the number of CD31+ microvessels and for EPO receptor expression [142].

3. Emerging tissue engineering strategies for heart regeneration after myocardial infarction

The combination of cells or protein with biomaterials has proved to be effective in preclinical animal models of MI. In brief, regarding cell therapy, it has been possible to enhance cell viability and engraftment. Biomaterials have enabled cells to assemble into effective tissue substitutes that may restore cardiac functions and structure. Concerning protein therapy, the use of DDSs has allowed researchers to protect growth factors against *in vivo* degradation and to achieve a controlled release over time, favoring important processes during cardiac healing such as angiogenesis or SC differentiation towards cardiac lineages. Moreover, SCs can directly benefit from the action of therapeutic GFs. For instance, SCs depend on GFs for correct survival and differentiation (Fig. 6). In addition, SC paracrine secretions together with therapeutic GFs may achieve a better regenerative effect. Thus, some authors have investigated the combination of both cellular and protein therapies together with biomaterial-based delivery systems. This integrated approach, known as the tissue engineering triad, has attracted considerable attention over the past years (Fig. 6).

Since tissue engineering is a novel approach only a few studies have been published so far, although showing interesting results. In the first study in 2005, a bioengineered NF scaffold made of polyglycolic acid succeeded in incorporating BMSC (1 × 107) and FGF (0.2 μg). When such systems were transplanted in a rat MI model, cells were detected inside the scaffold 4 weeks after implantation, and NFs were absorbed by the host tissue indicating good incorporation into the organism. Global cardiac function and capillary density were significantly improved in BMSC–FGF-NFs treated animals when compared to BMSC or FGF loaded NF groups [144]. Similarly, our group investigated the feasibility of using NRG-releasing PLGA-MPs (20 μm diameter, 1.8 μg NRG/mg of MP) combined with ADSC (2.5 × 105 or 5 × 105) as a multiple growth factor delivery-based tissue engineering strategy for

implantation in the infarcted myocardium [37]. ADSC–NRG-MPs proved to be compatible with intramyocardial injection in a rat MI model and systems were present in the peri-infarcted tissue 2 weeks after implantation [37]. Efficacy studies are currently being performed. Apart from those polymers, other synthetic materials have been employed for generating injectable DDSs. The group of Kraehenbuehl T.P. *et al*. formulated a three-dimensional metalloproteinase-sensitive PEG-based hydrogel, and used such systems to deliver thymosin ß4 (Tß4, 2.5 µg) in combination with ESCs (6.6 × 106) and smooth muscle stem cells (3.3 × 106) in ischemic injuries of a rat MI model. Tß4 protein activates the survival kinase Akt, protects cardiac muscle form death after ischemic damage and promotes angiogenesis, making it an interesting molecule for cardiac regeneration [145]. Thus, the cell seeded-Tß4-hydrogels effectively preserved contractile performance 6 weeks after myocardial infarction and attenuated LV dilation compared to controls and to the Tß4-hydrogel treated group [146]. Neovascularization and infarct size were also significantly improved in cell seeded-Tß4-hydrogels and Tß4-hydrogels groups compared to controls.

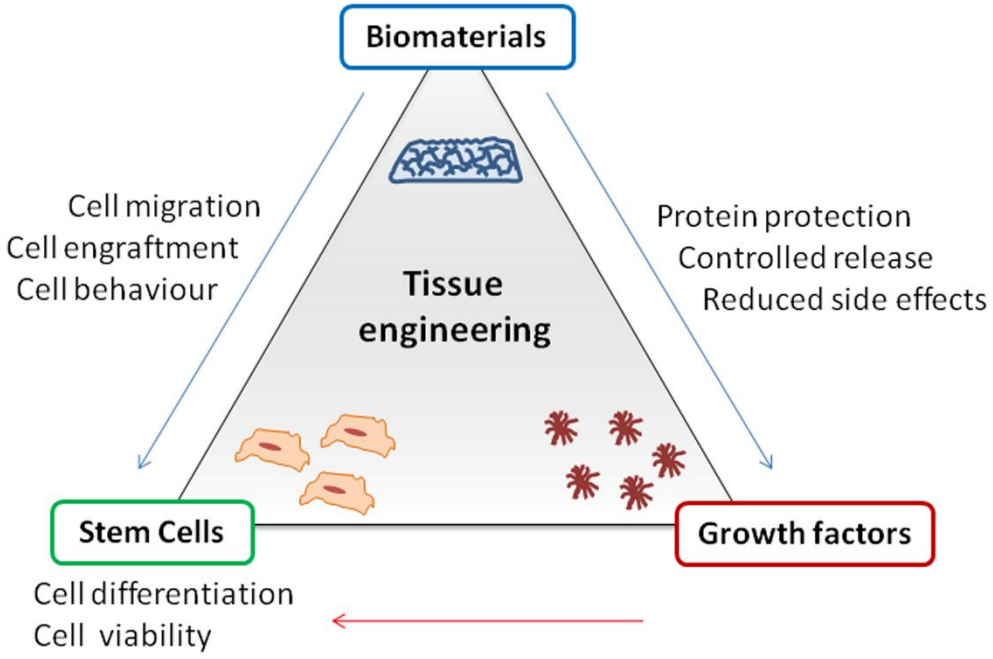

Fig. 6. Tissue engineering triad, with the benefits that each element (growth factors, SCs and biomaterials) gives to other element.

Concerning full synthetic biomaterials, self-assembling peptide RADA16-II has been used to create injectable hydrogels incorporating IGF (approximately 1 ng) in combination with CMCs (1 × 106) [147] or CPCs (1 × 105) [148] for cardiac repair. In both studies the administration of cell-seeded-IGF-hydrogels

significantly improved the recovery of myocardial structure and function in rats one month after treatment. Apoptosis was also reduced regardless of the cell type, but a reduced infarct size and increased capillary density were only reported when CPCs were co-injected with IGF [148]. In any case, the presence of IGF resulted in a protective environment that favored SC proliferation. In other study using the same RADA16-II peptides, Dubois G. *et al*. compared the efficacy of skeletal myoblasts (SMs) and PDGF therapies to SM–PDGF tissue engineering in a rat MI model. Significantly greater angiogenesis was observed in all GF-treated groups compared to controls one month after treatment. However, this was not correlated with an improved cardiac function. In fact, LV function was not improved in either of the treated groups compared to controls at the same time point. The lack of functional improvements observed *in vivo* was explained by an *in vitro* SM viability study. Authors concluded that specific tailoring of the biomaterial to the cell type is required for correct cell survival [149].

The combination of synthetic and natural biomaterials is common in tissue engineering, and relevant promising results have been obtained. For instance, semi-synthetic hydrogels made of PEGylated fibrin biomatrix efficiently bound HGF and entrapped BMSC (5 × 105). After administration in a mouse MI model, the systems allowed significant improvements in cell prevalence at the injection site for at least 4 weeks, compared to free cell administration. Interestingly, in BMSC– HGF-hydrogel treated animals, cell retention was accompanied by the lowest levels of apoptosis and the highest LV function recovery among all the groups, confirming that tissue engineering was more effective than protein or cell therapy alone [150].

In order to obtain a system inspired by tissue specific niches able to mimic the real biological process of heart healing, several DDSs have been combined. Thus, in the work of Holladay C.A. *et al*. MSCs were seeded onto semi-synthetic hydrogels of collagen, 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide and N-hydroxysuccinimide. 2 μg of interleukin-10 (i10), the most potent anti-inflammatory cytokine, was incorporated into dendrimer polyplexes and incubated with the hydrogels. Four weeks after treating MI rats with such systems, SC retention and FS were found to be significantly improved in animals which received MSC-i10-systems compared to the rest of the groups. Improved function was associated with increased infarcted wall thickness, decrease of cell death and a change in macrophage markers from mainly cytotoxic in the MSC-hydrogel group to mainly regulatory in MSC-i10-system treated group, confirming the success of tissue engineering over cell therapy [151]. Using another strategy, Miyagi Y. *et al*. combined both NFs and hydrogels. Thus, authors first synthesized a gelfoam/poly-(ε-caprolactone) NFs construct. Then, BMSC (1 × 106), stem cell factor and/or SDF (30 ng of each one) were incorporated in a polymeric temperature-sensitive hydrogel made

of valerolactone and PEG. MI was induced in rats, and after two weeks NFs were transplanted covering the infarcted area. At the same time, hydrogels were injected next to the NFs. Four weeks after treatment, those animals treated with NFs and SC–GF-hydrogels showed a significant global cardiac function improvement compared to animals treated only with the NFs. Although NFs in combination with SC–GF-hydrogel treatment resulted in better heart recovery compared to controls, no significant differences were observed when compared to animals that had received NFs and SC-hydrogel or GF-hydrogel. Finally, neovascularization and wall thickness were enhanced in all treated animals compared to controls [152].

3.1. Challenges ahead

The above examples represent some of the ways in which tissue engineering strategies are being investigated to address cell and protein hurdles. Interestingly, several synthetic biomaterial-based DDS have been explored although no one has proved to be better than the others. In any case, if we want to reach clinical applications, new techniques for treating MI must not be more invasive than the existing cardiac procedures. Concerning this aspect, hydrogels, NPs, MPs, and liposomes achieve this goal, and can be administered by trans-endocardial injection or via catheters. On the other hand, NFs need to be attached to the pericardium, so a more invasive administration technique is required. However, it is highly desirable that biomaterials should provide satisfactory mechanical support to the infarcted heart, in order to favor functional recovery of the damaged organ [33]. In this sense, NFs have proved to be able to contribute more efficiently to the heart's mechanical properties than other DDS. However, given the intricate anisotropic mechanical behavior of myocardium, it is not easy to produce a biomaterial that responds to mechanical stresses in a way that is similar to the heart itself. In this regard, PU seems to be the most promising biomaterial [91,92]. Another recommendable characteristic for DDS in cardiac repair is the ability to mimic the natural heart microenvironment [153]. This way, biomaterials are used as an alternative to extracellular matrix, being NFs and hydrogels the DDS that reproduce natural conditions in the best way possible. Taking all of this into account, we can say that the search for the optimum biomaterial-based delivery system still continues and further research in this area is guaranteed.

We cannot forget that the mammalian heart is a complex organ composed of a heterogeneous cell population. Consequently, the potential of a long list of SCs and GFs for regenerating the infarcted heart tissue has been investigated so far. Tissue engineering has proved to be useful in regenerative medicine in terms of high viability and long term engraftment of cells. In addition, cardiac repair and regeneration is favored by effective delivery of therapeutic GFs.

Nonetheless, although all tissue engineering strategies regardless of the therapeutic agent employed have enhanced myocardial functional, the repair mechanisms remain unclear at the moment. It is still unknown whether the repair of the infarcted heart is caused by the functional activity of the cells or by structural changes brought by biomaterials or proteins. Therefore, myocardial tissue engineering approaches have to be developed considering both cell and GF requirements of the heart for successful cardiac recovery. In addition, functional integration between the graft and the host tissue, in both electromechanical and vascular terms, still remains a major challenge that must be considered when designing new cardiac tissue engineering approaches [154]. The establishment of well-defined protocols and the optimization of the synergies between the different cells and GFs are required before clinical applications can be attained. In fact, tissue engineering is still at the development phase and the only clinical trial evaluating a tissue engineering strategy is the one called ALCADIA. In this ongoing trial CPCs and FGF are being combined in a gelatin hydrogel to treat ischemic cardiomyopathy (Clinicaltrials.gov identifier NCT00981006). Thus, all of these promising results should be considered preliminary, and further studies are needed to confirm the possible benefits of myocardial tissue engineering.

4. Conclusions and future prospects

New contributions to the advancement and optimization of classical treatments for MI have allowed a reduction in the number of death due to this pathology over recent decades. However, complications deriving from MI remain a big problem. Therefore, new strategies have been investigated to overcome such limitations, and the ones that have shown the most promising results so far are cell and protein therapies [14,15]. As this review has illustrated, both of these have encountered various challenges when tested in clinical trials, related to the low cell engraftment and the rapid degradation of therapeutic proteins once they are administered. Fortunately, it seems that nowadays we are close to reaching their full potential by combining them with biomaterials. Thus, this review has also demonstrated the relevance of biomaterials in the repair and regeneration of the damaged heart. Currently, synthetic hydrogels, NFs, NP, MP and liposomes are being investigated in depth in cardiac repair, in combination with cells and proteins. The capacities of these DDS to increase cell survival and engraftment, and to protect and control GF release are the main reasons for their success. However, the type of material, cell and GF sources, timing, dose and injection technique are still uncertain, and further investigation is mandatory in order to achieve the best patient outcomes. The current challenge is to establish a perfect combination of three components: biomaterials, cells and proteins. Tissue engineering is a

rapidly evolving discipline. In fact, it is expected that in the next 10–20 years, these therapies will account for more than half of the new drugs introduced on the market [155]. Great advances have been made in the last few years, although there are still several aspects to improve and current results should be considered preliminary. In the future, MI treatments will surely represent an amazing challenge in terms of biomaterials and delivery systems with the final goal of providing many benefits to MI patients.

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HYPOTHESIS AND OBJECTIVES

Protein therapies have failed to show consistent benefits in clinical trials of ischemic cardiovascular diseases. The main reason for this failure is thought to be the protein instability *in vivo* that makes the continuous administration of the protein necessary. However, this causes important side effects.

The hypothesis for this study is that encapsulation of therapeutic proteins in polymeric microparticles acting as delivery systems will help to increase protein bioavailability, to achieve a controlled release over time and to specifically deliver proteins to the targeted therapeutic sites, thus avoiding the toxicity caused by the drug at untargeted sites. Moreover, the loaded microparticles will modulate the inflammatory response of the cardiac macrophages favoring its polarization toward a reparative phenotype.

To test this, the following objectives were formulated:

1. To prepare and characterize PLGA and PEG-PLGA microparticles loaded with NRG1 and to study the *in vivo* release, bioactivity, biodegradation and phagocytosis of the protein delivery systems in a rat model of myocardial infarction.

2. To evaluate the efficacy of PLGA and PEG-PLGA microparticles loaded with NRG1 or FGF1 in inducing cardiac repair after a myocardial infarction in a rat model of the disease.

3. To study the interactions between PLGA microparticles loaded with NRG1 and the inflammatory response of the heart after a myocardial infarction induced in a mouse model.

CHAPTER 1

TRACKING THE *IN VIVO* RELEASE OF BIOACTIVE NRG FROM PLGA AND PEG–PLGA MICROPARTICLES IN INFARCTED HEARTS

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Abstract:

The growth factor neuregulin (NRG) is one of the most promising candidates in protein therapy as potential treatment for myocardial infarction (MI). In the last few years, biomaterial based delivery systems, such as polymeric microparticles (MPs) made of poly(lactic co glycolic acid) and polyethylene glycol (PLGA and PEG–PLGA MPs), have improved the efficacy of protein therapy in preclinical studies. However, no cardiac treatment based on MPs has yet been commercialized since this is a relatively new field and total characterization of polymeric MPs remains mandatory before they reach the clinical arena. Therefore, the objective of this study was to characterize the *in vivo* release, bioactivity and biodegradation of PLGA and PEG–PLGA MPs loaded with biotinylated NRG in a rat model of MI. The effect of PEGylation in the clearance of the particles from the cardiac tissue was also evaluated. Interestingly, MPs were detected in the cardiac tissue for up to 12 weeks after administration. *In vivo* release analysis showed that bNRG was released in a controlled manner throughout the twelve-week study. Moreover, the biological cardiomyocyte receptor (ErbB4) for NRG was detected in its activated form only in those animals treated with bNRG loaded MPs. On the other hand, the PEGylation strategy was effective in diminishing phagocytosis of these MPs compared to noncoated MPs in the long term (12 weeks after injection). Taking all this together, we report new evidence in favor of the use of polymeric PLGA and PEG–PLGA MPs as delivery systems for treating MI, which could be soon included in clinical trials.

Key words:

Myocardial infarction, Microparticles, Protein therapy, Phagocytosis, Bioactivity, Biotinylation.

1. Introduction

Considered globally, cardiovascular diseases are the leading cause of death worldwide, being responsible for 30% of total deaths every year, and myocardial infarction (MI) is the most common form that they take [1]. In spite of their clinical importance, these disorders are far from being controlled. Current treatments for MI have several limitations [2], because they are mainly palliative and not regenerative. Consequently, several alternative strategies have been developed over the last few years to regenerate the infarcted heart, the most relevant being those based on the administration of stem cells [3] or growth factors (GFs) [4]. These days, state-of-the-art of cardiac recovery suggests that the success of stem cell therapy is mainly due to a paracrine effect associated with the release of GFs [5–7]. In fact, direct GF administration to the infarcted tissue has proved to favor the regeneration of the damaged heart by inducing recruitment of endogenous progenitors, encouraging differentiation into functional cardiomyocytes and vascular cells, favoring angiogenesis, promoting perfusion, reducing fibrosis and inhibiting apoptotic processes [8].

To date, a number of GFs have been studied and their therapeutic effect in heart recovery has been reported (reviewed in [2]). Among them, neuregulin (NRG) is nowadays considered one of the most promising GFs in the field of cardiac regeneration [9]. The NRG pathway is known to be essential for the development of adult heart and the maintenance of cardiac function [10]. Interestingly, preclinical studies have already shown that NRG administration into infarcted hearts induces angiogenesis, favors cardiomyocyte proliferation and improves cardiac function [11]. NRG's potential for treating MI was also observed in clinical trials when NRG was administered daily through parenteral injection [12,13]. However, the clinical relevance of NRG is still unclear and must be confirmed, since other promising GFs such as vascular endothelial growth factor or fibroblast growth factor have failed to provide the desired effect when studied in larger scale trials [14,15]. This is due to major limitations resulting from low bioavailability, difficulties in targeting the heart, the lack of long term stability and the short half-life of the therapeutic proteins [2]. In an attempt to overcome such limitations, GFs have been combined with drug delivery systems (DDSs), which are biocompatible vehicles presenting different architectures and characteristics [16]. Among DDSs, microparticles (MPs) are solid particles in the micrometer size range in which drugs are generally entrapped, encapsulated or attached [17]. They have provided relevant benefits in cardiac repair, such as localized and sustained protein release over time and the protection of the therapeutic GFs against degradation [18,19].

Our group has proven experience in the synthesis of biodegradable polymeric MPs made of poly(lactic co glycolic acid) (PLGA) and poly-ethylene glycol (PEG)–PLGA, copolymers [20,21], approved by the FDA for their use in humans [22]. Recently, we have demonstrated the safety, biodegradation and heart retention of PLGA MPs intramyocardially injected in rats [23]. Moreover, when these systems were loaded with NRG, beneficial effects in tissue remodeling and cardiac function were observed in both rat and pig models of MI [21,24]. On the other hand, PEG coating of MPs has proved to be efficient in giving MPs a stealth character [20] and in preventing MP opsonization in the blood [25]. PEGylation also has other effects, such as altering MP biodegradation or modifying drug release [26]. Despite the studies using these systems performed to date, total characterization of polymeric MPs remains mandatory before they reach the clinical arena. With this aim, it is essential to specifically track the therapeutic GF and the DDSs within the organism, in order to determine their *in vivo* behavior. Nowadays, with the progress made in molecule labeling and imaging techniques, it is possible to differentiate injected proteins from the endogenous ones and to identify them in the biologic tissue once they are administered. Similarly, labeled DDSs can easily be followed through the organs after injection, which helps us to understand how both therapeutic agents and vehicles interact with each other and with the biological environment under *in vivo* conditions.

The goal of this study was thus to characterize the *in vivo* behavior of NRG loaded PLGA and PEG–PLGA MP in a rat model of MI. In order to distinguish between exogenous and endogenous molecules, therapeutic NRG was labeled with biotin (bNRG). PLGA and PEG–PLGA MPs were also labeled using rhodamine for *in vivo* follow up. Hence, besides the *in vitro* characterization of both DDSs, we further determine MP biodegradation in the cardiac tissue and *in vivo* bioactivity and release of bNRG after being encapsulated and administered in MPs into the heart. Finally, the MP phagocytosis rate was evaluated to assess the effect of PEGylation in the clearance of the particles from the cardiac tissue.

2. Materials and Methods

2.1. Materials

Recombinant human NRG-1b-iso was obtained from EuroBioSciencies (Friesoythe, Germany). Western blot (WB) gels NuPAGE® Bis–Tris and electrophoresis MOPS SDS running buffer were purchased from Novex® Life Technologies TM (Waltham, MA USA), transfer Tris/Glycine buffer and molecular weight standard Precision Plus Protein Standards Kaleidoscope were obtained from BioRad (Hercules, CA, USA). Horseradish peroxidase conjugated

goat anti biotin antibody, rabbit anti ErbB4-p-Y1284 antibody and Alexa 488 conjugated goat anti rabbit antibody were supplied by Abcam® (Cambridge, UK). Goat anti NRG was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase conjugated donkey anti goat IgG was provided from Invitrogen™ (Carlsbad, CA, USA). Lumilight Plus Western blotting Substrate for WB analysis was from Roche Diagnostics (Mannheim, Germany). PLGA Resomer® RG 503 H (MW 34 kDa) and PLGA:PEG (50:50) Resomer® RGP d 50105 (10% PEG with MW 5 kDa) were purchased from Boehringer-Ingelheim (Ingelheim, Germany). Dichloromethane, acetone and Tween 20 were obtained from Panreac Quimica S.A. (Barcelona, Spain). Bovine serum albumin (BSA), human serum albumin (HSA), rhodamine B and polyethylene glycol (PEG) (MW 400 Da) were supplied by Sigma-Aldrich (Barcelona, Spain). Poly(vinyl alcohol) (PVA) 88% hydrolyzed (MW 125 kDa) was obtained from Polysciences, Inc. (Warrington, PA, USA). EZ-Link Micro Sulfo-NHS-LC Biotinylation Kit (21935) was from Thermo Scientific (Waltham, MA USA). Vectastain ABC Kit was provided from Vector Laboratories Inc. (Barcelona, Spain). Dako Liquid DAB+ Substrate Chromogen System (k3468) was purchased from Dako Diagnósticos, S.A. (Barcelona, Spain). Vicryl and Prolene sutures were from Ethicon Inc. (Johnson & Johnson, Brussels). IsoFlo® employed for animal anesthesia was from Abbot Laboratories S.A. (North Chicago, IL, USA).

2.2. Neuregulin biotinylation process

NRG biotinylation was performed as described in the EZ-Link® Micro Sulfo-NHS-LC biotinylation kit protocol. Briefly, 50 µg of human recombinant NRG were dissolved in 200 µL of Sulfo-NHS-LC-Biotin solution and incubated for 2 h on ice. To remove the excess of biotin a desalting column was used. Biotinylation was confirmed by WB, as previously reported ([27]). Goat anti biotin antibody (1:2000) or goat anti NRG primary antibody (1:50) were used as primary antibodies.

2.3. Preparation of PLGA and PEG–PLGA microparticles containing NRG

bNRG loaded MPs were obtained by the solvent evaporation method using Total Recirculation One-Machine System® (TROMS) procedure [28]. Briefly, for bNRG–PLGA MPs, 50 mg of Resomer® RG 503 H were dissolved in 4 ml of dichloromethane/acetone (3:1) (phase O), injected in the inner aqueous phase and then recirculated together through the system for 90 s. The inner aqueous phase (phase W1) consisted of 20 µg of bNRG, 5 µL of PEG 400, 5 mg of HSA, 5 µL of rhodamine (20 mg/mL) and 75 µL of PBS. Late, W1/O emulsion was injected into the external aqueous phase (20 mL of PVA) (phase W2) and recirculated for 120 s. The multiple emulsion formed (W1/O/W2) was stirred at RT for a minimum of 2 h up to total solvent evaporation. Finally, MPs were washed with ultrapure water, frozen at −80 °C, lyophilized (Virtis Genesis Freeze Dryer 12 EL) and stored at 4 °C until their use. For PEGylated MP, 25

mg of Resomer® 503 H and 25 mg of Resomer® RGP d 50105 were dissolved in the organic phase and MPs were prepared as described above. Non-loaded PLGA and PEG–PLGA MPs were also formulated without including bNRG in the W1.

2.4. Microparticle characterization

2.4.1. Particle size, zeta potential and encapsulation efficiency analysis

Particle size and size distribution were determined by laser diffractometry using a Mastersizer-S® (Malvern Instruments, Malvern, UK). The zeta potential was measured using Zetaplus®, based on the analysis of complete electrophoretic mobility distributions (Brookhaven Instruments, NY, USA). In both cases a minimal amount of MPs was dissolved in deionized water before measurement.

bNRG content of the MPs was quantified by WB analysis. First, the GF was extracted from 1 mg of MPs through DMSO treatment. Secondly, WB was performed as mentioned in Section 2.2, using horseradish peroxidase (HRP) goat anti NRG as primary antibody. Quantitative analysis of the bNRG bands was performed by densitometry using ImageOne software (Bio-Rad Laboratories Inc., Munich, Germany).

2.4.2. *In vitro* degradation study

For the *in vitro* degradation study, 40 mg of PLGA MPs and 40 mg of PEG–PLGA MPs were suspended separately in 5 mL of PBS. Each suspension was divided in four tubes (1.25 mL/tube) and incubated under orbital shaking at 37 °C. At pre set time intervals (24 h, 1, 4 and 12 weeks) one tube of each type of MP was centrifuged at 15,000 g during 10 min. The precipitate was recovered, frozen at −80 °C and lyophilized. Then, particles were weighted and weight reduction was calculated as a percentage. The surface morphology of the MPs was observed by scanning electron microscopy (SEM). Briefly, 0.1 mg of MPs were resuspended in 50 µL of deionized water and sputtered with a thin metallic layer before analyzed.

2.5. *In vivo* experiments

2.5.1. Animal model of myocardial infarction

All animal procedures were approved by the University of Navarra Institutional Committee on Care and Use of Laboratory Animals. A total of 80 female Sprague Dawley rats at 8 weeks of age were obtained from Harlan-IBERICA (Barcelona, Spain). They were housed in appropriate cages, with controlled environment (12 h light/dark cycles at 21 °C), standard diet and water ad libitum during all the experiment. MI induction was performed as described elsewhere [29], after which a total number of 62 animals survived and were

included in the study. Briefly, anesthetized animals (inhalatory isoflurane) underwent endotracheal intubation for mechanical ventilation, and before the operation animals received ketoprofen and fentalyl. The surgical procedure consisted of a left thoracotomy through the fourth intercostal space, and the left anterior descending coronary artery was permanently blocked. Antiinflammatory ketoprofen administration was repeated 24 and 48 h after surgery.

2.5.2. Intramyocardial administration of microparticles

Intramyocardial injection of MPs was carried out one week after arterial occlusion following a well-established procedure [29]. Firstly, 1.2 mg MPs were dispersed in 100 µL of resuspension media (0.1% (w/v) carboxymethylcellulose, 0.8% (w/v) polysorbate 80 and 0.8% (w/v) mannitol in PBS, pH 7.4). Chests were opened and 100 mL of MP suspension was injected with a 29 gauge needle into 3–4 regions in the border zone surrounding the infarct. Finally, the chest was closed and rats were allowed to recover. Animals were divided into 5 groups: sham (PBS injection), animals treated with non-loaded PLGA MPs, animals treated with non-loaded PEG–PLGA MPs, animals treated with bNRG–PLGA MPs and animals treated with bNRG–PEG–PLGA MPs.

2.6. Histological studies

At defined time points (24 h, 1, 4 and 12 weeks), animals were sacrificed and their hearts were collected for histological analysis. Briefly, animals were anesthetized using intraperitoneal ketamine/xylazine (50/5 mg/kg) and chest was opened. Next, hearts were first perfused with PBS to eliminate blood content, then with paraformaldehyde to fix the tissue, and finally with PBS again. Hearts were then harvested, cleaned and submerged into 4% paraformaldehyde at 4 °C during 24 h, followed by dehydration in ethanol 70% at 4 °C at least during 48 h. Finally, they were embedded in paraffin. Transversal sections of 5 µm thickness were cut to carry out histological studies.

2.6.1. *In vivo* release studies

For the analysis of bNRG released from the PLGA and PEG–PLGA MPs, enzymatic immunohistochemistry was performed. 18 sections per heart and treatment were analyzed. First, samples were deparaffinized and hydrated. Then, samples were washed with water and the endogenous peroxidase was quenched by 3% hydrogen peroxide in deionized water. Antigen retrieval was done by incubating the tissue slides with trypsin (0.2% trypsin and 0.1% CaCl2 in deionized water). After this, enzymatic immunohistochemistry using ABC complex against biotin was done. Samples were then rinsed with TBS and incubated with Substrate Chromogen System. When observed under bright

field microscopy, therapeutic protein was detected as a green/brown precipitate. On the other hand, when samples were examined under fluorescence microcopy, MPs gave red fluorescence due to rhodamine. Images were acquired using the Zeiss Axio Imager M1 microscope equipped with a RGB filter. Overlaying and analysis of the images was performed using ImageJ and MicroManagement softwares.

2.6.2. Neuregulin bioactivity *in vivo*

The bioactivity of the bNRG released from the MPs was evaluated *in vivo* by double fluorescent immunohistochemistry against phosphorylated receptor of NRG (pErbB4) and cardiac troponin (cTnT), to identify cardiomyocytes. 12 sections per heart were studied. Briefly, samples were deparaffinized, hydrated and rinsed with water. Antigen retrieval was done by microwaving with 10 mM citrate buffer (pH 6) for 10 min. Samples were next washed with TBS-T and then incubated for 4 h with blocking solution (0.1% TBS-T, 5% BSA, 5% normal goat serum solution) at RT. Incubation with rabbit anti pErbB4 primary antibody was done overnight at 4 °C (1:50 in blockage solution). Samples were then incubated with goat anti rabbit IgG Alexa 488 secondary antibody for 1 h at RT. After washing the samples with TBS-T, mouse anti cTnT primary antibody was added and samples were incubated over night at 4 °C. Finally samples were incubated with Alexa 688 goat anti mouse IgG for 2 h at RT. Images were captured with a camera attached on the Zeiss Axio Imager M1 fluorescence microscope.

2.6.3. Microparticle phagocytosis by macrophages

In order to evaluate how PLGA and PEG–PLGA MPs are uptaken by macrophages, fluorescent immunohistochemistry against CD68+ cells was carried out. 12 heart sections per treatment and sacrifice time were included in the study. After deparaffinization, hydration and washing were performed as described above. Antigen retrieval was done by incubation with trypsin (0.2% trypsin and 0.1% CaCl2 in deionized water). After water and TBS rinsings, the sections were blocked as previously described and then incubated over night at 4 °C with mouse anti CD68 antibody. Goat anti mouse IgG Alexa 488 secondary antibody was utilized to amplify the signal (1 h at RT). Both antibodies were diluted 1:100 in blocking solution. Imaging analysis was done as described in Section 2.6.2. Determination of the extent of phagocytosis at different times was calculated as the ratio between the number of rhodamine labeled microparticles internalized in CD68+ macrophages and the total number of microparticles detected in each section [23].

3. Results

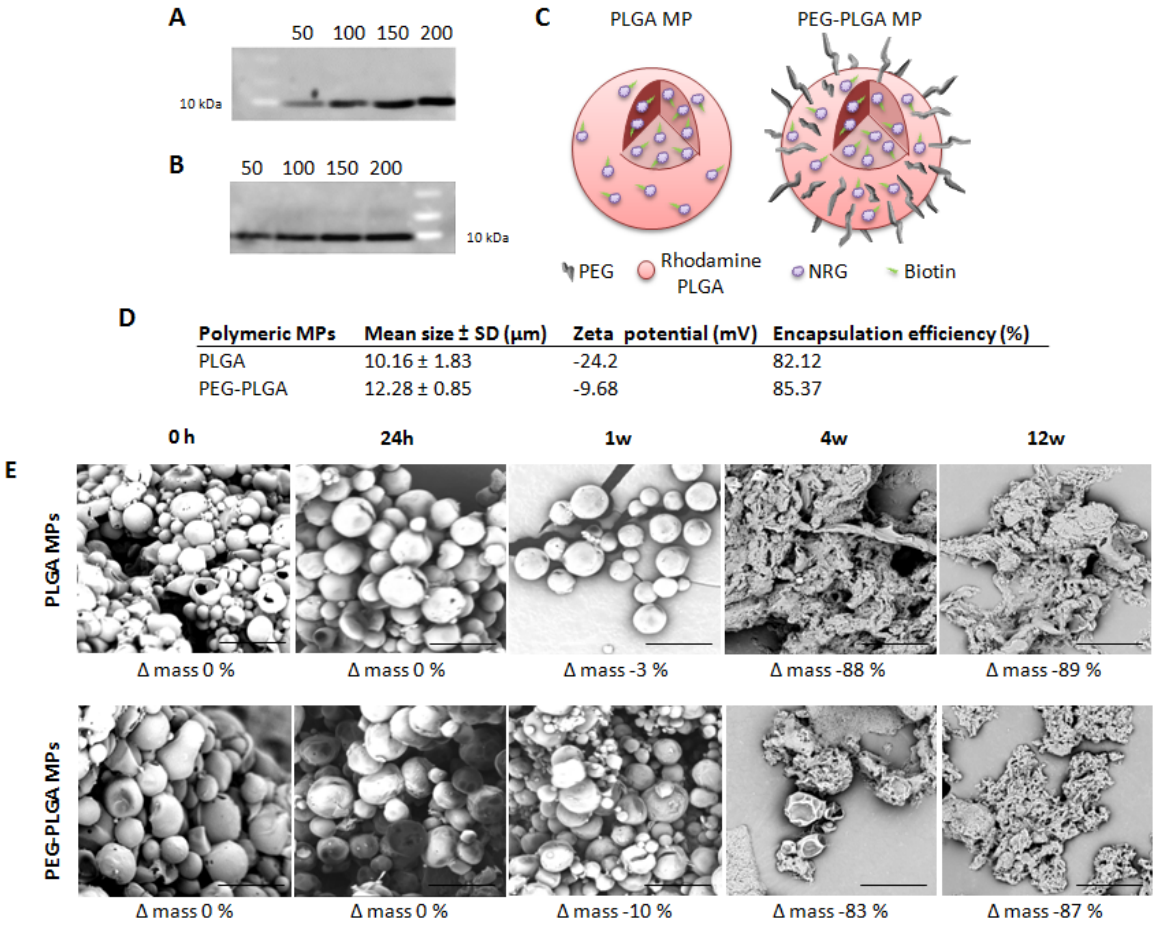
3.1. NRG

NRG was labeled with biotin to distinguish the exogenous from the endogenous molecule in the *in vivo* studies. The biotinylation process was confirmed by WB analysis. Gels were loaded with bNRG. When samples were incubated against biotin or NRG (Fig. 1A and B, respectively) 10 kDa band was observed, providing evidence for the presence of NRG linked to biotin.

3.2. Microparticle characterization

In this study TROMS® technology was used to synthesize spherical bNRG containing MPs, made of PLGA or PEG–PLGA (Fig. 1C shows a schema of PLGA and PEG–PLGA MPs loaded with bNRG). TROMS® is based on the multiple emulsion solvent evaporation technique, and varying parameters such as flow, recirculation times and inner diameters of the needles an adequate particle size for intramyocardial administration was achieved [23]. PLGA MP size was 10.16 ± 1.83 μm, with a Z potential of −24.2 mV (Fig. 1D). On the other hand, PEG–PLGA MPs resulted in spheres of 12.28 ± 0.85 μm with a Z potential of −9.68 mV (Fig. 1D). Encapsulation efficiency of bNRG was 82.12% for PLGA MPs and 85.37% for PEG–PLGA MPs, which corresponded to a final loading of 328 and 341 ng of NRG per mg of MPs, respectively. To sum up, although PEG coating neutralized MP zeta potential and slightly increased particle size, MPs conserve their negative zeta potential and their shape. Importantly, encapsulation efficiency remained at high level.

Regarding MP *in vitro* degradation, both types of particles presented similar degradation profiles (Fig. 1E). No degradation was detected in PLGA or PEG–PLGA MPs after 24 h under continuous agitation in PBS at 37 °C. The particles presented no loss of mass (Δ mass = 0%), and SEM showed that the initial spherical shape was well conserved, confirming that no degradation had taken place in the short term. These results were maintained after 1 week, when degradation remained still nonexistent. In this case, although MPs had lost some mass (Δ mass = −3% for PLGA MPs and −10% for PEG–PLGA MPs), the morphology of the particles was still maintained. However, MP degradation was found to be important at 4 weeks and beyond. PLGA and PEG–PLGA MPs presented a loss of mass of −88 and −83% respectively, indicating that MPs were practically degraded by week 4 in degradation medium. Their spherical shape was almost lost and MPs, mainly PLGA MPs, were observed as a clump with a undefined contour when viewed by SEM (Fig. 1E). Interestingly, PEG–PLGA MPs' initial morphology was still conserved in some cases in week 4, and some individual particles could still be identified, suggesting that PEG coating confers on MPs the ability to resist degradation in the long term.



D

Polymeric MPs	Mean size ± SD (µm)	Zeta potential (mV)	Encapsulation efficiency (%)
PLGA	10.16 ± 1.83	-24.2	82.12
PEG-PLGA	12.28 ± 0.85	-9.68	85.37

Fig. 1. NRG biotinylation and MPs characterization *in vitro*. To address NRG biotinylation two WB were performed, both loaded with bNRG (50, 100, 150 and 200 ng) and revealed against biotin (A) or NRG (B). C) Schematic representation of PLGA and PEG-PLGA MPs loaded with bNRG. D) Particle size with standard deviation, zeta potential and encapsulation efficiency of PLGA and PEG-PLGA MPs. E) Representative SEM images showing *in vitro* degradation of PLGA and PEG-PLGA MPs over time. Δ mass: % of grams lost at each time point. h: hours, w: weeks. Scale bar 10 µm.

In any case, both PLGA and PEG-PLGA MPs were totally degraded by week 12 *in vitro*. Mass reduction (Δ mass = −89% for PLGA MPs and −87% for PEG-PLGA MPs) had similar values to those found at 4 weeks. SEM confirmed that none of the MPs conserved its initial morphology. A jumble of shapeless polymer was observed in all the samples after 12 weeks (Fig. 1E).

3.3. Histological studies

3.3.1. *In vivo* release studies

For a complete MP characterization, we first carried out an *in vivo* study in which we analyzed how bNRG was released from MPs in the heart over time. Biotinylation of loaded NRG allowed us to specifically detect the therapeutic

cytokine in the cardiac tissue. First, we confirmed the absence of unspecific signals by observing samples from animals who received a sham procedure, blank-PLGA or blank-PEG-PLGA MPs. In bNRG-PLGA or bNRG-PEG-PLGA MPs treated animals bNRG could be observed from 1 week for up to 12 weeks post implantation (Fig. 2). Regardless of whether MPs were coated with PEG or not, the therapeutic protein was detected in an area next to the MPs, confirming the suitability of both types of MPs for the controlled delivery of therapeutic drugs in the heart. The *in vivo* release study also allowed us to conclude that MPs were present in the cardiac tissue from 24 h for up to 12 weeks after implantation.

During the follow up of the MPs *in vivo*, partial degradation of the DDSs could be observed over time due to the biodegradable nature of the copolymers that form the MPs. Thus, at one week, spherical, well defined MPs were present in the tissue, whereas at 4 and especially at week 12 after injection, smaller MPs with an ill defined shape were observed, indicating that they had undergone degradation processes (Fig. 2).

3.3.2. Neuregulin bioactivity *in vivo*

In the organism, NRG binds and activates, through phosphorylation, the ErbB4 receptor to trigger the cellular signals responsible for its biological activity. To test if therapeutic bNRG was bioactive after being encapsulated and administered into the cardiac tissue, we developed an *in vivo* bioactivity assay

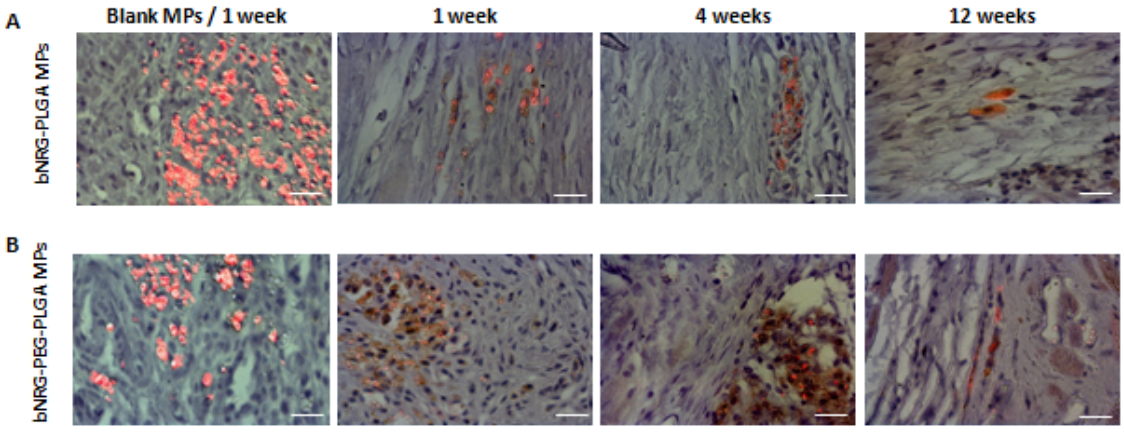


Fig. 2. Polymeric PLGA and PEG-PLGA MPs releasing therapeutic bNRG in the cardiac tissue. Representative images correspond to the merging of fluorescent (showing rhodamine MPs in red) and bright field images (showing cell nuclei in blue and bNRG in brown). A) bNRG-PLGA MPs treatment. B) bNRG-PEG-PLGA MPs treatment. First column of each row represents animals treated with blank MPs and sacrificed at 1 week. Scale bar 50 μm.

to detect ErbB4 receptor in its active form specifically, to prove phosphorylation at the Y1284 residue. Fluorescence microscopy revealed that whereas in animals injected with sham (data not shown) and blank MPs (Fig. 3A, B, E and F) ErbB4 receptor was not phosphorylated, its activation was clear in those animals treated with either bNRG–PLGA or bNRG–PEG–PLGA MPs from 1 week (Fig. 3C and G, respectively) to at least 12 weeks (Fig. 3D and H) after treatment administration. Interestingly, PEG coating of MPs did not affect NRG bioactivity, since in both treatments ErbB4 was phosphorilated (Fig. 3C, D, G and H). In addition, by double immunofluorescence against pErbB4 and cTnT, we could identify cells expressing pErbB4 as cardiomyocytes (Fig. 4). Thus, for both PLGA (Fig. 4A) and PEG–PLGA MPs (Fig. 4B) and for the short and long terms, signal corresponding to pErbB4 receptors colocalized with signal from cardiomyocytes (see Fig. 4). Therefore, MPs were not only able to maintain NRG bioactivity, but also to induce the activation of ErbB4 receptor in the cardiomyocytes. Importantly, cardiomyocytes have been defined as the principal mediator of cardiac tissue development and homeostasis of the structure and function of the adult heart [10]. Thus, these findings together suggest that synthesized polymeric MPs are an optimal approach for therapeutic cardiac protein delivery after MI.

3.3.3. Microparticle phagocytosis by macrophages

With the aim of evaluating how MPs are phagocyted by immune system cells in the cardiac tissue, an *in vivo* phagocytosis study was performed, in which the number of MPs located inside and outside the macrophages was quantified to determine the phagocytosis rates. In the short term (one week after treatment administration), fluorescent immunohistochemistry against CD68+ cells revealed that most of the MPs were located outside macrophages, regardless of whether they were coated with PEG or not (Fig. 5A and B). In fact, in animals sacrificed one week after treatment, the phagocytosis rates were 29% for PLGA MPs and 18% for PEG–PLGA MPs (Fig. 5C). Four weeks after treatment, MPs were more phagocyted, the phagocytosis rates being 40% for PLGA MPs and 29% for PEG–PLGA MPs (Fig. 5C). Images from animals sacrificed at 12 weeks revealed a big change regarding MP location, confirming the phagocytic trend observed from 1 to 4 weeks. Thus, in these animals, most of the MPs were phagocyted by immune system cells: PLGA MP phagocytosis rate was 80% and PEG–PLGA MP phagocytosis rate was 67% (Fig. 5C). Importantly, in all cases, differences between PLGA MPs and PEG–PLGA MP phagocytosis rates were statistically significant ($p<0.0001$), indicating that PEG coating was effective in reducing MP clearance by macrophages in both the short and long term.

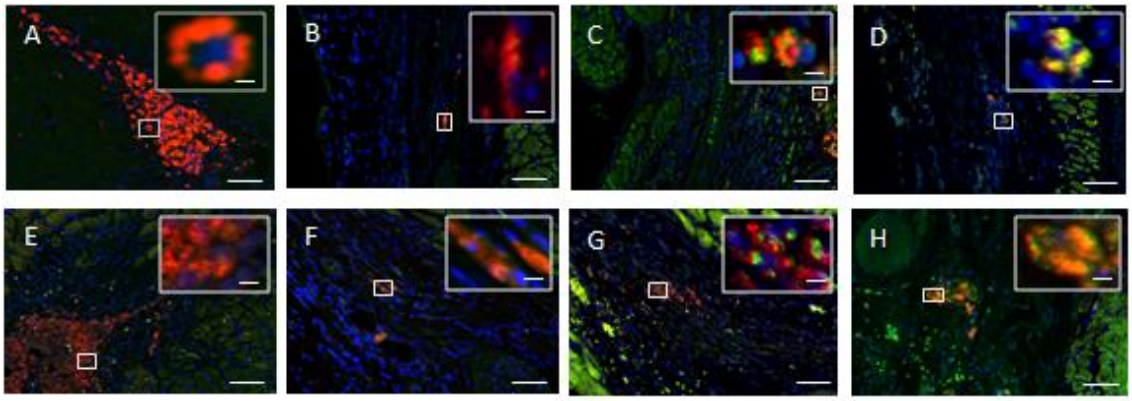


Fig. 3. *In vivo* bioactivity of bNRG. Fluorescence images of anti phospho-ErbB4 immunohistochemistry. A) Blank PLGA MPs animals sacrificed at 1 week. B) Blank PLGA MPs animals sacrificed at 12 weeks. C) bNRG–PLGA MPs animals sacrificed at 1 week. D) bNRG–PLGA MPs animals sacrificed at 12 weeks. E) Blank PEG–PLGA MPs animals sacrificed at 1 week F) Blank PEG–PLGA MPs animals sacrificed at 12 weeks. G) bNRG–PEG–PLGA MPs animals sacrificed at 1 week H) bNRG–PEG–PLGA MPs animals sacrificed at 12 weeks. MPs are seen in red (rhodamine), pErbB4 in green dots (Alexa 488) and cell nuclei in blue (DAPI). Heart sections gave auto fluorescence (green). Top right squares are a magnification of a section of the figure. Scale bar 50 μm. Scale bar of magnification insets: 8 μm.

As mentioned, MP phagocytosis occurred at all times and for both types of particles. Thus, the total number of PLGA and PEG–PLGA MPs in the tissue, considering MPs both inside and outside macrophages, significantly decreased over time. Although the total number of PLGA MPs at 12 weeks vs 1 week was significantly smaller ($p<0.01$), as well as for PEG–PLGA MPs ($P<0.05$) (Fig. 5B), both PLGA and PEG–PLGA MPs were present in the tissue for at least 12 weeks. In addition, when total PLGA and PEG–PLGA MPs were compared for each time point, statistical differences were found in the long term (12 weeks) ($p<0.05$) (Fig. 5B). Similarly, 12 weeks after treatment, the number of free PEG–PLGA MPs was significantly higher than the number of PLGA MPs ($p<0.05$) (Fig. 5B), confirming that PEG strategy is effective in reducing PLGA MP phagocytosis.

4. Discussion

State-of-the-art in cardiac repair has suggested that protein therapy is a promising strategy that may substitute conventional treatments in the near future [4]. Indeed, NRG is currently being tested in clinical trials [30]. However, further development is mandatory before protein therapy reaches routine clinical practice. In fact, important and controversial results have been observed in the clinical trials carried out to date [31]. The short half-life of the proteins administered and their fast degradation under biological conditions are key factors limiting the successful application of these

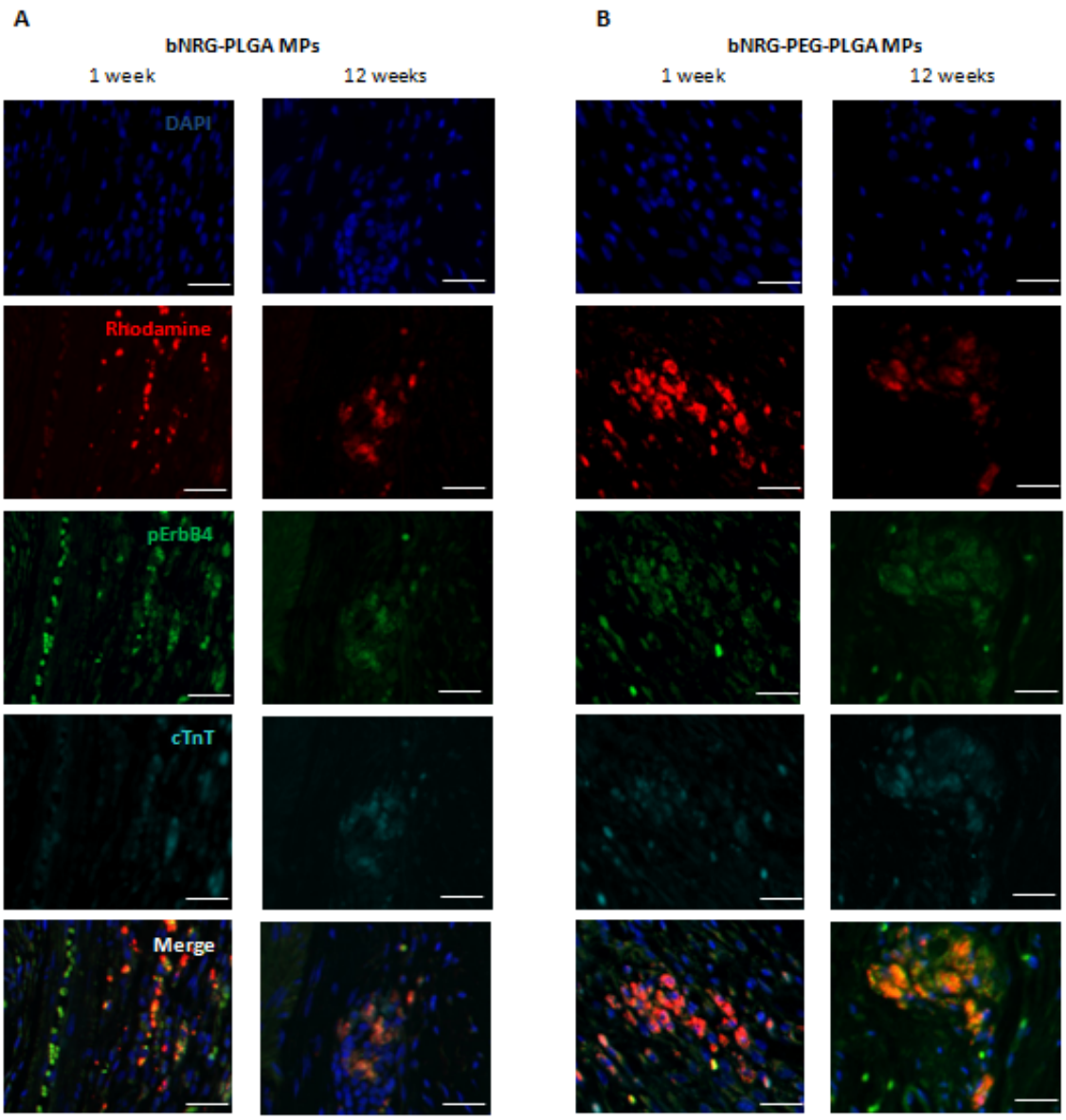


Fig. 4. NRG induces activation of receptor ErbB4 in cardiomyocytes. Representative fluorescent images are showed, indicating zones where polymeric particles (rhodamine), pErbB4 (Alexa 488) and cardiomyocytes (Alexa 688) colocalize. A) bNRG-PLGA MPs inducing activation of pErbB4. B) bNRG-PEG-PLGA MPs inducing activation of pErbB4. Scale bar 20 μm.

therapies [32]. In this regard, polymeric PLGA MPs have demonstrated great potential as delivery systems, allowing protection and sustained delivery of a list of proteins in the heart, such as VEGF [20,28], FGF and NRG [21], which facilitates the maintenance of protein therapeutic levels at their site of action [18].

Concerning NRG-1, the role of this cytokine in cardiac development has been well established, and there is a growing appreciation of the importance of the NRG/ErbB4 signaling pathway in the adult heart [10,33]. Specifically, from a therapeutic perspective, NRG-1 loaded MPs have shown promise in preserving

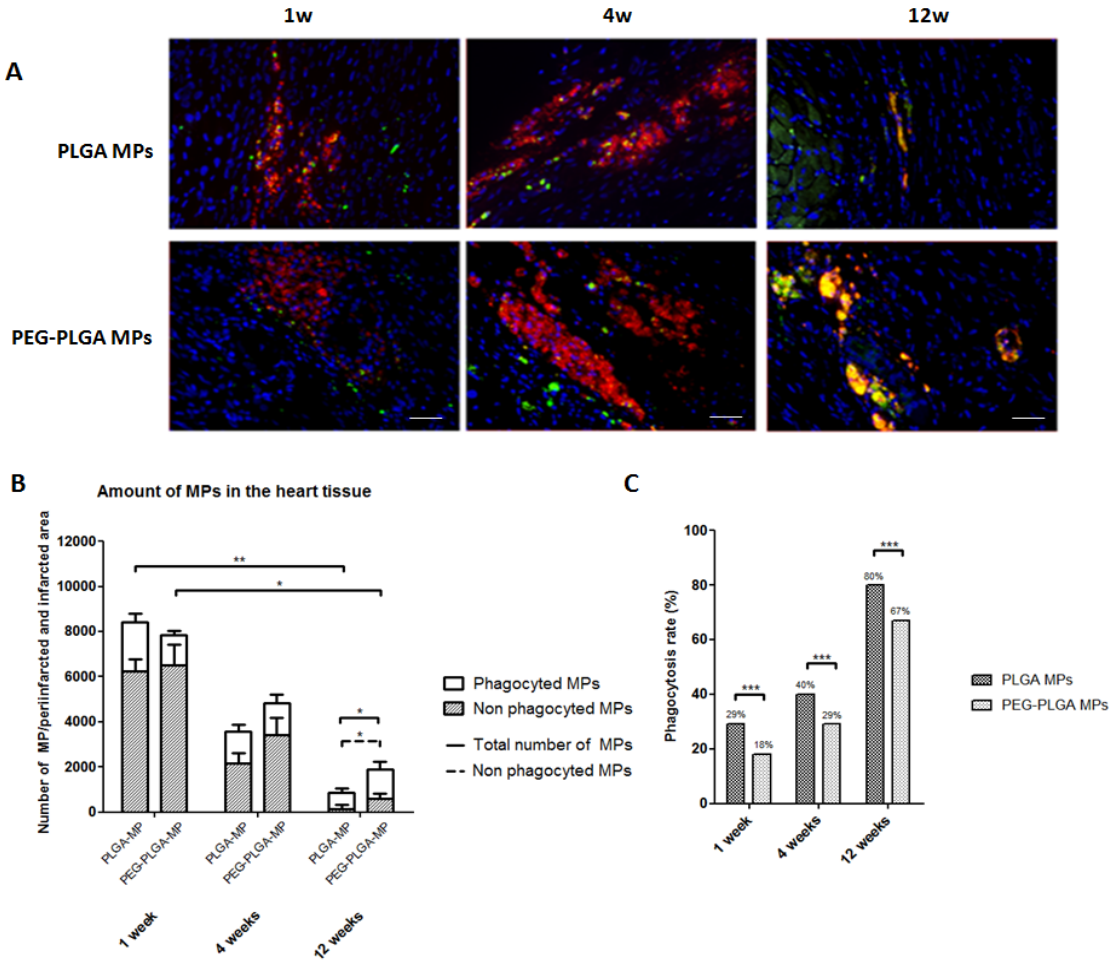


Fig. 5. *In vivo* phagocytosis study of PLGA and PEG-PLGA MPs in the heart tissue. A) representative fluorescence images of heart samples after immunohistochemistry against CD68+ cells. MPs are observed in red (rhodamine), macrophages in green (Alexa 488) and cellular nucleus in blue (DAPI). Free MPs are seen in red and phagocyted MPs are seen in yellow (overlap of red and green signals). B) Statistical analysis of the total amount of MPs in the cardiac tissue over time. C) Phagocytosis rates of PLGA and PEG-PLGA MPs. *: $p<0.05$, **: $p<0.01$, ***: $p<0.0001$. Scale bar 50 μm.

cardiac function and inducing regeneration after a MI injury [21]. Nevertheless, in order for MPs to become a viable clinical treatment further *in vivo* characterization is imperative to verify their efficacy and to better understand the mechanisms underlying their beneficial effects.

Our present findings confirm the suitability of PLGA MPs for cardiac protein delivery and provide relevant information in favor of the use of PEG coating for improving MP behavior *in vivo*. In particular, we show how PLGA and PEG-PLGA MPs were able to remain in the infarcted heart for at least 12 weeks releasing bioactive NRG. Moreover, we demonstrate that PEGylation strategy is effective in preventing MP phagocytosis in the infarcted myocardium.

Taking that into account, tracking vehicles and therapeutic agents once they are administered in the organism is mandatory to establish the interactions among delivery systems, drugs and biological structures. In this work, we labeled PLGA MPs with rhodamine, which is a non-toxic, easy and stable technique. NRG-1 was labeled with biotin, in order to differentiate between therapeutic and endogenous molecules. Biotinylation has been recently reported as a labeling technique that does not alter protein biological activities [34] and is safer than other common techniques such as radioactivity [35]. Interestingly, we report in the present work the presence of rhodamine labeled PLGA and PEG–PLGA MPs in the myocardium for at least 12 weeks after treatment administration. Whereas previous published data only reported the retention of PLGA particles in the heart for 7 [36] or 30 days maximum [28], we show how PLGA and PEG–PLGA MPs were retained in the myocardium for a prolonged period of time (12 weeks). This is fully in line with previous results obtained by our group [23] and confirms the ability of MPs to obtain long term benefits. Moreover, since MPs could be detected in the heart after 12 weeks, we could confirm that such devices were not totally degraded at that time. This is in contrast with the *in vitro* results, where after 4 weeks most of the MPs were practically degraded, which highlights the lack of reliable correlation between *in vitro* and *in vivo* experiments [37,38] and supports the need to study the *in vivo* behavior of drug delivery systems in greater depth. On the other hand, in this pioneer *in vivo* release study we also follow the distribution of the labeled NRG in the cardiac tissue over time, observing colocalization of MPs and bNRG from 1 week to 12 weeks (Fig. 2). Previously, our group had shown that PLGA MPs were able to release the therapeutic proteins for at least one month in a controlled manner [21], but here we could confirm this controlled release for up to three months. Thus, both PLGA and PEG–PLGA MPs were releasing NRG in a controlled manner until the end of the experiment. This indicates that PLGA MPs act as a controlled delivery system and, in addition, that polymeric MPs protect proteins from degradation for at least 12 weeks in the cardiac tissue. Therefore, the presence of bNRG releasing MPs in the heart for the entire 12 week experiment confirmed the feasibility of such systems for achieving therapeutic concentrations of the encapsulated drug at the desired site. Moreover, our defined size MPs of around 10 μm diameter did not induce inflammatory reactions [23] or alter maintained tissue physiological characteristics [27].

Although several studies have investigated the use of polymeric particles to deliver therapeutic proteins (reviewed in [39]), only a few products have reached the market [40]. Protein instability issues during MP preparation, storage and release may explain in part the commercial failure of MPs [41]. To face this challenge, in the present study we used TROMS® technology to prepare protein loaded MPs.

This technology allows the encapsulation of labile molecules into polymeric particles using nonaggressive techniques, maintaining their native properties and achieving higher encapsulation efficiency than other emulsion preparation methods [23]. In any case, protein bioactivity must be always tested in order to guarantee the therapeutic effect of final pharmaceutical product. Previously, we demonstrated *in vitro* that NRG retained its bioactivity after encapsulation into PLGA MPs [21]. However, no data confirming NRG bioactivity *in vivo* have been provided [42]. Here we report for the first time an *in vivo* bioactivity assay for PLGA and PEG–PLGA MPs releasing NRG. After injection in the hearts, bioactivity of the encapsulated protein was demonstrated both in the short (1 week) and in the long (12 weeks) term, at which the phosphorylated form of ErbB4 receptor was always detected. Going a step further, we determined the type of cells presenting pErbB4, identifying cardiomyocytes as the main cell lineage expressing the activated form of the NRG receptor. PLGA MPs and PEG–PLGA MPs therefore appear to be an optimal device for cardiac protein administration. Taking together the *in vivo* and the bioactivity results, we can conclude that MPs act as vehicles which obtain active concentrations of bioactive NRG at its site of action. This may explain our previous results in which NRG loaded PLGA particles led to heart regeneration and improvement in cardiac function and remodeling [21].

In order to go deeper in the characterization and optimization of the treatment with polymeric MPs, we also evaluated how PEGylation strategy may reduce MP phagocytosis by immune system cells. After MI, the inflammatory response of the myocardium is an essential component of the host response and plays an important role in cardiac repair [43]. Recruited macrophages at the infarcted zone phagocyte all strange molecules, including foreign structures such as MPs. To trigger the appropriate cell responses that help the heart to heal after MI, NRG needs to bind its cellular receptor ErbB4. However, if MPs are taken up by macrophages, therapeutic NRG would be degraded inside them without reaching its biological target, thereby reducing its therapeutic activity [23]. The phagocytosis process mainly depends on the presence of opsonins on the MP surface. If these proteins do not bind to MPs, macrophages will typically not be able to bind to or recognize the foreign particles [44]. After PEGylation, PEG chains form a coat around MPs interfering opsonin binding to their surface and conferring stealth behavior on MPs, decreasing the phagocytosis of PEGylated MPs [45]. Besides, PEG chains mask the MPs' superficial charge, resulting in more neutral MPs. As a consequence, recognition by the immune system through opsonization is reduced [45]. Such concepts were previously confirmed by our group *in vitro* [20]. Nevertheless, almost all *in vivo* studies carried out so far concerning how PEG can reduce opsonization have been performed in the blood stream [26,46].

Only in a few cases has the PEG immunological response been studied in other body compartments, exclusively oral and dermal applications, leading to reports of hypersensitivity and allergic reactions respectively (reviewed in [45]). Here we report for the first time the effects of PLGA MPs PEGylation on the residence time of the particles in the heart and on the macrophage mediated phagocytosis of such DDSs in the cardiac tissue. After inducing MI in rodents, modulation of cardiac macrophages and phagocytosis are regulated by the expression of intramyocardial cytokines, which start to decrease toward baseline after 1 week [23]. However, we found that the phagocyted PLGA and PEG–PLGA MPs rates augmented over time. One week after administration in the cardiac tissue MPs are practically nondegraded, conserving their spherical morphology and size and thus presenting some resistance to phagocytosis. After longer periods of time (for instance 4 weeks), MPs are generally degraded into smaller particles, facilitating macrophage uptake. This process is even more evident 12 weeks after injection, when all MPs have lost their initial morphology and size. Consequently, these small particles were more susceptible to phagocytosis [47], which explains why the phagocytosis rates increased over time. This is in accordance with other previous results obtained by our group [23]. Finally, we also observed that PEGylation diminished PLGA MP phagocytosis in the infarcted hearts from 1 week for up to 12 weeks after MP administration. These differences led to a significantly higher amount of PEG–PLGA MPs in the tissue compared to PLGA MPs in the long term (12 weeks).

5. Conclusions

Our present findings confirm the potential of PLGA MPs for treating MI. MPs have proved to allow local, controlled release of bioactive NRG *in vivo* for at least 12 weeks. At this time both bioactive NRG and MPs were still detected in the cardiac tissue. Indeed, PEGylation proved to be effective in preventing PLGA MP phagocytosis 12 weeks after particle administration, suggesting that PEG coating may enhance the efficacy of the treatment. The ability to improve the treatment in the long term is a highly desirable characteristic of any cardiac treatment, since the longer the life of the therapeutic agents in the organism, the fewer patient heart interventions would be needed. Consequently, patient compliance and outcomes would be improved. Taking all this together, we have reported new evidence in favor of the use of PLGA and PEG–PLGA MPs as delivery systems for treating MI, which could soon be successfully included in clinical trials. For this, we have to bear in mind the main limitations that have to be addressed, such as issues related to translation to bigger animals or humans: the microparticle size, loading and release of the cytokines will need to be adjusted. Presently, we are

addressing these points in order to administer the microparticles in a clinically relevant pig model of MI.

Acknowledgments

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CHAPTER 2

CYTOKINE-LOADED PLGA AND PEG-PLGA MICROPARTICLES SHOWED SIMILAR HEART REGENERATION IN A RAT MYOCARDIAL INFARCTION MODEL

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Abstract:

Neuregulin (NRG1) and fibroblast growth factor (FGF1) are well known growth factors implicated in cardiomyocyte proliferation and survival, as well as in angiogenesis, the development of adult heart and the maintenance of cardiac function. NRG1 and FGF1 have become promising therapeutic agents to treat myocardial infarction (MI) disorder. Unfortunately, clinical trials performed so far reported negative efficacy results, because growth factors are rapidly degraded and eliminated from the biological tissues once administered. In order to increase their bioavailability and favor their therapeutic effects, they have been combined with poly(lactic-co-glycolic acid) and polyethylene glycol microparticles (PLGA MPs and PEG-PLGA MPs). Here we compare both types of microparticles loaded with NRG1 or FGF1 in terms of efficacy in a rat MI model. Our results showed that intramyocardial injection of NRG1 or FGF1-loaded PLGA and PEG-PLGA MPs brought about similar improvements in the ejection fraction, angiogenesis and arteriogenesis after administration into the infarcted hearts. PEG coating did not add any effect regarding MP efficacy. Both PLGA and PEG-PLGA MPs were equally phagocyted in the heart. To our knowledge, this is the first study analysing the opsonisation process in heart tissue. The results allow us to conclude that the opsonisation process is different in heart tissue compared to blood.

Key words:

Myocardial infarction, Protein therapy, Microparticles, Controlled release, Efficacy, PEGylation.

Biodegradable and biocompatible biomaterials have emerged as essential bricks to develop drug delivery systems (DDSs), and constitute an active and up-to-date field of research. DDSs have demonstrated promising potential in medical therapeutic applications, and protein therapy for myocardial infarction (MI) is a good example of a disorder that can directly profit from DDS technology. The success of DDSs is due to their ability to increase protein bioavailability and protection, and control their release, thus enhancing the therapeutic effects of cytokines (Segers and Lee, 2010). Growth factors such as neuregulin (NRG1) or fibroblast growth factor (FGF1) have been found to induce angiogenesis, cardiomyocyte proliferation and cardiac function in preclinical studies (Odiete *et al.*, 2012; Palmen *et al.*, 2004). However, both resulted in disappointing outcomes when translated to clinical applications. The main reasons for this failure can be attributed to low bioavailability, lack of long term stability and short half-life of the therapeutic proteins (Pascual-Gil *et al.*, 2015a). As pointed out before, DDSs are thought to protect and accumulate the protein cargo at its site of action. Therefore, some of the most promising DDSs involve the entrapment of drugs in biocompatible polymeric devices (Gainza *et al.*, 2015). This is the case of poly(lactic-co-glycolic acid) and polyethylene glycol microparticles (PLGA MPs and PEG-PLGA MPs). PLGA is one of the most widely used polymers in controlled drug delivery, and PLGA MPs and PEG-PLGA MPs have proved their efficacy in protecting growth factors against degradation once administered into the heart, and in releasing cytokines in a controlled manner for up to three months *in vivo* (Formiga *et al.*, 2014; Pascual-Gil *et al.*, 2015b; Simón-Yarza *et al.*, 2013).

Several products based on PLGA MPs are available on the market, such as Zoladex, Nutropin Depot and Telstar Depot and Decapeptyl (Gasmi *et al.*, 2015). Nevertheless, regarding MI, despite various advances, incomplete understanding of the interactions between biomaterials and biological systems still limits progress using DDSs in clinical settings (Tallawi *et al.*, 2015). In addition, a number of PLGA-based MPs have been developed so far with different sizes, encapsulated therapeutic proteins, coatings, co-polymers and degradation profiles (Allison, 2008; Pascual- Gil *et al.*, 2015a). In fact, there is a lack of standardisation regarding PLGA MP synthesis, and it is difficult to find any study where several types of PLGA MPs are compared, which finally hinders the choice of the more suitable MP design for MI treatment.

The aim of this paper is therefore to elucidate if PEG-PLGA MPs loaded with NRG1 or FGF1 would increase the efficacy of PLGA MPs in a rat MI model by decreasing the opsonisation process.

For this purpose, Total Recirculation One Machine System1 (TROMS) was used to formulate both PLGA and PEG-PLGA MPs. This technique is based on the

multiple emulsion and solvent evaporation method (Garbayo *et al.*, 2011; Simón-Yarza *et al.*, 2013). Briefly, 50 mg of Resomer1 RG 503 H for the PLGA MPs or 25 mg of Resomer1 503 H and 25 mg of Resomer1RGP d 50105 for the PEG-PLGA MPs were dissolved in the organic phase (phase O, 4 ml of dichloromethane/acetone (3:1)), injected in the inner aqueous phase (phase W1, containing 50 mg of the therapeutic protein and 5 mL of rhodamine for fluorescent labelling) and then recirculated together through the system. Later, the W1/O emulsion formed was injected into the external aqueous phase (phase W2, 20 mL of PVA 0.5%) and recirculated once. The multiple emulsion (W1/O/W2) was stirred at room temperature until total solvent evaporation. Finally, MPs were washed three times with distilled water and lyophilised, obtaining a final size of 5.01 T 0.23 mm (Mastersizer-S1, Malvern Instruments, Malvern, UK) and encapsulation efficiency of 84.47 T4.24% (corresponding to 844.7 ng of protein per 1 mg of MP, calculated by Western Blot using a goat anti NRG1 antibody (sc-1793 Santa Cruz, dilution 1:50)). Importantly, PEGylation did not modify in a significant way the *in vitro* release profile of MPs (data not shown). Only a higher burst release was observed during the first three days for PEG- PLGA MPs. Blank PLGA and PEG–PLGA MPs were formulated in the same way but without including cytokines in the W1.

Then the *in vivo* efficacy of MPs was tested in Sprague Dawley rats (200 g, 8 weeks of age, Harlan). Ethical Protocol was approved by the University of Navarra Institutional Committee on Care and Use of Laboratory Animals. For MI induction, anaesthetised animals underwent endotracheal intubation for mechanical ventilation. The surgical procedure consisted of a left thoracotomy through the fourth intercostal space, and the left anterior descending coronary artery was permanently blocked. A total of 42 animals with a left ventricular ejection fraction (LVEF) below 50% at 2 days post MI induction were included in the study. 4 days post MI, rats' chests were reopened and animals were treated and divided into seven groups: sham (no treatment), blank PLGA MPs, blank PEG-PLGA MPs, NRG1 loaded PLGA MPs, NRG1 loaded PEG- PLGA MPs, FGF1 loaded PLGA MPs and FGF1 loaded PEG-PLGA MPs. The final amount of growth factor administered was in all cases around 1675 ng. At 1 and 12 weeks post treatment the LVEF was assessed and finally animals were sacrificed for histological studies of angiogenesis, arteriogenesis and location of MPs in the heart tissue. To that end, immunofluorescence techniques were per- formed against caveolin (3238S Cell Signaling, dilution 1:125), smooth muscle actin (C6198 Sigma Aldrich, dilution 1:500) and CD68+ cells (MCA341R AbDSerotec, dilution 1:100). Results are expressed as mean SEM. Statistics were calculated using Prism 5.0 software (Graphpad Software Inc., San Diego, CA, USA). The differences among the groups were evaluated using the unpaired t- test (two-tailed).

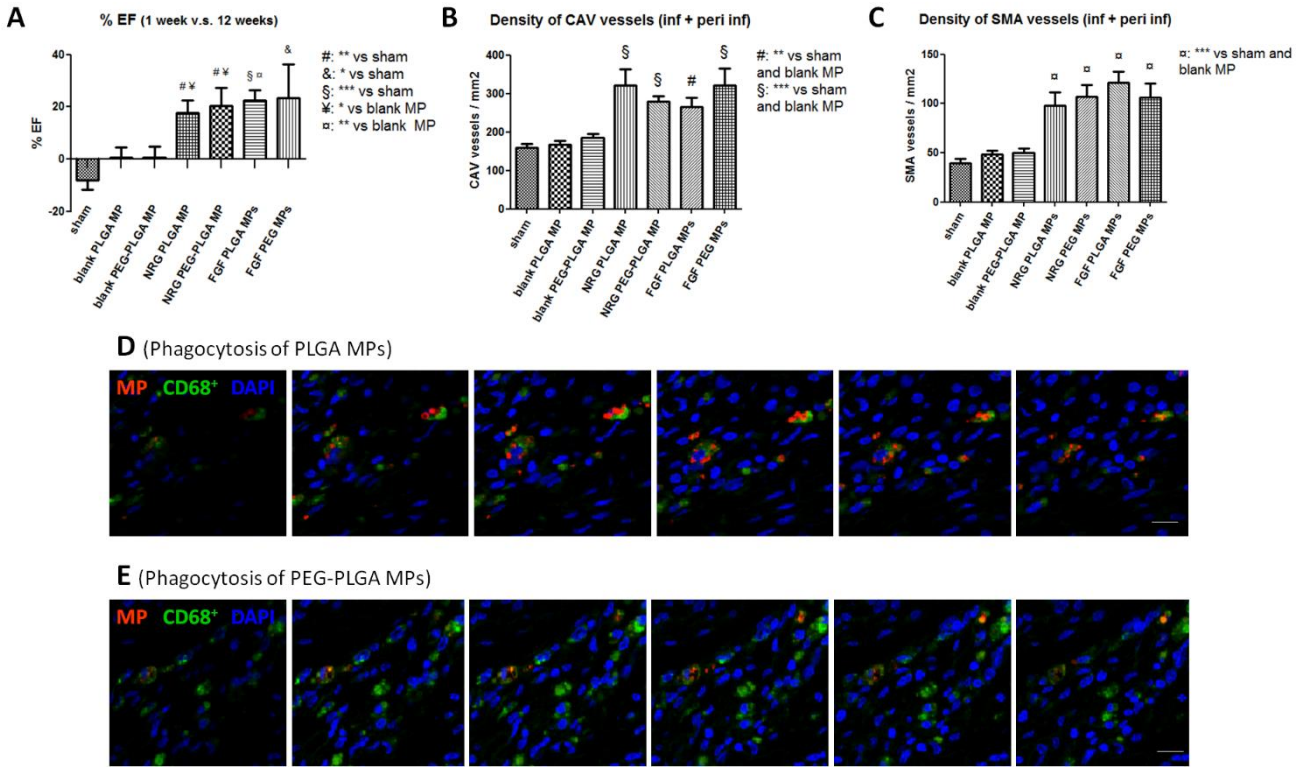

Fig. 1. Efficacy of PLGA and PEG-PLGA MPs loaded with NRG1 or FGF1 as treatments for MI. A) Variation of the ejection fraction of infarcted hearts from 1 week and 12 weeks after MI induction. B) Angiogenesis. Measurements were performed 12 weeks after MI induction. C) Arteriogenesis. Measurements were performed 12 weeks after MI induction. D) Confocal images showing PLGA MP phagocytosis in the infarcted tissue. E) Confocal images showing PEG-PLGA MP phagocytosis in the infarcted tissue. Each set of panels are different stacks of the same heart section. Inf: infarcted area. Peri-inf: peri-infarcted area. Scale bar 10 mm.

In line with other studies carried out by our group (Formiga *et al.*, 2014), NRG1 and FGF1-loaded MPs enhanced the ejection fraction 12 weeks after treatment (Fig. 1A). In addition, our results add new evidence about the ability of PLGA and PEG-PLGA MPs for protecting encapsulated cytokines from degradation in the heart, confirming previous results published by our group (Formiga *et al.*, 2014; Garbayo *et al.*, 2016; Simón-Yarza *et al.*, 2013).

Concerning angiogenesis and arteriogenesis, both cytokines proved to be equally effective in promoting the formation of new vessels (Fig. 1B) and their maturation (Fig. 1C). Thus, in animals treated with loaded MPs regardless the growth factor encapsulated, a significantly higher number of new caveolin rich vessels and developed smooth muscle actin rich vessels were detected when compared to animals who received blank MPs or sham procedures. Blank MPs had no positive effects in improving heart irrigation and blood flow, which confirms the main role of NRG1 and FGF1 in heart regeneration after MI.

Interestingly, we could not establish any difference between PLGA and PEG-PLGA MPs in terms of cardiac function or blood vessel growth. It could be expected that PEGylation would protect MPs against *in vivo* phagocytosis, but PEG coating only reduced MP uptake by macrophages in the long term (12 weeks) (Pascual-Gil *et al.*, 2015b). Taking into account that during the days following a MI event a potent inflammatory response is triggered in the heart that will contribute to a great extent to heart recovery (Uygur and Lee, 2016), avoiding phagocytosis 12 weeks after such event does not have any repercussion on the efficacy of the MPs because the therapeutic time window has already finished (Maxwell, 1999). Importantly, from this study it can be deduced that opsonins and phacogytosis processes do not work in the same way in the heart as in the blood tissue. In the blood stream, PEGylation of MPs that are intravenously injected reduces their elimination rate (augmenting their half-life) (Wattendorf and Merkle, 2008), which is translated into higher bioavailability and efficacy of therapeutic drugs. However, here we have proved that the coating of MPs with PEG administered intramyocardially does not improve the efficacy of the treatment. It may be explained by the fact that PEG-PLGA MPs are equally phagocyted as PLGA MPs at least during the first month after treatment (Pascual-Gil *et al.*, 2015b), when the cytokines released may trigger their therapeutic activity.

Whereas it is well known that PLGA MPs are phagocyted by immune cells after their *in vivo* administration (Fischer *et al.*, 2007), there are only few studies where PEG-PLGA MP phagocytosis has been studied (Pascual-Gil *et al.*, 2015b). Moreover, MP phagocytosis has been extensively studied in the blood but not in tissues. Here we show for the first time how PLGA MPs injected into the myocardium can be phagocyted by CD68+ cells and can be found inside macrophages 1 week after administration (Fig. 1D), sharing their location with PEG-PLGA MPs (Fig. 1E). This result confirms that the PEGylation strategy is not effective in preventing MP uptake in the short term in the heart, which can definitively explain why PLGA and PEG-PLGA MPs have similar efficacy.

In short, NRG1 and FGF1 have similar effects in terms of improving heart function recovery and blood vessel formation and maturation after MI. In addition, although PEGylation strategy is a promising approach for enhancing the therapeutic efficacy of DDSs when administered directly into the bloodstream, it does not add any benefit for MPs injected directly into the myocardium.

Acknowledgments

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CHAPTER 3

NRG1 PLGA MP LOCALLY INDUCE MACROPHAGE POLARIZATION TOWARD A REGENERATIVE PHENOTYPE IN THE HEART AFTER ACUTE MYOCARDIAL INFARCTION

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Abstract:

Neuregulin-1 loaded PLGA microparticles hold great promise for treating acute myocardial infarction, as they have proven to recover heart function and induce positive heart remodeling in preclinical studies. More recently, the inflammatory response of the heart after acute myocardial infarction (AMI) has been identified as one of the major mechanisms in cardiac tissue remodeling and repair. However, the connection between neuregulin-1 PLGA microparticles and inflammation is still not well characterized. In the present study we assessed this relationship in a mouse AMI model. Firstly, *in vitro* evidence indicated that neuregulin-1 PLGA microparticles induced a macrophage polarization toward a regenerative phenotype ($CD206^+$ cells), preventing macrophages from evolving toward the inflammatory phenotype ($B7\text{-}2^+$ cells). This correlated with *in vivo* experiments, where neuregulin-1 PLGA microparticles locally improved the $CD206^+/B7\text{-}2^+$ ratio. Moreover, neuregulin-1 PLGA microparticles were administered at different time points (15 minutes, 24, 72 and 168 hours) after infarction induction without causing secondary inflammatory issues. Thus, the time of treatment administration did not alter the inflammatory response. Taken together these results suggest that neuregulin-1 PLGA microparticles can be administered depending on the therapeutic window of the encapsulated drug and that they enhance the reparative inflammatory response of the heart after acute myocardial infarction, helping cardiac tissue repair.

Key words:

Acute myocardial infarction, heart attack, neuregulin, cytokine, PLGA, microparticles, macrophage polarization, inflammation, tissue repair.

1. Introduction

Following acute myocardial infarction (AMI), the heart undergoes a tissue remodeling process that ultimately determines patient outcomes and prognosis. This cardiac remodeling consists of a group of molecular, cellular and interstitial changes that clinically manifest as changes in the size, shape and function of the heart [1]. Among the broad spectrum of local and systemic mechanisms initiated after AMI that contribute to cardiac remodeling, inflammation has been recently identified as the most conditioning process [2,3]. Macrophages are the main mediators of inflammation, and are abundant during all stages of tissue remodeling, influencing the progress and resolution of tissue damage. In vertebrates, macrophages adopt distinct phenotypes and functions that are shaped by the environment of the organ of residence [4,5]. Regarding the heart, several types of heart macrophages have lately been described, which can be divided into two main polarized groups: anti-inflammatory and pro-inflammatory macrophages (aiMac and piMac, respectively) [6,7]. aiMac are from embryonic origin, retain angiogenic properties, are able to induce cardiomyocyte proliferation and inhabit the heart during steady state [6,7]. However, right after AMI, huge amounts of monocytes and macrophages derived from the bone-marrow (piMac subtype) enter the heart, replacing aiMac and creating a pro-inflammatory environment [8]. Importantly, this burst of inflammatory response is distinct in adult mammals [9]. In grown-up humans, the inflammatory response has evolved to act against bacteria and other infectious pathogens, losing its reparative pathway. This may lead to negative changes in the heart tissue and thus have deleterious effects on cardiac function in the long term [10]. On the other hand, neonatal mammals and lower vertebrates such as zebrafish and salamanders react to an AMI by enhancing the reparative response (reviewed in [11]). In this scenario, piMac are not recruited after AMI and aiMac are responsible for cardiac remodeling and recovery of heart function [12,13].

As a consequence, the ideal treatment for AMI needs to address inflammation, enhancing the reparative response for inducing positive heart tissue remodeling and repair. Among the new treatments currently under development for AMI, protein therapy is probably the most promising approach [14–17]. Protein therapy consists of the administration of growth factors (GF) into the heart. Several GF have been tested so far in the cardiovascular field for tissue repair [18]. Among them, neuregulin-1 (NRG1) holds huge potential for cardiac repair, since it promotes cardiomyocyte proliferation, angiogenesis, arteriogenesis, embryonic stem cell differentiation into cardiac lineage and stem cell survival [12,19,20]. Importantly, NRG1 is not only effective in the early development of the heart, but also prevents severe dysfunction of the adult heart and improves cardiac

performance in rodent models of ischemic, dilated and viral cardiomyopathy [21], making NRG1 a broad-spectrum therapeutic agent for the treatment of heart failure. In addition, our group has shown NRG1 efficacy in a large pre-clinical model of AMI in pigs [22]. Nevertheless, the effects NRG1 may have on inflammatory response remain unknown. Moreover, NRG1 is a labile molecule with an extremely short circulating half-life of 30 minutes [23]. Therefore, it is encapsulated into delivery systems such as poly (lactic-co-glycolic acid) microparticles (PLGA MP) to protect it against *in vivo* degradation and to improve its bioavailability [18,24]. It is worth noting that although PLGA is a biodegradable and biocompatible polymer approved by the Food and Drug Administration for use in humans, inflammatory responses associated with PLGA administration have been reported in some cases [25]. In particular, predicting the behavior of the phagocytic cells after particle uptake may be challenging, and is extremely important to evaluate the contributions of both polymer and GF to the inflammatory response [26]. Finally, inflammation in adult mammals is a progressive process in which macrophage populations evolve considerably over time [27]. piMac and aiMac have different genotypes and functions, and may respond differently to the presence of NRG1 loaded PLGA MP. As a consequence, the time of treatment administration could affect the polarization and resolution of inflammation, which could condition final heart repair. Taking all this into account, the objective of this study is to assess the relationship between NRG1 PLGA MP and the heart's inflammatory response after AMI.

2. Materials and methods

2.1. Materials

Recombinant human NRG-1b-iso was obtained from EuroBioSciencies (Friesoythe, Germany). Western blot (WB) gels NuPAGE® Bis–Tris and electrophoresis MOPS SDS running buffer were purchased from Novex® Life Technologies TM (Waltham, MA, USA), transfer Tris/Glycine buffer and molecular weight standard Precision Plus Protein Standards Kaleidoscope were obtained from BioRad (Hercules, CA, USA). Goat anti NRG1 antibody, B7-2 rat anti mouse IgG antibody and CD206 rat anti mouse IgG antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase conjugated donkey anti goat IgG and Alexa 488 conjugated goat anti rat antibody were provided by Invitrogen™ (Eugene, OR, USA). Lumi-light Plus Western blotting Substrate for WB analysis was from Roche Diagnostics (Mannheim, Germany). PLGA Resomer® RG 503 H (MW34 kDa) was purchased from Boehringer-Ingelheim (Ingelheim, Germany). Dichloromethane, acetone and Tween 20 were obtained from Panreac Quimica S.A. (Barcelona, Spain). Human serum albumin (HSA) and polyethylene glycol (PEG) (MW 400 Da) were

supplied by Sigma-Aldrich (Barcelona, Spain). Poly (vinyl alcohol) (PVA) 88% hydrolyzed (MW125 kDa) was obtained from Polysciences, Inc. (Warrington, PA, USA). Vicryl and Prolene sutures were from Ethicon Inc. (Johnson & Johnson, Brussels). IsoFlo® employed for animal anaesthesia was from Abbot Laboratories S.A. (North Chicago, IL, USA). DMEM, FBS, glutamine and penicillin/streptomycin were from Gibco®. INF-γ was purchased from Immunotools (Friesoythe, Germany). IL-4 was from PeproTech (Rocky Hill, NJ, USA.). LPS, TRIzol, chloroform, isopropanol and RNAase free water were provided by Sigma-Aldrich (Barcelona, Spain). High-Capacity cDNA Reverse Transcription Kit was from Applied Biosystems (Foster City, CA, USA). Primary antibodies for flow cytometry Alexa Fluor 488 anti-mouse CD68, PE anti-mouse CD206, Alexa Fluor 647 anti-mouse CD197 and anti-mouse CD16/32 were from BioLegend (San Diego, CA, USA).

2.2. Synthesis of NRG1 PLGA MP

NRG1 loaded PLGA MP were obtained by the multiple emulsion and solvent evaporation method using Total Recirculation One-Machine System® (TROMS) procedure [28]. Briefly, 50 mg of Resomer® RG 503 H were dissolved in 4 ml of dichloromethane/acetone (3:1) (organic phase), injected in an inner aqueous phase and then recirculated together to form a simple emulsion. The inner aqueous phase consisted of 50 µg of recombinant human NRG-1b-iso, 5 µL of PEG 400, 5 mg of HSA and 195 µL of PBS. Later, simple emulsion was injected into the external aqueous phase (20 mL of PVA 0.5%) and recirculated again. The multiple emulsion formed was stirred at RT up to total organic solvent evaporation. Finally, MP were washed with ultrapure water, frozen at −80 °C, lyophilized (Virtis Genesis Freeze Dryer 12 EL) and stored at 4 °C. Non-loaded PLGA MP were formulated without including NRG1 in the inner aqueous phase.

2.3. MP characterization

Particle size and size distribution were determined by laser diffractometry using a Mastersizer-S® (Malvern Instruments, Malvern, UK). The zeta potential was measured using Zetaplus® (Brookhaven Instruments, NY, USA). In both cases around 0.5 mg of MP were dissolved in deionized water before measurement. Encapsulation efficiency was quantified by WB analysis. First, NRG1 was extracted from 1 mg of MP through DMSO treatment. Secondly, WB was performed using goat anti NRG as primary antibody, horseradish peroxidase donkey anti goat as secondary antibody and Lumi-light Plus substrate. Quantitative analysis of the NRG1 bands was performed by densitometry using ImageOne software (Bio-Rad Laboratories Inc., Munich, Germany).

2.4. Cell lines and culture conditions

J774A.1 cell line (ATCC® TIB-67™) was used as monocyte/macrophage *in vitro* model, and was purchased from the American Type Culture Collection (Sigma Aldrich, Barcelona, Spain). Cells were maintained in DMEM medium supplemented with 10% FBS 1% penicillin/streptomycin and 1% glutamine in an incubator at 37 ºC and a humidified atmosphere with 5% carbon dioxide. Cells for RT-PCR were pre-treated with INF-γ (100 ng/mL) and LPS (50 ng/mL) for 24 hours. Then, medium was changed and cells were treated for another 24 hours with 1) medium, 2) LPS, 3) free NRG1, 4) non-loaded PLGA MP or 5) NRG1 PLGA MP. Cells for flow cytometry were treated 24 hours with INF-γ (100 ng/mL), IL-4 (20 ng/ml) or NRG1 PLGA MP. NRG1 dose was in all cases 25 ng/mL.

2.5. Real-time polymerase chain reaction

After treatment, J774A.1 cells were dissociated with trypsin and then prepared for RT-PCR assay. First, total RNA was extracted from J774A.1 cells using the TRIzol-Chloroform-Isopropanol method. Briefly, 5x106 cells were lysed with 1.5 mL of TRIzol, then 300 µL of chloroform were added and aqueous phase containing total RNA was recovered. 700 µL of isopropanol were added and samples were incubated at -20 ºC overnight. After centrifugation, pellet was washed three times with ethanol 75% and resuspended in RNAase free water. Second, total RNA was concentrated at 600 ng/µg and cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit. Third, RT-PCR was carried out employing 7300 System SDS RQ Study Software (Thermo Fisher). RT-PCR reactions in a 96-well microamp plate consisted of 1.2 µL of cDNA, 10.05 µL of Sigma water, 12.5 µL of PCR Master Mix and oligonucleotide primers for TNF-α and IL-6 (supplied by Thermo Fisher). GADPH expression was used as control. 2-ΔΔCT method was used to calculate the relative expression levels of the cytokines.

2.6. Flow Cytometry

After treatment, J774A.1 cells were trypsinized and resuspended into 0.5 mL of FACS buffer (PBS, 2.5 mM EDTA, 0.5% FBS and 0.1% NaN3 sodium azide). 5x105 were incubated with Fc block and CD68 (common marker for monocytes and macrophages), CD197 (marker for piMac) and CD206 (marker for aiMac) primary antibodies (final volume of 100 µL) during 30 minutes in ice. Fc block, CD68 and CD197 antibodies were at the concentration of 0.5 mg/mL. CD206 antibody was used at 0.2 mg/mL. Then, samples were washed with FACS buffer and resuspended into 0.5 mL of FACS buffer before analyzed using a FACSCANTO (BD BioSciences) and FACS Diva v6.1.3 as acquisition software.

2.7. Animal model of acute myocardial infarction

All animal procedures were approved by the University of Navarra Institutional Committee on Care and Use of Laboratory Animals. A total of 64 male C57BL/6J mice were included in the study (Harlan-IBERICA, Barcelona, Spain). They were housed with standard diet and water ad libitum during the whole experiment. Animals were anesthetized (inhalatory isoflurane) and underwent endotracheal intubation for mechanical ventilation. Before the operation animals received buprenorphine and fentalyl. AMI induction was done by left thoracotomy through the fourth intercostal space, and the left anterior descending coronary artery was permanently blocked. Intramyocardial injection of the treatments was carried out after 15 minutes, 24, 72 or 168 hours after arterial occlusion (Figure 1). For each sacrifice group there were a total of four animals. NRG1 PLGA MP were dispersed in 12 µL of resuspension medium (0.1% (w/v) carboxymethylcellulose, 0.8% (w/v) polysorbate 80 and 0.8% (w/v) mannitol in PBS, pH 7.4) at a final dose of 170 ng of NRG1 per 12 µL. Treatment (NRG1 PLGA MP) or control (resuspension medium alone) was administered into 3 regions in the border zone surrounding the infarct. Anti-inflammatory ketoprofen administration was repeated 24, 48 and 72 hours after surgery; and enrofloxacin was given to animals dissolved in water for five days after surgery. Finally, animals were sacrificed 2 or 5 days after treatment administration (Figure 1). Briefly, animals were anesthetized using intraperitoneal ketamine/xylazine (50/5 mg/kg) and chests were opened. Next, hearts were perfused with PBS and then with paraformaldehyde. Hearts were harvested, cleaned and submerged in 4% paraformaldehyde at 4 °C during 24 h, followed by dehydration in ethanol 70% at 4 °C at least during 48 h. Finally, they were embedded in paraffin and stored at room temperature.

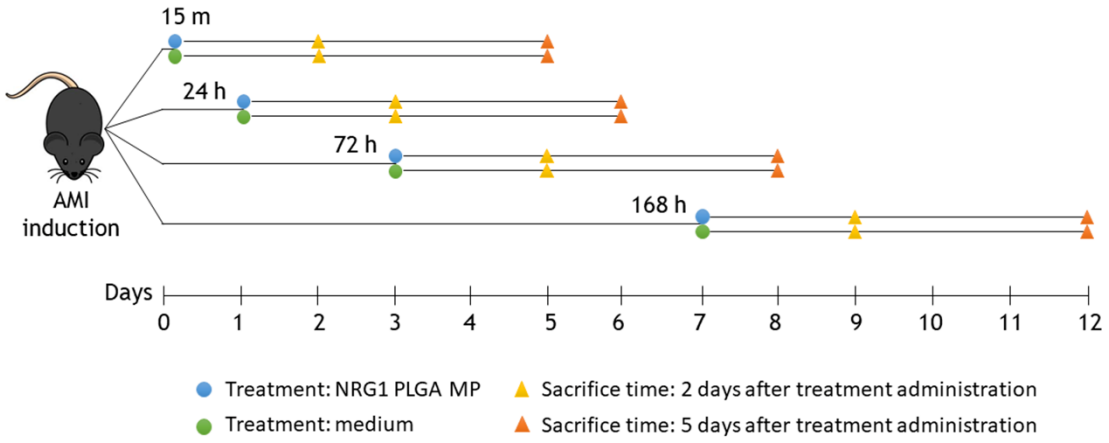

Figure 1: Layout of the animal groups used for this experiment. n=4 for each one of the sixteen sacrifice groups was assured. m: minutes. h: hours.

2.8. Histological studies

Transversal sections of 5 μm thickness were cut from heart paraffined samples to carry out histological studies. In order to evaluate how NRG1 PLGA MP are taken up by macrophages in the heart, fluorescent immunohistochemistry against B7-2$^+$ (marker for piMac) and CD206$^+$ cells was performed. 8 heart sections per treatment and sacrifice time were included in the study. After deparaffinization with xylol and hydration of the samples, antigen retrieval was performed by incubation with trypsin (0.2% trypsin and 0.1% CaCl2 in deionized water). Samples were blocked with TBS-Tween20 0.1% and BSA 5%. Then samples were incubated overnight at 4 ºC with rat anti mouse B7-2 (1:100) or rat anti mouse CD206 (3:200) antibodies. All primary antibodies were diluted in blocking solution. Goat anti rat IgG Alexa 488 secondary antibody at concentration 1:100 was added for 1 hour at room temperature after incubation with B7-2 or CD206 antibodies. Images were captured with a camera attached on the Zeiss Axio Imager M1 fluorescence microscope.

2.9. Statistical analysis

Statistical analyses were performed using a multi-factorial three-way ANOVA, complemented with a contrasts test. Results are expressed as mean ± standard deviation. For CD206$^+$/B7-2$^+$ ratios, a student t test for independent samples was used, and results are expressed by mean ± standard deviation.

3. Results

3.1. NRG1 PLGA MP characterization

In this study TROMS® was used to synthesize spherical NRG1 PLGA MP. Importantly, TROMS® technology allowed a high encapsulation efficiency of NRG1 into the PLGA MP, calculated to be 76.93 ± 1.93 by WB (Figure 2A). This corresponded to 769.3 ng of NRG1 per mg of PLGA MP. Final NRG1 PLGA MP size was 8.03 ± 0.95 μm (Figure 2B), and Scanning Electron Microscopy images confirmed the spherical shape of the MP (Figure 2C). Z potential had a negative value of −23.3 mV due to PLGA charge.

3.2. *In vitro* evidence of the effect of NRG1 PLGA MP on inflammation

J774A.1 cell line consisting of monocyte/macrophage culture derived from reticulum sarcoma mice cells was used to evaluate the effects of NRG1 PLGA MP on macrophage activation and polarization. RT-PCR assay showed that NRG1 did not cause any significant increase in the expression of the well-established pro-inflammatory cytokines TNF-α nor IL-6 on J774A.1 cells (Figure 3A). In a similar way, PLGA MP did not have any effects on the cytokine expression profile either (Figure 3A). Although not statistically

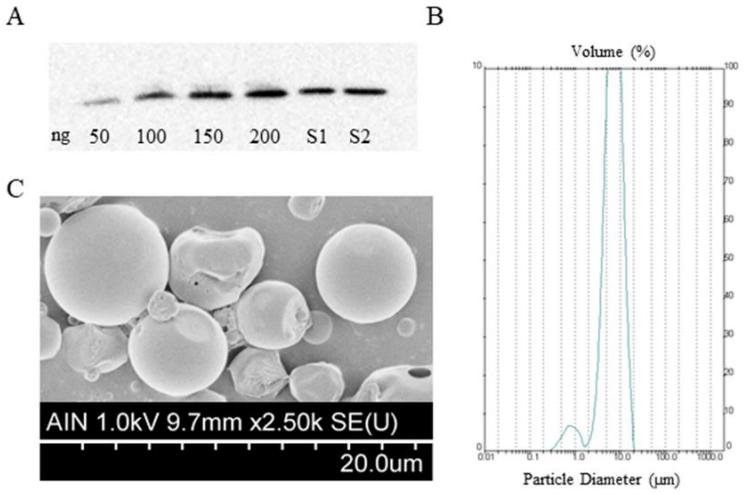

Figure 2: NRG1 PLGA MP *in vitro* characterization. A) WB analysis showing calibration curve from 50 to 200 ng of NRG1 and two bands corresponding to NRG1 PLGA MP (S1 and S2). B) NRG1 PLGA MP size and size distribution graph. C) Representative Scanning Electron Microscopy image illustrating shape and external structure of NRG1 PLGA MP.

different, encapsulation of NRG1 into PLGA MP showed a trend in reducing IL6 expression, confirming correct NRG1 incorporation into the vehicle.

For the flow cytometry study, lymphoid cells were first selected using SSC-H vs FSC-H gate, and then macrophages were identified using a $CD68^+$ antibody. Macrophages were finally gated according to CD197 and CD206 markers. J774A.1 cells treated with INF-γ showed a shift toward a pro-inflammatory phenotype, as indicated by an augment in the receptor CD197 (also known as CCR7) (Figure 3B). On the other hand, J774A.1 cells that had IL-4 at their culture medium changed toward an anti-inflammatory phenotype, confirmed by the increased expression of the CD206 receptor (Figure 3B). Importantly, when cells were co-cultured with NRG1 PLGA MP, macrophages clearly swapped into an anti-inflammatory phenotype (Figure 3B).

3.3. Time of treatment administration does not alter inflammatory response in the heart after AMI

In order to evaluate how administration of the treatment itself could influence the inflammatory response of the heart after AMI, we administered NRG1 PLGA MP or control treatment at different times after AMI induction (see Figure 1). $B7\text{-}2^+$ and $CD206^+$ cells were analyzed in the infarcted and peri-infarcted areas. When NRG1 PLGA MP treated animals were compared to control animals, no statistical differences could be found between the two macrophage populations, indicating that the time of treatment administration did not alter the inflammatory response. As an example, Figure 4A shows representative images of immunofluorescent detection of $B7\text{-}2^+$ and $CD206^+$ macrophages in the peri-infarcted tissue of animals treated 24 hours after AMI induction and sacrificed 2 days after treatment administration. Consequently, both NRG1 PLGA MP and control animals followed a normal inflammation curve. In this sense, while pro-inflammatory macrophages ($B7\text{-}2^+$) were high after AMI and decreased over time, anti-inflammatory macrophages ($CD206^+$)

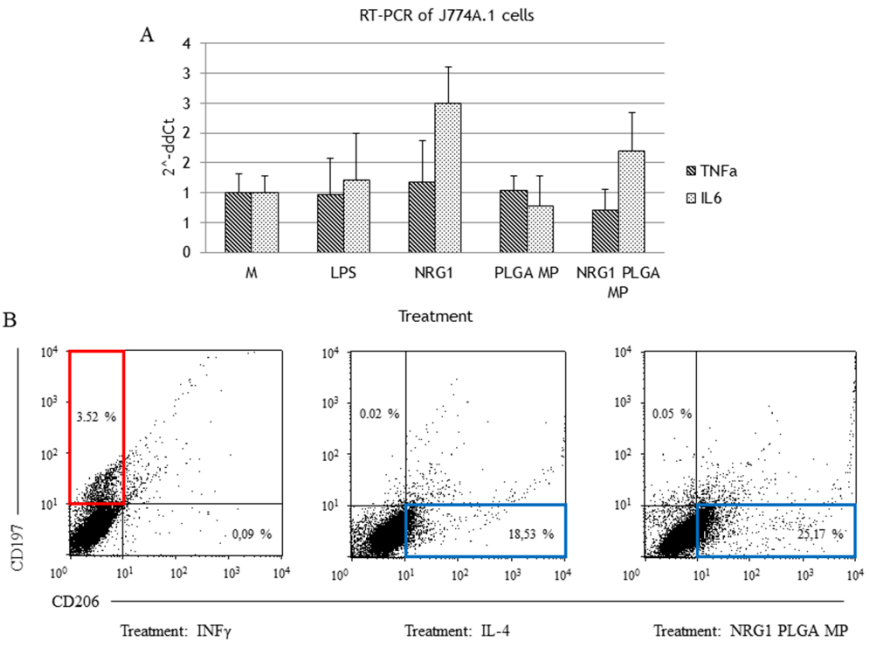

Figure 3: Effects of NRG1 PLGA MP on J774A.1 cells. A) RT-PCR showing that NRG1 does not induce the expression of TNF-α or IL-6. B) Flow cytometry indicating the polarization of J774A.1 cells after being treated with INF-γ (pro-inflammatory polarization), IL-4 (anti-inflammatory polarization) and NRG1 PLGA MP (anti-inflammatory polarization). α: $p<0.05$ versus M, LPS and PLGA MP. β: $p<0.05$ versus M and LPS.

evolved from small amounts right after AMI to higher numbers one week after (Figure 4B). Interestingly, administration of NRG1 PLGA MP 15 minutes after AMI induction showed a trend to increase of CD206$^+$ and reduce B7-2$^+$ macrophages after 5 days (Figure 4B (red circle)). This suggests a positive role of NRG1 PLGA MP in modulating the immune response toward a reparative polarization.

3.4. NRG1 PLGA MP locally induce a positive shift in macrophages toward a reparative phenotype

Heart immunofluorescence assay against B7-2$^+$ and CD206$^+$ cells showed that when hearts were studied as a whole, there were no significant differences between animals treated with NRG1 PLGA MP or controls (Figure 5A and 5B). Interestingly, when macrophage count was performed only at the injection track (area surrounding NRG1 PLGA MP), not taking into account other infarct and peri-infarct areas without MP, an increase in both types of macrophages was found. In particular, the total amount of B7-2$^+$ cells significantly increased in the injection track compared to more distal zones, for both NRG1 PLGA MP and control injections ($p<0.05$ for all time points, Figure 5A and 5C). On the other hand, CD206$^+$ macrophages markedly increased in number only in the areas close to where NRG1 PLGA MP were placed ($p<0.001$ for all time points, Figure 5A and 5C). Such CD206$^+$ increase could not be found in control

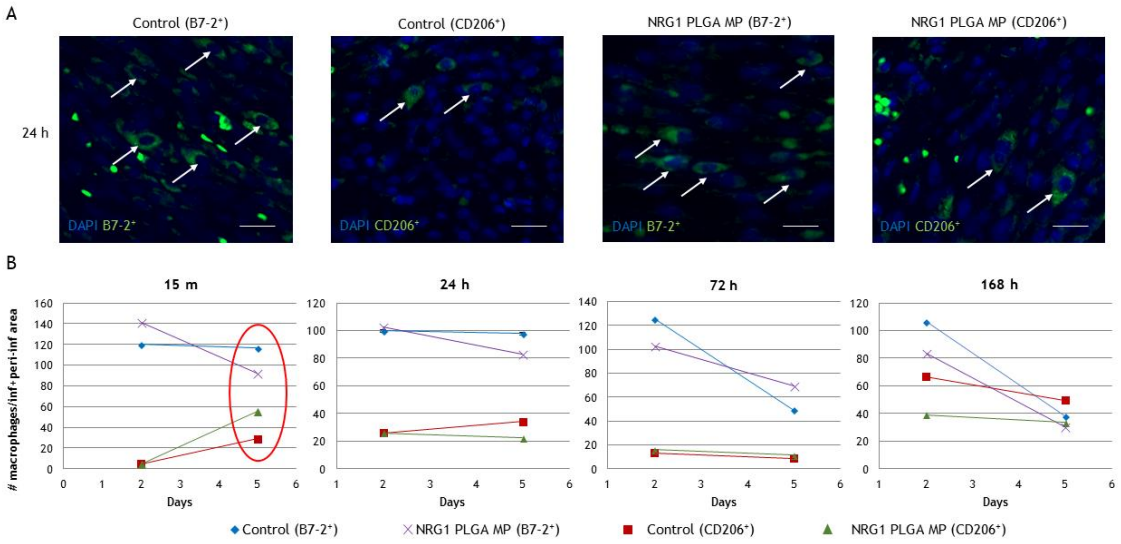

Figure 4: Evolution of B7-2⁺ and CD206⁺ macrophage populations depending on the time of NRG1 PLGA MP administration. A) Representative images of immunofluorescent detection of B7-2⁺ and CD206⁺ macrophages in the peri-infarcted tissue of animals treated 24 hours after AMI induction and sacrificed 2 days after treatment administration. B) Quantitative analysis of B7-2⁺ and CD206⁺ macrophages in the heart 2 or 5 days after treatment administration (15 m, 24 h, 72 h or 168 h after AMI induction). Red circle indicates a trend of NRG1 PLGA MP to enhance CD206⁺ over B7-2⁺ macrophages in the short term. m: minutes. h: hours. Scale bar: 15 µm.

animals, indicating that NRG1 PLGA MP are responsible for the macrophage swap into the reparative phenotype. Importantly, CD206⁺/B7-2⁺ ratio significantly improved close to the injection track of NRG1 PLGA MP compared to control animals ($p<0.01$ for all time points, Figure 5D). This improvement on CD206⁺/B7-2⁺ ratio could not be observed when all heart tissue was analyzed together, suggesting that NRG1 PLGA MP only induce a positive inflammatory shift locally (Figure 5D and 5E).

4. Discussion

The inflammatory response of the heart after AMI has been identified as a major mechanism in cardiac tissue remodeling and thus one of the most important mediators involved in restoring heart function [2,3]. This recent realization has revolutionized the way heart repair is understood, making it mandatory for treatments under development to address inflammation in order to achieve total cardiac muscle repair. In particular, a growing amount of data indicate that aiMac are the main mediators of this tissue regeneration [12,13]. Treatments for AMI should therefore be focused on leading the inflammatory response toward a regenerative polarization.

A

# macrophages / 0.001 mm²			Sacrifice days after AMI						
			2	3	5	6	8	9	12
	Global heart	Control (B7-2⁺)	121 ± 5.9	100 ± 6.9	120.9 ± 4.3	98 ± 3.4	49 ± 4.9	106 ± 1.8	38 ± 4.3
		Control (CD206⁺)	21 ± 8.3	26 ± 10.2	21.2 ± 7.8	34.3 ± 8.8	28.75 ± 6.9	66.6 ±4.6	49,5 ± 3.7
		NRG1 PLGA MPs (B7-2⁺)	109.3 ± 10.2	102.5 ± 4.1	97.5 ± 5.5	82.75 ± 2.1	69.5 ± 4.4	83.3 ± 8.5	30,6 ± 1.9
		NRG1 PLGA MPs (CD206⁺)	15.7 ± 4.2	26 ± 3.7	35.7 ± 19.7	22.25 ± 6.4	11.5 ± 1.2	39.2 ± 4.9	33,6 ± 5.4
	Injection track	Control (B7-2⁺)	198 ± 11.6$^{\alpha}$	175 ± 5.6$^{\alpha}$	245 ± 19.8$^{\alpha}$	250 ± 15.9$^{\alpha}$	180 ± 9.5$^{\alpha}$	106 ± 10.4$^{\alpha}$	103 ± 8.6$^{\alpha}$
		Control (CD206⁺)	19.6 ± 9.6	37 ± 5.5	33.3 ± 10.4	40.1 ± 8.4	51.75 ± 12.8	61.6 ± 3.7	52.1 ± 10.0
		NRG1 PLGA MPs (B7-2⁺)	214 ± 2.8$^{\beta}$	163 ± 10.2$^{\beta}$	235.4 ± 12.2$^{\beta}$	190 ± 23.9$^{\beta}$	206 ± 14.7$^{\beta}$	107 ± 15.5$^{\beta}$	91 ± 12.8$^{\beta}$
		NRG1 PLGA MPs (CD206⁺)	224.8 ± 17.4$^{\gamma}$	235.3 ± 4.8$^{\gamma}$	215.4 ± 6.9$^{\gamma}$	238.9 ± 11.8$^{\gamma}$	198 ± 6.1$^{\gamma}$	170 ± 10.4$^{\gamma}$	164 ± 11.9$^{\gamma}$

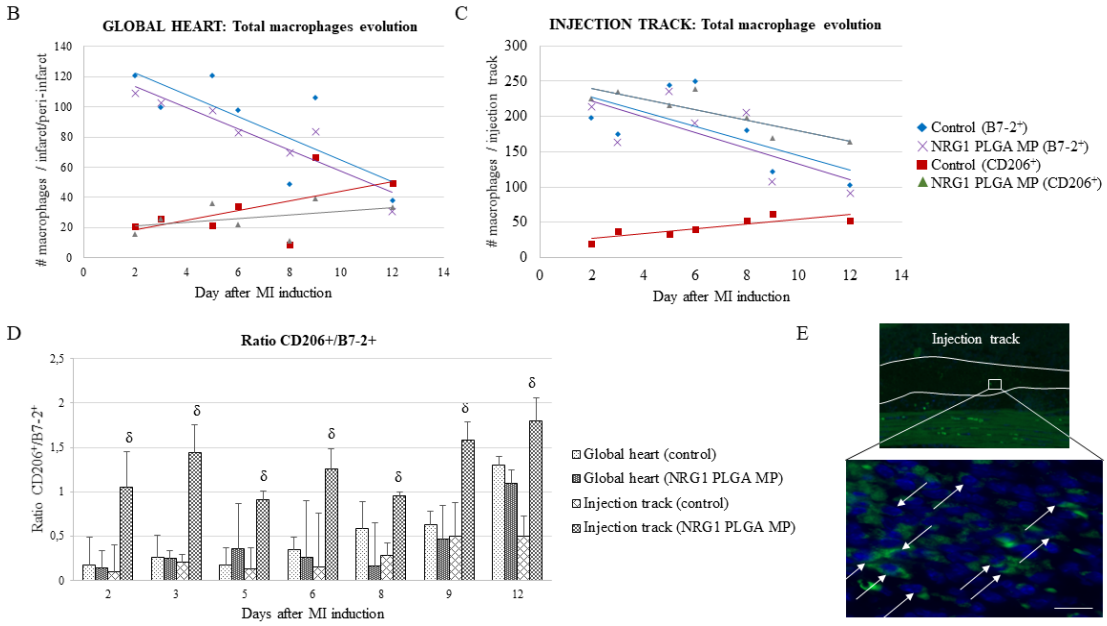

Figure 5: Evolution of B7-2⁺ and CD206⁺ macrophage populations in distal and close areas with respect to NRG1 PLGA MP injection track. A) Count of total number of B7-2⁺ and CD206⁺ cells per 0.001 mm2 of heart tissue. B) B7-2⁺ and CD206⁺ macrophage populations' evolution when all infarcted and non-infarcted regions were analyzed together. C) B7-2⁺ and CD206⁺ macrophage populations' evolution when only proximal regions to the injection track were analyzed. D) Ratios CD206⁺/B7-2⁺. Data are shown as mean ± SD. E) Immunofluorescence images showing NRG1 PLGA MP injection track and CD206+ macrophages. α: $p<0.05$ versus 'Global heart: Control (B7-2⁺)'. ß: $p<0.05$ versus 'Global heart: NRG1 PLGA MP (B7-2⁺)'. γ: $p<0.001$ versus 'Global heart: NRG1 PLGA MP (CD206⁺)'. δ: $p<0.01$ versus 'Injection track (control)'. Scale bar: 30 μm.

Regarding PLGA MP and inflammation, the first thing that must be taken into account is the size and shape of the MP. Although it has been suggested that small particles of around 1 μm accumulate more in cells than larger MP [29], this is only true for opsonized particles [30]. Importantly, we have previously demonstrated that phagocytosis in the heart tissue is not dependent on opsonization [31]. In this scenario, larger microparticles such as the ones used in this study (8 μm) may be more efficient at delivering a greater therapeutic payload to macrophages than smaller microparticles [30]. The size of the MP may also have an effect on cytokine production by macrophages, as shown by Nicolete *et al*. [32]. In particular MP with a diameter of 6.5 μm induced the expression of TNF-α and IL-1ß in J774A.1 cells [32]. However, these

increments in cytokine expression are in the order of pg per mL, which may not have any biological effect. Moreover, we have shown in this chapter that PLGA MP of 8 μm do not induce any significant effect on the expression of TNF-α in the same cell line, or other pro-inflammatory cytokines such as IL6 (Figure 3A). In any case, how macrophages react against the presence of MP must always be taken into consideration, since changes in cytokine expression profiles have been reported for other types of MP [33]. Concerning shape, smooth spherical particles such as the ones used in this chapter (Figure 2C) do not induce the pro-inflammatory response observed with rough surface particles [34]. Last but not least, biomaterials such as PLGA may be non-immunogenic at one time point, but as the material degrades, the physicochemical properties can change in ways that alter the immunogenicity. Our group showed in previous papers that PLGA MP of 5-10 μm size did not induce inflammatory reactions when compared to the injection of resuspension medium [28]. Although it is still not well known how PLGA degradation products could interact with immune cells and modify their behavior [34], there is evidence that indicates that PLGA MP are neutral with respect to inflammation [28], which suggests that this type of MP is suitable for delivering GF or other therapeutics into the heart after AMI without hampering a proper inflammatory response.

Another factor that is mandatory to consider regarding MP administration is when to administer the treatment. On the one hand, piMac are predominant during the first days after AMI and aiMac exert their role afterwards [27]. On the other hand, each type of macrophage may respond in a different way to NRG1 PLGA MP. Moreover, one of the main NRG1 therapeutic actions is the promotion of cardiomyocyte proliferation [35]. After AMI, although the majority of the cardiomyocyte deaths occurs during ischemia, necrosis and apoptosis can continue for up to 3 days [36]. This creates a therapeutic window for arresting cell death using NRG1. In addition, patients promptly treated after the onset of symptoms of AMI will have the best outcomes [37]. In this study we have evaluated how NRG1 PLGA MP administration could diversely affect heart inflammatory response depending on the time of treatment administration (15 minutes, 24, 72 and 168 hours after AMI induction). We selected these time points because they are clinically relevant and cover the therapeutic window of NRG1. Surprisingly, we demonstrated that the inflammatory response was not modified by the time at which the treatment was administered (Figure 4B), and NRG1 PLGA MP treated animals showed a similar macrophage polarization to control animals regardless of the time of treatment administration. Clinically, this means that PLGA MP could be administered whenever needed in order to meet the therapeutic window of the encapsulated GF, without undesired inflammatory issues.

Apart from MP, the nature of the encapsulated GF could also have a huge impact on inflammation. In particular, NRG1 have proved to have anti-inflammatory effects in brain diseases [38,39], multiple sclerosis [40] and sepsis cardiomyopathy [41]. Relevantly, parenteral administration of NRG1 (10 μg/kg) significantly reduced the serum levels of TNF-α and IL-6 in septic rats, suggesting suppression of the inflammatory response of the immune system [41]. The amount of NRG1 administered in this study was higher than that used for this work, which could explain why we did not observe any suppression effects on pro-inflammatory cytokine expression. In any case, our flow cytometry results correlate with the immune suppressive effects since we showed that treatment of J774A.1 cells with NRG1 PLGA MP induced macrophage differentiation toward the aiMac phenotype (Figure 3B). In fact, NRG1 have been shown to reduce the expression of pro-inflammatory cytokines such as IL1-ß and nitic oxide synthase on macrophages [42], and to induce apoptosis of piMac [43]. Taking together, these results strongly suggest that the final effect of NRG1 on inflammation is positive, inducing macrophages to polarize toward a regenerative phenotype (aiMac), as we could confirm in this study (Figure 3B). Anyhow, assessing the effects of NRG1 on the production of anti-inflammatory cytokines on macrophages represents an interesting approach to further characterize the exact role of NRG1 on inflammation.

In this chapter we have demonstrated that NRG1 PLGA MP enhances aiMac over piMac polarization in an AMI model in mice. However, it is important to note that this shift toward a regenerative phenotype was only observed in the vicinity of the injection track, where NRG1 PLGA MP were concentrated. In distal regions, although still infarcted, macrophage populations were not altered compared to control animals. This suggests local polarization of the inflammatory response, but not a global effect of the treatment. In fact, in this study we showed that $CD206^+/B7\text{-}2^+$ ratio only improved in the injection track, whereas in other infarcted regions no significant differences were observed with respect to controls (Figure 5D). This correlates with other observations by other groups, who also detected a positive $CD206^+/B7\text{-}2^+$ ratio increase around the polymeric MP implant [44,45]. In addition, GF released from PLGA MP can only be found next to PLGA MP in the tissue, and not far away [31]. Therefore, both NRG1 and aiMac, which are ultimately responsible for inducing heart repair [6,7,12,46], are located close to PLGA MP, and do not diffuse through the cardiac tissue. On the other hand, other beneficial effects derived from NRG1 PLGA MP treatment such as cardiomyocyte proliferation, angiogenesis, arteriogenesis, embryonic stem cell differentiation into the cardiac lineage and fibrosis reduction can also be observed throughout the infarcted and peri-infarcted regions [16,34,47–49]. This could be explained by the fact that NRG1 induces production and secretion of cytokines and pro-reparative factors (angiopoietin-2, brain-

derived neurotrophic factor, crypto-1 and so on), supporting their role in cardiac repair through the activation of paracrine signaling [50]. Similarly, aiMac release IL-10, TGF-ß [51] and other anti-inflammatory cytokines [52], creating a reparative environment which spreads from the injection track to more distal areas [53], thus justifying the positive and global tissue remodeling observed after NRG1 PLGA MP administration.

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Declaration of Interest Statement

The authors report no conflict of interest.

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GENERAL DISCUSSION

As the reader already knows, the knowledge society has about MI evolved notably over the last century. Advancements in technology and medicine have led to a better understanding of the biology and mechanisms underlying MI, allowing for the development of better and more efficient treatments. Apart from conventional clinical interventions such as bypass or stent approaches, pharmacological treatments have also contributed to the improvement of MI patients' outcomes. As a result, nowadays MI prognosis is the best ever [1,2] and MI mortality is now falling in most European countries [3]. It is important to note that, apart from better treatments, this decrease in the number of deaths was achieved in part thanks to a reduction in smoking [4], which is one of the most important risk factor for this pathology. Nevertheless, it is estimated that other risk factors such as alcohol, diabetes and sedentary life-style will significantly increase in the near future [3,5]. More importantly, many cancer treatments carry a risk of late effects including cardiovascular diseases, possibly leading to significant morbidity and mortality [6]. Better knowledge is needed regarding the late effects of modern systemic cancer treatments and the effects radiotherapy may have on critical structures of the heart. For instance, some chemotherapy drugs can damage the heart muscle provoking congestive heart failure, tachycardia, cardiotoxicity or changes to the heart muscle, as is the case with anthracycline [7]. Consequently, although the current MI mortality is reduced compared to that of some decades ago [8], the total number of MI events reported per year is dramatically increasing [5]. In this sense, hospitalizations due to MI are likely to reach 23 million cases per year in 2030 [5], causing a major annual burden to countries' economies [9].

Concerning conventional MI treatments, these are unable to prevent the negative LV remodeling process that leads to heart failure [10]. Their palliative but not reparative nature contributes greatly to the high secondary economic costs associated with MI [11,12]. A clinical need therefore exists for new and better MI treatments which should ideally be able to repair the infarcted cardiac tissue, thus restoring heart functionality [13]. Moreover, in order to achieve complete heart repair, new treatments must address the mechanisms underlying LV remodeling, in particular, inflammation [14,15]. aiMac, the macrophage subtype with the strongest regenerative properties, has been suggested as a key mediator of tissue repair [16]. In this scenario, protein therapy in combination with biomaterials has emerged as a promising approach to deal with heart remodeling and to recover organ functionality [17–20]. As a matter of fact, recent findings have revealed the importance of the paracrine effect of stem cells [21,22]. This means that when stem cells are administered to treat MI (an approach known as cell therapy), the differentiation of stem cells into cardiac lineage cells is almost irrelevant [23,24]. The benefits observed are mainly due to the GF and other molecules stem cells release into the environment [25], adding support in favor of the

direct administration of therapeutic proteins. Coming back to protein therapy and biomaterials, on the one hand, GF protein allows targeted interventions with well-defined regulatory pathways (such as cardiac microvasculature formation, cardiomyocyte proliferation, etc.), improved scalability, and potentially reduced overall cost [26]. It is relevant to note that a long list of GF with cardioreparative and cardioprotective effects have been tested so far, with promising results (reviewed in [12]). On the other hand, biomaterials' main function is to protect GF against degradation, augmenting their bioavailability and enhancing their therapeutic effects [12]. Biomaterials can be of synthetic, natural or mixed origin, each type providing unique characteristics [27]. In some cases, even the biomaterial itself can have therapeutic effects, but generally speaking they act as vehicles or delivery systems, being used for enhancing the therapeutic actions of the encapsulated drug [10]. Interestingly, both GF and biomaterials cad modulate inflammation [28–30], making them optimal choices for polarizing the inflammatory process of the heart after MI toward a reparative response. Taking all this together, there is strong evidence in favor of using protein therapy to address the heart after a MI event.

To make a brief summary, in this study the potential of the combination of PLGA based MP with GF as treatment for MI has been shown. More precisely, poly (lactic-co-glycolic acid) microparticles loaded with GF such as neuregulin-1 (NRG1 PLGA MP) or fibroblast growth factor-1 (FGF1 PLGA MP) were used. First, the suitability of PLGA MP for encapsulating, protecting and releasing in a controlled manner therapeutic GF *in vivo* was demonstrated (Chapter 1) [31]. Importantly, GF could be detected in the infarcted tissue for up to three months after treatment administration. The phagocytosis profile of PLGA MP and PEGylated PLGA MP (PEG-PLGA MP) in the cardiac tissue was also assessed, bringing to light no differences between them. Secondly, the efficacy of PLGA MP and PEG-PLGA MP loaded with NRG1 or FGF1 in a rat MI model was evaluated (Chapter 2). The study of ejection fraction (EF), angiogenesis and arteriogenesis showed that both types of MP and GF were equally efficient in terms of heart repair and cardiac function recovery after MI [32]. Taking into account that PEGylation did not avoid MP phagocytosis and clearance from the cardiac tissue, and that NRG1 can potentially trigger a more robust positive heart remodeling than FGF1 [33,34], only PLGA MP were employed for the next set of experiments. Finally, in Chapter 3, the interaction between NRG1 PLGA MP and the inflammatory process of the heart after MI was evaluated. Importantly, it was demonstrated that NRG1 PLGA MP locally induce a macrophage shift from the piMac to aiMac phenotype. In turn, this caused an increase in aiMac/piMac ratio, which generates a more favorable environment for cardiac repair [35].

Why NRG1 and FGF1?

As mentioned above, several GF have been shown to influence CMC survival and proliferation, as well as play a role in activating other cardiac repair mechanisms by improving the neovascularization of the infarcted tissue and limiting fibrosis [36]. Such is the case of vascular endothelial growth factor, insulin-like growth factor, hepatocyte growth factor, epidermal growth factor, transforming growth factor beta 1, NRG1, FGF1 and so on [26,37]. Some of them have already been tested in clinical trials (Table 1), and others are being tested currently (as is the case of FGF1; NCT00117936). Each GF has specific characteristics. For instance, NRG1 is the most potent enhancer of CMC proliferation, whereas FGF1 plays a key role in angiogenesis. In any case, particular attention must be placed on NRG1. This GF has been shown to be one of the most efficient in promoting cardiac repair after MI in clinical trials (Table 1), and this has promoted its inclusion in more clinical trials (NCT01439893) that have no published results yet.

Table 1: Clinical trials using growth factor as experimental treatment for acute or chronic myocardial infarction in the last decade. Only clinical trials with available results were included. IGF: insulin-like growth factor. NRG1: neuregulin 1. rh: recombinant human. EDV: end-diastolic volume. ESV: end-systolic volume. LVEF: left ventricular ejection fraction. SDF1: stromal cell-derived factor 1. NYHA: New York Heart Association Functional Classification. G-CSF: granulocyte colony stimulating factor.

Growth factor	Administered agent	Trial status	Main results	Country	Reference
IGF	IGF-1 (mecasermin®)	Completed	Improved remodeling of heart muscle two month after heart attack.	Ireland, Netherlands	NCT01438086
NRG1	rhNRG1	Terminated	Improved LVEF and decreased ESV and EDV	China, United States	NCT01214096
NRG1	rhNRG1	Completed	Increased cardiac output	Australia	ACTRN12607000330448
SDF1	JVS-100	Completed	Improved quality of life, minute walk distance and NYHA.	United States, India	NCT01410331
G-CSF	G-CSF (filgrastim)	Completed	Coronary collateral growth promotion	Switzerland	NCT00596479
Epoetin	Erythropoietin	Completed	Higher rates of adverse cardiovascular events	United States	NCT00378352
Immuno globulin	**I**mmunoglobulin	Completed	Improved LVEF, decreased scar area	Norway	NCT00430885

Importantly, very recent studies have demonstrated that NRG1 "makes" the heart muscle (reviewed in [34]). Whether mature CMC, the main cardiac cells, can proliferate to make new muscle has been an area of contention for many years. However, it has been established that NRG1 promotes heart regeneration in zebrafish, and cardiomyocyte proliferation in mammalian hearts [38–40]. Nowadays, NRG1 is commonly accepted as the most powerful GF for directly inducing CMC proliferation, in part because its biological receptors ErbB4 and ErbB2 are expressed in the cardiac cells [31]. Such receptors are found in both neonatal and adults animals, indicating that NRG1 is a valid therapeutic agent to treat grown-up individuals [41–44]. In this regard, NRG1 prevents severe dysfunction of the adult heart and improves cardiac performance in rodent models of ischemic, dilated and viral cardiomyopathy [45], making NRG1 a broad-spectrum therapeutic agent for the treatment of heart failure. This cytokine has also strong neovascularization effects [46], as confirmed by our group [47,48]. Other GF with cardioreparative properties such as vascular endothelial growth factor do not possess the same angiogenic effects [49], and may need to be combined with other cytokines that cooperate with vessel maturation. Moreover, NRG1 induces mature ventricular cardiac differentiation from induced pluripotent stem cells, which contributes to cardiac tissue repair [50]. In parallel, NRG1 reduces cell apoptosis [14], which could reduce CMC loss after MI. Therefore, NRG1 possesses direct effects on the main cardiac cells, and heart function overall (Figure 1). Taken together, all this strongly supports the use of NRG1 for addressing heart repair and remodeling after MI. Relevantly, all these mechanisms in which NRG1 is involved take place within the first hours and days after the MI event. Therefore, the therapeutic window of NRG1 corresponds with the typical time frame in which patients suffering from MI undergo an in-hospital intervention.

Early in the discussion it was stated that inflammation of the heart after MI is the most conditioning process in cardiac repair [15,52]. Although the relevance of inflammation will be discussed in the following sections, it is essential to note here that this inflammatory response determines the final heart recovery [53–55], and that this fate is decided during the first week after MI [54]. This corresponds to the therapeutic window of NRG1. Interestingly, aiMac stimulation and piMac inhibition during this early stage may have a high impact on final heart remodeling [16,35]. In addition, ErbB4 is expressed in immune system cells as well [18,56,57]. As a consequence, NRG1 can be used to modulate the immune response. In fact, parenteral administration of NRG1 resulted in significant reduction of the pro-inflammatory cytokines TNF-α, IL-6 and IL-ß1, thus suppressing the pro-inflammatory response [58]. In Chapter 3 it was shown that NRG1 PLGA MP did not increase or reduce TNF-α or IL-6 expression.

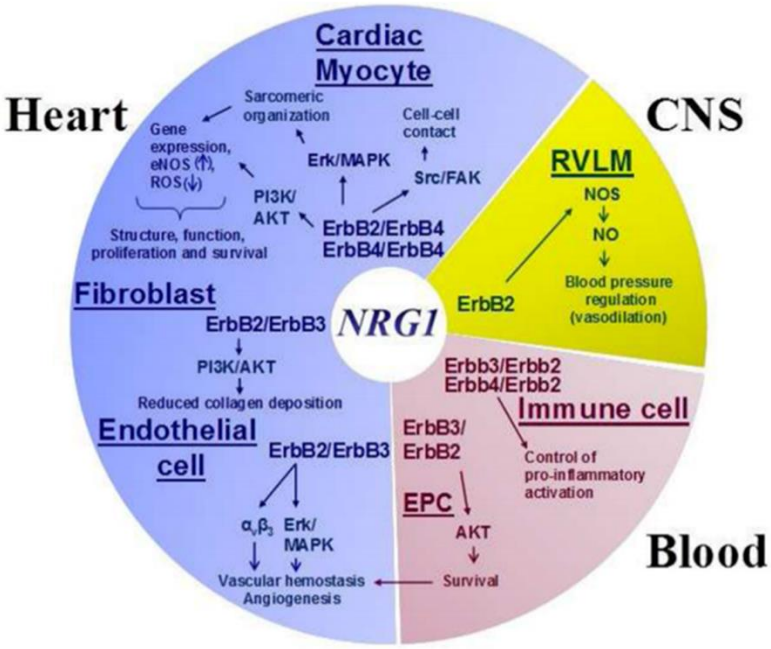

Figure 1: Role of NRG1/ErbB signaling in the adult cardiovascular system. AKT: protein kinase B. CNS: central nervous system. EPC: endothelial progenitor cells. ERK: extracellular signal-regulated kinases. FAK: focal adhesion kinase. NO: nitric oxide. NOS: nitric oxide synthase. PI3K: phosphoinositide-3-kinase. RVLM: rostral ventrolateral medulla. Src: proto-oncogene tyrosine-protein kinase. This Figure is kindly provided by [51].

This may be due to dose issues, because normally when administered intravenously a higher dosage is used. In any case, all this adds more evidence in favor of using NRG1 as a cardioreparative agent, converting this GF into one of the most promising approaches for addressing heart repair [34,41].

Apart from NRG1, another GF that is attracting research interests is FGF1. FGF1 is well-known for its potent activation effects on vascular smooth muscle and endothelial cells [59]. As a result, FGF1 exerts a leading role in controlling blood vessel formation under pathological conditions [60], a function that is preserved during adult life [61]. Importantly, whereas other GF induce angiogenesis under any circumstances, FGF1 only trigger vascular smooth muscle cell hyperplasia under ischemic conditions [62]. This offers the opportunity of a more localized and safer treatment since cytokines spread to other body compartments will not have undesired side effects. What is more, independently from angiogenesis, FGF1 provides cardioprotection at the level of the cardiac myocytes via activation of the mitogen activated protein kinase ERK1 and 2 [63], avoiding programmed myocyte cell death triggered by I/R injury [64] and upregulating cardiogenesis through the protein kinase C signaling [65]. Concerning inflammation, FGF1 has been reported to reduce neutrophil infiltration and tissue damage in rat

I/R heart model [66], which results in a good environment conditioning for cardiac tissue repair. Even though FGF1 has been demonstrated to be efficient in recovering cardiac function after MI in preclinical studies carried out by our group [47,48], positive heart remodeling is enhanced when it is co-administered with p38 MAP kinase inhibitor [67] or enoxaparin [68], as confirmed by reduced scarring and wall thinning, markedly improved cardiac function and higher regional myocardial blood flow.

After MI, scar tissue in the ventricle wall represent one major challenge [69]. The shaped scar initially represents a functional solution to the heart, avoiding LV wall rupture. However, if not restored, it may lead to LV negative remodeling and dilation, causing eventual death [18,52,70]. In this regard, taking into account that FGF1 attenuates fibrosis in pulmonary [71] and liver [72] diseases, FGF1 may play a role in preventing scar tissue spreading in the heart. Indeed, FGF1 induces apoptosis of fibroblast and inhibits myofibroblast differentiation [73], which could prevent heart tissue contractibility loss. Since almost all cell death after MI occurs in the hours and days following the infarct, FGF1 therapeutic window is right after MI, when there is still an opportunity for avoiding cardiac cell leakage and scar formation. Together, all these facts encourage the use of FGF1 as a potential therapeutic drug for heart repair after MI.

Finally, the dose of each GF was calculated based on previous experiments by our group, where we showed that administration of around 1500 ng of NRG1 or FGF1 in a rat heart is enough to induce cardiac repair [48]. These doses were obtained after using *in vitro* models for establishing potential therapeutic doses. When *in vitro–in vivo* correspondence was carried out, a lack of correlation was found, due to the intrinsic differences between the two models [74]. In the same way, huge differences can be found when preclinical and clinical assays are compared. Moreover, in clinical studies the standard dosage is not maintained among trials, being 0.6-2.4 μg/Kg/day for 10 days for NRG1 (clinical trials references: NCT01214096, ACTRN12607000330448) and 2-40 μg/Kg in a single dose for FGF1 (clinical trial reference: NCT00117936). Hence biological doses for each organism must be reviewed for each GF in order to administer a clinically relevant therapeutic dose.

Why PLGA based MP?

Notwithstanding the evidence in favor of NRG1 and FGF1 as ideal candidates for addressing cardiac repair after MI, there is still one major limitation to their application. Both are labile proteins that are rapidly degraded after *in vivo* administration and are removed from the site of injection [75]. In fact

this is the reason why some clinical trials in the past have failed in proving the efficacy of protein therapy [12]. Nowadays delivery systems made of biomaterials are well established as the best solution to this problem [27]. Among all delivery systems, MP are solid particles in the micrometer size range in which the active principle is dissolved, entrapped, encapsulated or adsorbed [76]. MP have been largely used as vehicles for delivering drugs into the heart after MI (reviewed in Annex 1) because they can be administered using non-invasive techniques such as catheter technology, serve as a matrix to support cell growth and integration and act as a controlled release reservoir to locally deliver bioactive molecules (Annex 1). For instance, some tumorigenic effects of NRG1 [41] and FGF1 [59] have been reported in certain cases. Fortunately, localized delivery of such therapeutics would prevent this undesired side effect and can ensure the safety of the treatments.

Regarding biomaterials, the synthetic polymer PLGA was approved by the Food and Drug Administration of the United States for its use in humans and thus its applications in biomedicine have increased enormously [77]. PLGA is a synthetic aliphatic polymer with a polyester backbone that is formed through the copolymerization of lactic and glycolic acid monomers (Figure 2A).

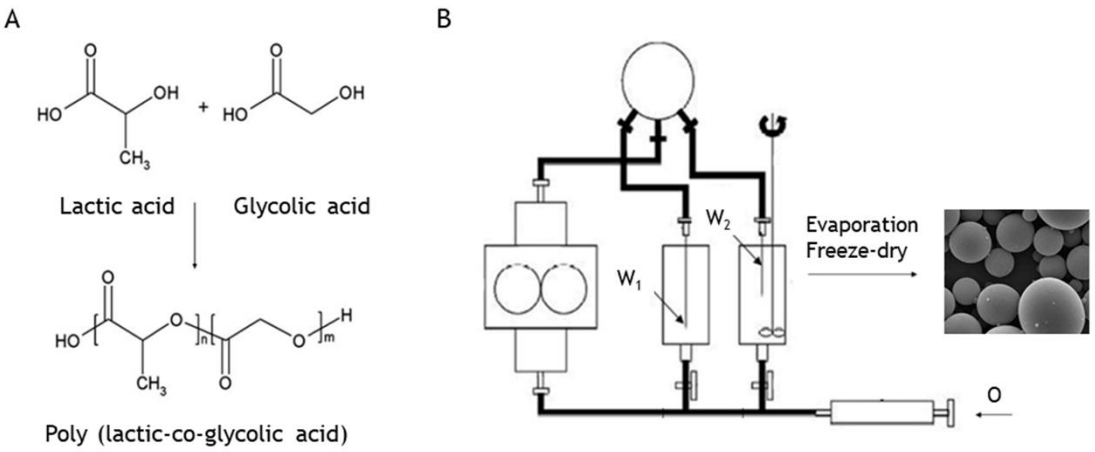

Figure 2: Poly (lactic-co-glycolic acid) microparticles structure and synthesis. A) Synthesis of PLGA as of its two monomers, lactic and glycolic acid. B) Total Recirculation One-Machine System (TROMS®). W_1: inner aqueous phase containing the GF. W_2: external aqueous phase. O: organic phase containing the polymer.

Because PLGA is a copolymer, it can be polymerized into many different formulations based on the composition of lactic and glycolic acid monomers [78]. This means that PLGA offers attractive characteristics such as biodegradability, biocompatibility, adjustable controlled release profiles, and encapsulation of different therapeutics, and it can be easily modified (refer to Annex 2 and ref [79]). With respect to cardiac applications, several

international groups including our own have tested PLGA MP in mice [80–82], rat [48,74,83–91] or pig models [47] for the delivery of proteins. In general, encouraging observations were reported and the role of PLGA MP proved to be essential for obtaining good efficacy results. However, the encapsulation efficiency of proteins into PLGA devices was low or not indicated in the majority of studies. Importantly, our group uses TROMS® technology for the encapsulation of therapeutic GF into PLGA MP, which allows high encapsulation efficiency of around 80% [87,88,92]. TROMS® is based on the multiple emulsion and solvent evaporation method, in which GF are incorporated in the inner aqueous phase (W_1, Figure 2B). This process does not imply high temperatures or aggressive solvents, thus ensuring conservation of GF activity. The PLGA MP obtained by TROMS® have a spherical shape and smooth surface, making them ideal for targeting inflammation in the heart. The spherical smooth particles of 5-10 μm employed in our experiments are efficient at delivering the therapeutic payload to macrophages [93] and do not induce any significant increase in the expression of pro-inflammatory cytokines [28,88]. Interestingly, MP of that size were found to remain at the site of the injection after administration in the cardiac tissue [88]. By contrast, nanoparticles may diffuse to other body compartments due to their reduced size, which means that they do not allow such a localized treatment [94]. MP were also found to be degraded over time under *in vivo* conditions (Chapter 1). However, other delivery systems made of PLGA, such as nanofibers, remain in the cardiac tissue for prolonged periods of time [95]. This could cause chronic inflammation characterized by the presence of giant cells, which might compromise tissue repair. Therefore, PLGA MP were selected to deliver NRG1 or FGF1 into the heart with the aim of developing an optimal treatment for arresting infarct progression and achieving full heart repair after MI.

Why NRG1 PLGA MP?

In Chapter 1 we showed how PLGA MP are able to release therapeutic NRG1 in a controlled manner over time. In addition, we also demonstrated that the delivery of NRG1 was local, as NRG1 could only be found next to where PLGA MP were located in the cardiac tissue, with no spread of NRG1 to more distal areas. Interestingly, the GF could be detected in the heart for up to three months after NRG1 PLGA MP administration, confirming the suitability of PLGA MP for protecting and releasing proteins for cardiac repair. Importantly, the released NRG1 proved to be biologically active, as demonstrated by the activation of Erb4 receptor in CMC.

As cited previously, PLGA MP are of synthetic origin, and thus can be modified in a variety of ways in order to change properties such as degradation,

release, targeting, stability and so on [96]. One elegant strategy is to enhance the permanence of the PLGA MP in the tissue by PEGylating them (adding a coating of PEG on the surface). This protocol is known to augment the half-life of MP in the blood [97]. In blood, opsonins bind and attach to circulating particles, thus favoring macrophage action and uptake of opsonin-marked bodies. Consequently, we hypothesized that PEGylation of PLGA MP could also improve the permanence of the systems in the heart tissue, thus enhancing the efficacy of the treatment. The set of experiments from Chapter 1 were also performed with PEGylated PLGA MP, obtaining similar results. However, when phagocytosis was assessed, no significant differences between PLGA and PEG PLGA MP were found in the short and medium term (up to one month after treatment administration). It is true that after three months there were more PEG-PLGA MP than PLGA MP in the tissue. Nevertheless, this is not relevant in terms of treatment efficacy since heart remodeling and the mechanisms that determine cardiac tissue repair occur within the first week after MI (Chapter 2). Fluorescent (Chapter 1) and confocal microscopy (Chapter 2) confirmed that both PLGA and PEG PLGA MP have been uptaken by macrophages. Consequently, phagocytosis in the heart may not depend on opsonization as it does in the blood tissue.

Chapter 2 revealed that similar heart function recovery, angiogenesis and arteriogenesis were obtained with PLGA based MP, regardless of whether they were made of PLGA or PEG PLGA, or they were loaded with NRG1 or FGF1. In other words, there are no significant differences in efficacy among NRG1 PLGA MP, NRG1 PEG PLGA MP, FGF1 PLGA MP and FGF1 PEG PLGA MP treatments. This, together with the phagocytosis studies, led us to select PLGA MP for further studies, since the fewer external compounds are administered to the organism, the likelier it is to get better outcomes. On the other hand, taking stock of NRG1 and FGF1, it is interesting to note that NRG1 is the most appropriate GF for cardiac repair [34]. Previously we discussed the unique and inherent properties that make NRG1 the ideal candidate to directly induce CMC proliferation and favor positive LV remodeling (reviewed in [40,51,56]). We have also confirmed that FGF1 is a potent angiogenic factor that possesses cardioprotective effects [60,63]. However, the full potential of FGF1 is only reached when it is combined with other drugs [67,68], whereas NRG1 itself triggers global heart repair. Moreover, NRG1 reduces cardiac fibrosis to a higher degree than FGF1 [47]. This is due to the fact that FGF1 reduces fibrosis by inhibiting Pdlim3 gene, which constitutes its main route of action. However, FGF1 can also favor collagen deposition by enhancing the expression of the Gpr158 pathway [33].

Taking all this into account, as well as the fact that all treatments showed the same efficacy in a rat model of MI as reported in Chapter 2, we selected NRG1

PLGA MP for the next experiments. This choice was made in order to minimize the complexity of the treatment while maximizing its safety.

Why inflammation?

As explained above, inflammatory response triggered in the heart after MI is considered nowadays the process that conditions cardiac repair most. During this inflammatory process macrophages, the main mediators of inflammation, suffer profound restructuring [54,98]. It has been demonstrated that aiMac, derived from yolk sac, retain important regenerative capacities and generate minimal inflammation while promoting cardiac recovery through cardiomyocyte proliferation and angiogenesis [16] (Figure 3). On the other hand, piMac are associated with pro-inflammatory activities and lack reparative properties [16] (Figure 3). It is important to note that this is a general classification, and inside aiMac and piMac other subtypes can be identified. Interestingly, from a functional perspective, when all cardiac macrophages are depleted after ischemic injury, a net beneficial role in scar formation is revealed, given that macrophage-depleted mice demonstrate poor infarct healing and myocardial rupture [99,100]. Hence, the solution is not to totally deplete macrophages but to enhance the reparative response of inflammation (governed by aiMac). For instance, piMac are responsible for debris clearance, a pathway that must be maintained for proper tissue regeneration afterwards. To date, several independent studies [101–106] and reviews [107,108] have confirmed this statement, leading to the conclusion that increments in aiMac/piMac ratio result in better cardiac outputs. All this has prompted a current large scale clinical trial testing the effects of reducing the pro-inflammatory response of the heart (NCT01327846).

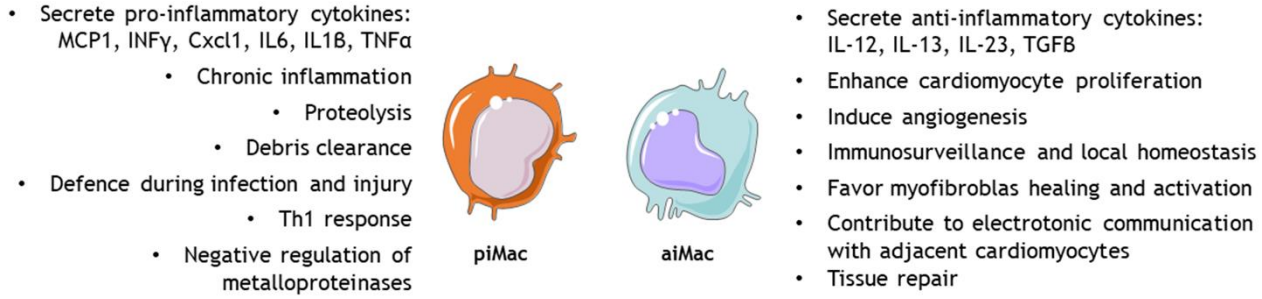

Figure 3: Two main polarizations of heart macrophages and their main functions: piMac or por-inflammatory (also known as M1 polarization) and piMac or anti-inflammatory (also known as M2 polarization).

However, none of the studies carried out so far assesses the administration of therapeutics directly into the heart tissue using delivery systems. In addition,

most of the approaches are based on reducing the pro-inflammatory response, whereas few of them focus on enhancing aiMac performance [101]. Thus, Chapter 3 constitutes a novel approach, in which we showed how NRG1 PLGA MP do not interfere with piMac while they do have a positive effect on aiMac (increase in the aiMac/piMac ratio at the track of injection of the treatments). This result was initially suggested by flow cytometry assays in J774A.1 cells. Since depletion of macrophages may cause negative results, strategies like ours focused on improving anti-inflammatory instead of suppressing pro-inflammatory response are more likely to achieve more complete tissue repair.

The animal model itself is also directly related to inflammation. There are several animal models that have been employed so far for the study of MI, which can be divided into three main kinds: those in which MI is surgically induced; those in which MI is produced due to hypertensive conditions; and those in which MI is the result of virus-induced myocarditis. In an attempt not to alter inflammation, the last two are the most indicated models since MI is obtained by minimally invasive techniques, mimicking clinical reality. However, it is also impossible to control the infarct size and magnitude of the final infarct. Therefore, they are not the best way for assessing treatment efficacy, since high inter-variability among animals could mask or impair the final observations. On the other hand, surgically induced MI offers a total control of infarct size through direct visualization of artery occlusion and infarcted tissue. In any case, it is important to consider that open chest surgeries induce a high secondary inflammatory response apart from the one caused by the infarct itself. This may have as a consequence a higher pro-inflammatory polarization of macrophages in the heart, which could hamper the effects of treatment. On account of this, for the *in vivo* experiments in Chapters 1 and 2 we used the permanent blockage model, even though we were conscious of its limitations.

Finally, regarding Chapter 3, instead of the permanent MI model, an I/R model was used. This is also surgically induced, but the occlusion of the coronary artery is temporary (45 minutes), and then blood flow is restored for tissue reperfusion. This way clinical interventions are perfectly reproduced in the animal model (patients normally underwent a bypass or stent procedure), making it optimal for the study of the interactions between inflammation and NRG1 PLGA MP. Although I/R allows the cell oxygen supply to be restored, it has been described that the final outputs of this procedure are negative for cardiac repair [109]. I/R is responsible for tissue damage mediated by oxidative stress, increase in the intracellular calcium levels and aggressive pH modifications [110,111]. However, taking into account that in Chapter 3 the aim was to assess how NRG1 PLGA MP were able to polarize and modulate the immune response, we selected this model for our *in vivo* experiments.

In short, this manuscript supports the use of NRG1 PLGA MP for heart repair after MI. PLGA MP proved to efficiently protect and release the bioactive therapeutic GF in the cardiac tissue for up to three months after treatment administration. Moreover, the efficacy of NRG1 PLGA MP was tested in a relevant animal model of MI, demonstrating their ability to enhance heart function and to induce positive heart remodeling through angiogenesis, arteriogenesis and positive modulation of inflammation. In particular, NRG1 PLGA MP improved the aiMac/piMac ratio, which creates an environment that is more conductive to repair. All this makes NRG1 PLGA MP a potential candidate for inclusion in clinical trials. However, there are some considerations that must be addressed beforehand. In particular, issues related to the translation from rodent models to humans. MP size and cargo will have to be adjusted to meet the conditions of human hearts, which are bigger and possess a lower heart rate. Taking into account the similarities between humans and pigs with regard to the cardiovascular system, the study and evaluation of these systems in clinically relevant pig models of MI is mandatory before moving to the clinical arena. Notwithstanding these concerns, it must not be forgotten that PLGA MP hold the potential to overcome the bioavailability and high-dose related problems observed in clinical trials so far when therapeutic proteins were administered intravenously (ClinicalTrials.gov identifiers: NCT01539590, NCT01438086, NCT00756756, NCT00378352), subcutaneously (NCT02419937, NCT00789724, NCT00670228) or orally (NCT01176968, NCT02145468, NCT01175018, NCT00684203, NCT00344019). In addition, NRG1 is a new molecule that is only now being tested in clinical trials (Table 1), and the combination of NRG1 and MP has not yet been evaluated. Nonetheless, the advantages it presents clearly outweigh the disadvantages, and we can propose NRG1 PLGA MP as one of the most promising and attractive approaches for addressing heart repair after MI.

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CONCLUSIONS

The results achieved during this PhD project have led us to the following conclusions:

1. Poly (lactic-co-glycolic acid) microparticles were successfully synthetized using TROMS® technology, obtaining microparticles of a final size compatible with intramyocardial injection and negative Z potential. PEGylated microparticles were similarly synthetized. PEG PLGA MP had the same size and negative but slightly higher Z potential due to PEG coating. NRG1 or FGF1 were encapsulated into PLGA and PEG PLGA microparticles, obtaining high encapsulation efficiencies (over 75% in all experiments).

2. Both types of microparticles were shown to be efficient in protecting NRG1 against degradation and in delivering the load for up to three months in a rat model of myocardial infarction. Moreover, NRG1 maintained its bioactivity during the entire encapsulation process and once in the cardiac tissue, as demonstrated by the specific activation of ErbB4 receptor in cardiomyocytes three months after administration by immunofluorescent studies.

3. Therapeutic NRG1 was located next to microparticles and did not spread to other cardiac tissues far away from the injection track. PLGA microparticles, NRG1 and cardiomyocytes could be detected together (fluorescent microscopy showed overlapping of all the three elements). Therefore, microparticles were able to carry therapeutic proteins to their biological site of action. All treatments significantly increased the angiogenesis and arteriogenesis of the infarcted and peri-infarcted areas compared to controls and non-loaded microparticle-treated animals.

4. Both types of microparticles loaded with NRG1 or FGF1 were able to similarly improve cardiac function in a rat model of myocardial infarction. Such improvements in ejection fraction parameter could not be observed in controls or non-loaded microparticle-treated animals.

5. PEGylation strategy did not avoid phagocytosis of microparticles in the heart during the first month, as confirmed by fluorescent and confocal microscopy. This suggested that phagocytosis in the heart is not mediated by opsonins. However, PEG coating of microparticles resulted in a larger amount

of PEGylated microparticles in the cardiac tissue than non-PEGylated microparticles three months after administration.

6. NRG1 non-pegylated PLGA microparticles were selected for further characterization. On the one hand, NRG1 directly trigger cardiomyocyte replication and potentially have a more direct role in cardiac repair than FGF1. On the other hand, PEG coating did not add any benefit to the treatment in the short and medium term, thus proving to be irrelevant for the efficacy of the treatment.

7. NRG1 PLGA MP induced a shift in macrophage polarization in the heart toward a regenerative phenotype. This effect was only detected in the injection track, corresponding to the area in which microparticles and NRG1 were located and was always observed regardless of the time of treatment administration. This indicates that PLGA MP can be administered depending on the therapeutic window of the encapsulated GF, without any inflammatory side effects.

CONCLUSIONES

Los resultados obtenidos en este trabajo nos permiten extraer las siguientes conclusiones:

1. Mediante la tecnología TROMS®, se formularon micropartículas de PLGA y PLGA-PEG con un tamaño compatible con la administración intramiocárdica y carga superficial negativa. La NRG1 o el FGF1 se encapsularon en ambos tipos de micropartículas, obteniéndose eficacias de encapsulación superiores al 75%.

2. La NRG1 mantiene su actividad biológica en ambos tipos de micropartículas tras su encapsulación y liberación en el tejido cardíaco, activando el receptor ErbB4 en cardiomiocitos.

3. Las micropartículas transportaron la proteína terapéutica hasta su lugar de acción; la NRG1 se detecta en la zona de inyección y no difunde a tejidos cardíacos lejanos a la misma.

4. Ambos tipos de micropartículas cargadas con NRG1 ó FGF1 mejoran de manera significativa la angiogénesis y la arteriogenesis de las áreas infartadas y peri-infartadas en un modelo de infarto de miocardio en rata. Además, todos los tratamientos mejoran de manera similar la función cardíaca en un modelo de infarto de miocardio en rata. Dicha mejora no se observa en los controles ni en los animales tratados con micropartículas vacías.

5. La PEGilación no evita la fagocitosis de las micropartículas en el corazón durante el primer mes tras el implante, tal y como se confirmó mediante microscopía confocal y de fluorescencia. Ello sugiere que la fagocitosis en el corazón es un proceso independiente de opsoninas. Sin embargo, el recubrimiento con PEG resulta en un número significativamente mayor de micropartículas en el tejido cardíaco tres meses después de la administración del tratamiento.

6. Las micropartículas de PLGA cargadas con NRG1 promueven directamente la división de cardiomiocitos y por ello pueden tener una mayor contribución en la reparación del tejido cardíaco que el FGF. El recubrimiento

de las micropartículas con PEG no supone ninguna mejora para la eficacia del tratamiento.

7. Las micropartículas de PLGA cargadas con NRG1 inducen un cambio en la polarización de los macrófagos del corazón hacia un fenotipo regenerativo. Este efecto solo se observa en la zona próxima al tracto de inyección, área en la cual se encuentran las micropartículas y la NRG1. Además, este efecto beneficioso en la respuesta inflamatoria es independiente del tiempo de administración del tratamiento. Ello indica que las micropartículas de PLGA pueden ser administradas en función de la ventana terapéutica del agente encapsulado, sin que se produzca una reacción inflamatoria no deseada.

ANNEXES

ANNEX 1

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ANNEX 2

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Chapter: 19

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Bioresorbable polymers for next- generation cardiac scaffolds

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Abstract

Biomaterial science may offer new approaches to cardiac tissue engineering. Several bioresorbable polymers already possess the characteristics necessary to be administered into a damaged heart for cardiac regeneration after myocardial infarction (MI). This chapter focuses on the principal preclinical studies performed in the past 10 years with natural and synthetic bioresorbable polymers for myocardial repair. Alginate, chitosan, collagen, fibrin, poly(lactic-*co*-glycolic acid), and polyethylene glycol, among others, have been explored to synthesize cardiac scaffolds such as nanofibers, nano- and microparticles, and hydrogels that have been injected either alone or combined with therapeutic cells and/or proteins into the infarcted heart tissue. Many positive advances, such as cardiac function improvement, infarct size reduction, and increase in neovascularization, have been observed after using such bioresorbable devices. However, although cardiac tissue engineering approaches have been shown to improve heart recovery after MI, there is still no approved treatment available on the market. More research is needed on cardiac tissue engineering strategies to optimize these promising treatments.

Keywords:

Biodegradable, Biomedical applications, Bioresorbable, Myocardial repair, Next-generation scaffolds, Tissue engineering.