Biodegradation and heart retention of polymeric microparticles in a rat model of myocardial ischemia

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ABSTRACT

Poly-lactide-*co*-glycolide (PLGA) microparticles emerged as one of the most promising strategies to achieve site-specific drug delivery. Although these microparticles have been demonstrated to be effective in several wound healing models, their potential in cardiac regeneration has not yet been fully assessed. The present work sought to explore PLGA microparticles as cardiac drug delivery systems. PLGA microparticles were prepared by Total Recirculation One-Machine System (TROMS) after the formation of a multiple emulsion. Microparticles of different size were prepared and characterized to select the most suitable size for intramyocardial administration. Next, the potential of PLGA microparticles for administration in the heart was assessed in a MI rat model. Particle biodegradation over time and myocardial tissue reaction were studied by routine staining and confocal microscopy. Results showed that microparticles with a diameter of 5 μm were the most compatible with intramyocardial administration in terms of injectability through a 29-gauge needle and tissue response. Particles were present in the heart tissue for up to three months post-implantation and no particle migration towards other solid organs was observed, demonstrating good myocardial retention. CD68 immunolabeling revealed 31%, 47% and below 4% microparticle uptake by macrophages one week, one month and three months after injection, respectively ($P<0.001$). Taken together, these findings support the feasibility of the developed PLGA microparticles as vehicles for delivering growth factors in the infarcted myocardium.

Keywords: Myocardial infarction, PLGA microparticles, biocompatibility, phagocytic uptake, growth factors.

1. Introduction

Myocardial infarction (MI) is a great threat to life in developed countries, and so research efforts are being focused on the development of new therapies. Therapeutic angiogenesis induced by exogenous administration of growth factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) has been considered a promising strategy to treat patients with MI. However, although many pre-clinical studies have reported beneficial effects of angiogenic growth factor administration after MI, neither VEGF nor FGF have demonstrated efficacy in double-blinded clinical trials [1, 2]. These disappointing results were attributed, at least partially, to the high intrinsic instability of the protein when systemically administered and the short half-life during which growth factors retain their biologic activity *in vivo*.

Current methods for growth factor delivery require administration of high protein concentration and repeated injections, which may result in abnormal vessel formation and unwanted side effects such as hypotension [3, 4]. Targeted delivery of angiogenic proteins into the ischemic heart could therefore be useful. Delivery strategies that provide sustained local release of growth factors would not only control protein concentration, but could also minimize systemic exposure. A number of approaches have been designed to deliver growth factors in the heart in a controlled fashion. These include hydrogels, peptide nanofibers, liposomes, nano- and microparticles mainly for delivery of VEGF [5-9], FGF-1 [10] and FGF-2 [11, 12]. While each delivery platform has both merits and drawbacks in the controlled delivery of angiogenic growth factors, there are few reports about the feasibility of these approaches via the intramyocardial route in relation to injectability, local retention and tissue response.

Polymeric microparticles encapsulating protein drugs offer the possibility of controlling the release of macromolecules over extended time periods [13]. Copolymers of lactic and glycolic acids (PLGAs) have been studied most commonly for this purpose because of their proven safety record and established use in marketed products for controlled delivery of several peptide drugs [14, 15]. Nevertheless, PLGA microparticles have not been thoroughly investigated as a feasible delivery system for growth factors into the myocardium.

In this study, the compatibility of PLGA microparticles with intramyocardial administration was evaluated in a rat model of myocardial ischemia. To this end, PLGA microparticles were prepared using Total Recirculation One-Machine System (TROMS), a technique based on the multiple emulsion solvent evaporation method which is suitable for the encapsulation of labile molecules like proteins [7, 16]. Physical characteristics of the microparticles such as morphology, size or surface charge were primarily investigated. Furthermore, flow properties such as dispersability and injectability of microparticle suspension were analyzed to avoid complications during their administration. The *in vivo* biodegradation of the particles in the infarcted tissue and the evaluation of the myocardial responses to PLGA microparticles were finally evaluated using a rat model of MI to ensure safety and biocompatibility requirements.

2. Materials and Methods

2.1 Materials

PLGA with a monomer ratio (lactic acid/ glycolic acid) of 50:50 Resomer® RG 503H (M_w: 34 kDa) was provided by Boehringer-Ingelheim (Ingelheim, Germany). Polyethylene glycol (PEG; M_w: 400), human serum albumin (HSA) and rhodamine B

isothiocyanate were provided by Sigma-Aldrich (Barcelona, Spain). Dichloromethane and acetone were obtained from Panreac Quimica S.A. (Barcelona, Spain). Poly(vinyl alcohol) (PVA) 88% hydrolyzed (M_w: 125,000) was obtained from Polysciences, Inc. (Warrington, PA, USA). Dubelcco's Modified Eagle Medium (DMEM) was provided from Gibco-Invitrogen (Carlsbad, CA, USA). Mouse monoclonal anti-rat CD68 antibody (MCA341R) was purchased from Serotec (Oxford, UK). Alexa Fluor 488 goat anti–mouse IgG antibody was provided by from Molecular Probes (Eugene, OR, USA).

2.3 Microparticle preparation

PLGA microparticles were obtained after the preparation of a multiple emulsion by solvent evaporation method using the Total Recirculation One-Machine System (TROMS) [7, 16, 17]. Briefly, the organic phase (O) composed of 50 mg of PLGA dissolved in 2 ml of a dichloromethane/acetone mixture (ratio 3:1) was injected into the inner aqueous phase (W_1) containing 5 mg of HSA and 5 μl of PEG 400 dissolved in 200 μl of phosphate-buffered saline (PBS pH 7.9). Next, the previously formed inner emulsion (W_1/O) was recirculated through the system under a turbulent regime maintained by a pumping flow through a needle with an inner diameter of 0.17 mm. After this homogenization step, the W_1/O emulsion was injected into the outer aqueous phase (W_2) composed of 20 ml of a 0.5%$_{w/v}$ PVA solution. The turbulent injection through a second needle resulted in the formation of a multiple emulsion ($W_1/O/W_2$), which was allowed to circulate through the system to become homogeneous. The multiple emulsion was stirred for 3 h to allow solvent evaporation. Microparticles were washed three times with ultrapure water by consecutive centrifugation at 4 °C (20,000×g, 10 min). Finally, the particles were resuspended in 1 ml of ultrapure water, frozen at −80 °C, lyophilized (Genesis 12EL, Virtis) and stored at 4 °C. In order to obtain batches with different particle sizes, the following TROMS parameters were adjusted during microparticle preparation: pumping flow, recirculation times to form W_1/O and $W_1/O/W_2$ emulsions, and the inner diameter of the needle used to prepare the $W_1/O/W_2$ emulsion. A needle with an inner diameter of 0.17 mm was used to form the primary W_1/O emulsion of all microparticle batches.

For fluorescence-labeled microparticle formulation, rhodamine B isothiocyanate (0.5 mg/mL) was added to the inner aqueous phase and microparticles were prepared as described.

2.4 Size, surface charge analysis and morphological observation of PLGA microparticles

Particle size and size distribution of the microparticles were measured by laser diffractometry using a Mastersizer® (Malvern Instruments, UK). The average particle size was expressed as the volume mean diameter in micrometers and samples were measured in triplicate.

For rhodamine-labeled microparticles, particle size was estimated using the software imaging system Cell* connected to the camera fluorescence microscopy system CH40 (Olympus GmbH, Münster, Germany). The morphology of the particles was characterized by scanning electron microscopy (SEM). Briefly, the lyophilized microparticles were mounted on carbon conductive disks attached to aluminum stubs. Samples were then coated with gold to a 16-mm thickness (Emitech K550 equipment). Microparticles were randomly scanned using SEM (Zeiss DSM 940A, Germany) and photomicrographs were taken.

Particle surface charge was determined by zeta potential measurement (Zeta Plus Potential Analyzer, Brookhaven Instruments Corp., New York, USA). A dilute suspension (0.5 mg/ml) of microparticles was prepared in 1 mM KCl (pH = 7.6) and the zeta potential measurements were performed after 10 cycles in the high precision mode.

Rhodamine-labeled microparticles were imaged at high-power by fluorescence microscopy. Microparticles were resuspended in water, mounted on a microscope slide, and visualized using a camera microscopy system (Olympus CH40).

2.5 Determination of microparticle dispersability and injectability

Prior to *in vivo* studies, microparticle dispersability was tested in three different resuspension media: PBS, DMEM and DMEM supplemented with a surfactant mixture composed of 0.1%$_{w/v}$ carboxymethyl cellulose, 0.8% $_{w/v}$, polysorbate 80 and 0.8% $_{w/v}$ mannitol in PBS, pH 7.4 (DMEM-S). Microparticle suspension injectability was assessed by its ability to pass through a 29-gauge needle, since these needles are used for heart injection [11]. Particle concentration and particle size were evaluated as injectability parameters in order to define the optimal microparticle formulation for heart injection.

2.6 Quantification of residual PVA content

The residual PVA associated with microparticles was determined by a colorimetric method [18]. Two milligrams of dry microparticles were hydrolyzed with 2 ml of 0.5 M NaOH for 15 min at 60ºC. The solution was then neutralized with 900 µl of 1 N HCl and the volume was adjusted to 5 ml with distilled water. Next, 3 ml of a 0.65 M solution of boric acid, 0.5 ml of a solution of I_2/KI (0.05 M/0.15 M) and 1.5 ml of distilled water were added. These conditions allowed the formation of a colored complex between two adjacent hydroxyl groups of PVA and an iodine molecule. After 15 min of incubation, the absorbance was measured at 690 nm using an Agilent 8453 UV–visible spectrophotometer (Agilent technologies, Palo Alto, CA, USA). A standard plot of PVA was prepared under identical conditions and measurements were performed in triplicate.

2.7 In vivo studies using PLGA microparticles

2.7.1 Induction of myocardial infarction

All animal procedures were approved by the University of Navarra Institutional Committee on Care and Use of Laboratory Animals as well as the European Community Council Directive Ref. 2010/63/EU. Animal experiments were carried out using a rat model of cardiac acute ischemia-reperfusion. Rats were initially anesthetized with 4% isoflurane in an induction chamber. Prior to surgery, animals received analgesic drug ketoprofen 5 mg/kg subcutaneously, fentanyl 0.15 mg/kg and heparin 0.1 mg/kg both administered by intraperitoneal route. The rats were then intubated and ventilated at 90 cycles/min (1.5–2% isoflurane was maintained for continuous anesthesia). A left thoracotomy through the fourth intercostal space was performed, and the left anterior descending (LAD) coronary artery was occluded 2–3 mm distal from its origin for 1 h and then re-opened. The chest was then closed in layers and rats allowed to recover on a heating pad.

2.7.2 Intramyocardial administration of microparticles

Four days after LAD coronary artery occlusion, animals were assigned to receive microparticles of different sizes (2, 5, 14 and 30 µm) or medium alone.

Microparticle suspensions (2 mg/100 µl) were injected with a 29-gauge needle into 4 regions of the border zone surrounding the infarct while the heart was beating. A total of 22 animals were used in the *in vivo* experiments. The chest was closed and rats were allowed to recover on a heated pad.

2.7.3 Histological assessment of myocardial tissue after microparticle administration

Animals were sacrificed at different times after microparticle injection and their hearts were collected for histology. After being harvested, the hearts were perfused-fixed in 4% paraformaldehyde at 4 °C, and sliced in three 4-mm-thick segments from apex to base. The hearts were dehydrated in ethanol 70% at 4 °C, embedded in paraffin and cut 5-µm-sections. Hematoxylin–eosin (HE) staining was carried out to localize the microparticles and to visualize tissue structure. Samples from control zone (non-injected tissue), right ventricle, and other organs (kidney, liver and spleen) were also analyzed.

2.7.4 In vivo biodegradation, tissue retention and phagocytic uptake of microparticles

A group of infarcted animals (n=8) was injected four days after LAD coronary artery occlusion with 5 µm-sized fluorescence-labeled microparticles and sacrificed 8, 30 and 90 days later. Rhodamine B was used as a fluorescent marker to localize the injected microparticles by confocal microscopy in the heart tissue. After the hearts were frozen in OTC compound, frozen sections were prepared. In order to assess the phagocytic uptake of microparticles after their intramyocardial delivery, rat macrophage staining was carried out. CD68 immunofluorescence was performed as follows: slides were dried for 15 min at room temperature. Next, tissue sections were hydrated by passing through a graded ethanol series for 2 min each from absolute ethanol, 96%, 80%, and 70% followed by washing in running tap water, and subsequently in distilled water. Prior to blocking with 5% bovine serum albumin (BSA) for 30 min, sections were rinsed with Tris Buffered Saline (TBS) plus 0.05% Tween 20 (TBST). Labelling with primary antibody was performed using a mouse anti-rat CD68 antibody (diluted 1:100 in TBS) by incubating at 4 ºC overnight. After three consecutive washings with TBST (5 min each), fluorescent Alexa Fluor 488 goat anti–mouse IgG secondary antibody (1:100 dilution) was applied to sections for 1 h in the dark followed by nucleus staining with TOPRO-3 (diluted 1:50 in PBS-glycerol). For confocal microscopy, a LSM 510 META (Carl Zeiss, Minneapolis, USA) microscope was used. Digital images at 40× were analyzed in order to quantify the cardiac phagocytic uptake of injected microparticles. The extent of phagocytosis was expressed in terms of microparticle phagocytosis index determined as the ratio between the number of rhodamine-loaded microparticles internalized in CD68-positive macrophages and the total number of microparticles detected in each section. Eight serial sections of each rat were analyzed.

2.7.5 Statistical analysis

Data are presented as mean ± S.D. Statistics was calculated with Prism 5.0 software (Graphpad Software Inc., San Diego, CA, USA). The differences among mp batches or groups of animals were first evaluated using the Kruskal–Wallis Test, followed by Mann–Whitney U-test when values followed a non-parametric distribution. The differences among the batches of microparticles or the groups of animals were assessed by ANOVA with a Tukey post hoc correction when the measured values were normally distributed. Shapiro–Wilk test was used to justify the use of a parametric test. A value of $P<0.05$ was considered statistically significant.

3. Results and discussion

3.1 PLGA microparticles prepared by TROMS

A wide range of formulation methods have been used for encapsulating drugs into PLGA microparticles. These include solvent extraction [19], phase separation [20], spray drying [21], solid encapsulation [22], static mixer extrusion [23], and expansion in a supercritical fluid [24]. But the most frequently utilized method for the entrapment of fragile molecules is the water/oil/water (W/O/W) multiple emulsion solvent evaporation method [25]. Based on this method, TROMS has the advantage, over the conventional solvent evaporation techniques, of encapsulating compounds without the need for aggressive techniques or heating during the emulsification process. Thus, the method is especially useful for the encapsulation of fragile molecules such as growth factors. Previously, we successfully encapsulated VEGF and GDNF into PLGA-MP using TROMS, which maintained their biological activities [7, 16, 26]. In fact, higher encapsulation efficiencies were found when using TROMS. For instance, VEGF encapsulation efficiency up to 83% were achieved by TROMS [7]. In contrast, Cao *et al.* reported an entrapment efficiency of 14.5% of VEGF in PLGA-MP, employing the solid-encapsulation method [27]. In turn, Kim *et al.* proposed a combination of the multiple-emulsion technique and the atomization-freeze process into a unique solid-encapsulation/single-emulsion/solvent extraction method, but the entrapment efficiency of VEGF into PLGA microparticles using this manufacturing strategy was not improved, achieving a 16% entrapment efficiency [22]. Furthermore, another significant benefit of TROMS in view of its industrial application is the consistent production of very homogeneous batches of microparticles allowing for an easy scale-up of the manufacturing process.

In this study, TROMS produced microparticles in the size range of 2-30 µm varying apparatus conditions during microparticle production. Particle size remained unchanged after lyophilization. The needle diameter for $W_1/O/W_2$ emulsion formation, pumping flow and recirculation times of both W_1/O and $W_1/O/W_2$ emulsions yielded batches with different particle sizes (Table 1). The inner diameter of the needles is a critical factor determining the final size of microparticles prepared by TROMS [17]. Microparticles with a diameter around 30 µm (batch 1) were obtained using the largest needle diameter to form the multiple emulsion (0.50 mm). A significant reduction in particle size was observed with needle diameters of 0.25 mm and 0.12 mm (batches 2 and 3, respectively, compared with batch 1, $P<0.01$). Table 1 also shows the influence of recirculation times of both W_1/O and $W_1/O/W_2$ emulsions on the final size of microparticles, which was strongly dependent on the recirculation time of the primary W_1/O emulsion. A reduction of 1 min on the recirculation time of this emulsion increased significantly the particle size from 4.1 µm (batch 4) to 14.7 µm (batch 5) ($P<0.01$). The pumping flow also played a key role in the final size of the microparticles, whereas increasing flows led to more turbulent regimes to form both primary and multiple emulsions. Consequently, smaller microparticles were formed under higher homogenization energies supplied by more vigorous flows (batches 7 and 9, $P<0.05$).

Colloidal stability was analyzed by measuring the zeta potential of PLGA microparticle surface. Particles were negatively charged (around −30 mV at pH 7.6) and no significant differences in zeta potential values were observed among all TROMS-produced microparticles batches. The morphology of the microparticles was examined by fluorescence microscopy (Fig. 1A). Microparticles appeared spherical in shape. Red fluorescence was distributed in the polymer matrix, indicating good rhodamine encapsulation. SEM visualization of the microparticles confirmed these findings,

revealing a spherical shape with a smooth surface and few small pores in some particles (Fig. 1B). Physical characteristics of microparticles such as morphology, size or surface charge were investigated as well as quality controls for batch-to-batch consistency. Concerning the residual PVA content, the percentage of PVA recovered in the microparticles ranged from 1.1% to 1.6% depending on the formulations. These values are several times lower than 13%$_{w/w}$ PVA content previously reported [28]. The differences could be explained by the polarity of organic solvent that was used by Zambaux *et al.*, [28] compared to that used in our study (dichloromethane vs. dichloromethane-acetone). Probably, the co-solvent system composed by a dichloromethane-acetone mixture led to less PVA partitioned into the polymeric phase, resulting in the lower deposition of PVA on the surface of TROMS-produced microparticles. Residual PVA differences could also be attributed to other formulation parameters such as PVA concentration in the external aqueous phase and PVA molecular weight.

3.2 Dispersability and injectability of microparticle suspension

Injectable microparticles encapsulating therapeutic agents require that the microparticles be well dispersed in the media used to deliver the agent. Therefore, good flow properties of the microparticles are necessary to ensure dose uniformity and safety requirements. In the present work, dispersability and injectability of TROMS produced microparticles were assessed to select an adequate injectable PLGA microparticle suspension for local myocardial injection. These flow properties describe the ability of the microparticle suspension to pass easily through a needle. Dispersability of freeze-dried microparticles was tested in 3 different saline solutions: PBS, DMEM and DMEM-S. PBS and DMEM are solutions commonly employed to inject drugs/cells into the infarcted heart [29, 30] and the carboxymethyl cellulose solution containing polysorbate 80 and mannitol has been previously used to suspend PLGA microparticles prior to intracerebral implantation [26]. Carboxymethyl cellulose is a wetting and biocompatible agent that prevents particle aggregation and makes easier their injection through a thin needle. Furthermore, carboxymethyl cellulose solution is a viscosity building agent commonly used in the formulation of oral and injectable pharmaceutical suspensions [31, 32]. Microparticles were better dispersed in DMEM-S, probably due to the surfactant mixture added to medium, which increased DMEM viscosity and reduced particle aggregation and sedimentation. Thus, DMEM supplemented with carboxymethylcellulose, polysorbate 80 and mannitol was selected as injection medium for animal experiments. Microparticle suspension in the range of 2−5 mg/100 µl was found to be both dispersible and injectable. Particle batches of 14 µm and 30 µm exhibited some resistance to resuspension in DMEM-S compared to smaller ones, probably due to sedimentation of particles with a diameter higher than 14 µm. Concerning microparticles' ability to pass through a 29-gauge needle, a typical needle size for heart injection [11], moderate levels of sedimentation could also explain the resistance observed when 30 µm-sized particles passed through the needle. In contrast, microparticles of 2 µm and 5 µm were flowable. They were easily injected through a 29-gauge needle.

3.3 Histological evaluation of myocardial tissue after microparticle injection

Microparticle batches of 2, 5, 14 and 30 µm were tested according to their compatibility with an intramyocardial injection. Microparticles were intramyocardially injected in the infarcted beating heart (Fig. 2). As mentioned above, large microparticles

did not exhibit suitable resuspension in the injection medium. Indeed, blockages in the 29-gauge needle were detected during the administration of 30 µm-sized particles in the myocardium. Probably, the presence of aggregates obstructed the flow through the needle, which limited the injection of large microparticles in the rat heart. Despite the obstruction, a residual amount of 30 µm-sized particles reached the infarcted area. HE staining showed a more consistent accumulation of inflammation-mediated cells (IMCs) after injection of large microparticles (30 µm-injected particles, Fig. 3C and 3D) than in smaller ones (2 µm-injected particles, Fig. 3A-B). In fact, it has been described that large microparticles (75 µm) caused extensive myocardial necrosis in a porcine model [33]. On the other hand, there were fewer IMCs in the surrounding areas of 2 µm-sized particles, which did not induce severe responses. However, these 2 µm diameter particles exhibited a low retention in the heart, probably due to local phagocytic activity (results not shown). Taken together, these observations demonstrated that particles with an intermediate diameter could be adequate for heart injection. Consequently, 5 µm-sized particles were selected for long-term tissue retention and response studies. Interestingly, 5 µm-sized particles did not induce inflammatory reactions when compared to the injection of resuspension medium. The response of the heart was the typical reaction observed following mechanical trauma and exposure to a foreign body. Slightly inflamed areas provoked by the needle during the myocardial administration of DMEM-S or suspension of microparticles with 5 µm diameter were observed. This finding correlates with the report that 7 µm resin particles encapsulating FGF-2 did not cause myocardial damage [34]. However, the clinical application of these microparticles is limited because the resin material is non-biodegradable, unlike PLGA which degrades generating monomeric acids (lactic and glycolic acids) that are consequently eliminated from the body as carbon dioxide and water [35]. Importantly, no myocardial hemorrhage was observed in our HE sections. Additionally, no signs of physiological disturbances such as fibrillation upon microparticles injection and no adverse cellular reactions in the tissue adjacent to the implanted microparticles were observed.

3.4 Biodegradation, tissue retention and phagocytic uptake of microparticles in the infarcted heart

Confocal microscopy and HE staining were carried out to study biodegradation of 5 µm-sized particles over time. The results are illustrated in Fig. 4. Fluorescent microparticles were always grouped at the implantation site and they remained at the site of injection during the entire 90-day experiment. At the beginning of the study microparticles showed a spherical shape but they underwent progressive changes in size and morphology over time. Between the first and second week there was a decrease in the microsphere size that continued to diminish, at a lower rate, over time. Most of the microparticles lost their spherical shape and showed a reduction of their size two months after implantation. Importantly, three months after implantation microparticles were not totally biodegraded and a significant amount of them were still detectable (Fig. 4D and 4E).

Some studies have demonstrated a rapid loss of intramyocardially implanted cells or drugs which is biologically caused by the contracting myocardium [36, 37]. In the present paper we have demonstrated that microparticles were not rapidly washed out from the infarcted myocardium (Fig. 2). Immediately after microparticle administration, a persistent blanching surrounding the injection point and a change of tissue color from dark pink to light pink after polymer injection was observed. There was no microparticle loss or leakage during the intramyocardial implantation, indicating a

localized retention of the microparticle suspension in the epicardial zone. Confocal microscopy and HE staining was performed to evaluate the temporal retention of fluorescence labeled PLGA microparticles in the heart tissue. Fluorescent microparticles were found in the injection sites up to three months post-implantation (Fig. 4). Whereas Sy et al. reported retention of 20 µm-sized poly(cyclohexane-1,4diyl acetone dimethylene ketal) (PCADK) microparticles for up to 10 days in the myocardium [38], and another study carried out by our group showed retention of 5 µm-sized PLGA microparticles encapsulating VEGF for at least 30 days [7], the results of the present study showed retention of 5 µm-sized PLGA particles for up to 90 days. These tissue retention results indicate the capacity of PLGA microparticles to remain in the myocardium for a prolonged period of time, a requirement for sustained growth factor treatment. Correlating with histological observations of HE stained sections, no fluorescent signal of rhodamine-loaded microparticles was observed in other tissues such as kidney, liver and spleen, indicating no migration of the microparticles towards solid organs. This is an important feature of PLGA microparticles for local delivery of therapeutics into myocardium, preventing systemic side effects of the loaded drugs.

While there are reports that have described the phagocytic uptake of PLGA microparticles in macrophage cultures [39, 40], there is no detailed *in vivo* study on the macrophage-mediated phagocytosis of PLGA microparticles in the heart tissue. As the macrophage is a primary responder cell involved in the regulation of post-MI wound healing, eliminating apoptotic/necrotic myocytes and other debris [41], phagocytic activity of cardiac macrophages upon injected microparticles was further assessed. Quantification of phagocytic uptake of rhodamine-loaded microparticles was carried out by detection of CD68 macrophages. The extent of phagocytosis measured as the ratio between rhodamine-loaded microparticles internalized into CD68-positive macrophages and the total number of microparticles detected in each section was assessed in three groups of animals: rats sacrificed one week, one month and three months after intramyocardial administration of fluorescent microparticles. CD68 immunolabeling revealed a microparticle uptake of 31% one week after microparticle injection (Fig. 5A, D). An increase in the phagocytic activity of macrophages upon microparticles was detected one month after drug administration, with a microparticle phagocytosis index of 47% (Fig. 5B, D). In contrast, a very low microparticle uptake (below 4%) was found three months after implantation (Fig. 5C, D).

In rodent models of MI, within the first hours to 1 day, there are robust up-regulations of intramyocardial cytokines including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6). These cytokines mediate the acute remodeling process in the infarcted myocardium, which includes modulation of cardiac macrophages and phagocytosis [42]. After the initial increase of gene expression in the infarcted region, the cytokines normally begin to decrease toward baseline after 1 week [43]. Therefore, a decrease in the phagocytic uptake of microparticles 1 month after injection must be expected, because of decreased phagocytic activity of cardiac macrophages during the chronic remodeling post-MI. However, a larger number of microparticles internalized into CD68-positive macrophage was quantified in injected animals after 1 month compared with 1 week. One potential explanation is that microparticles maintained their spherical shape and diameter around 5 µm, presenting some resistance to phagocytosis one week after injection, a very short period for polymer degradation. In contrast, one month after their injection, particles originally 5 µm in size were observed as smaller ones (< 2 µm) due to higher polymer degradation. Consequently, these small particles in the heart tissue were more susceptible to

phagocytosis, whereas particle size around 1 μm is suitable for efficient uptake by macrophages [44]. Three months after microparticle injection, microparticles were visible in heart tissue. However, a low colocalization degree of microparticles with CD68-positive macrophage was observed. It is possible that macrophage response decreases considerably after this period, owing to very low levels of intramyocardial cytokines which modulate macrophage activation. At this time, damage due to the injection is repaired and the inflammatory response is ended.

On the other hand, in addition to particle size, other formulation parameters could affect the phagocytic uptake of microparticles. For example, particle hydrophobicity decreases with the amount of residual PVA associated with microparticles, reducing their recognition by macrophages [18]. We used a 0.5% PVA solution as stabilizer, which prevents microparticle aggregation during solvent removal. Using this PVA concentration, microparticles with a minimal content of residual PVA were obtained. Moreover, microparticles presented a high negative charge, which is associated with a stable colloid nature. An increase in PVA concentration used for microparticle formulation would result in the increase in the residual PVA content. However, as PVA is a potentially toxic non-biodegradable polymer, its administration should be minimized as much as possible [45]. Therefore, changes in formulation parameters must be rationally performed to alter microparticle hydrophobiticy, with the aim of controlling its phagocytic uptake during the first month after implantation.

4. Conclusions

In this study, a PLGA microparticle formulation was developed that was compatible with intramyocardial injection in terms of particle size, injectability and tissue response. In addition, these particles exhibited the capacity to remain in the myocardium for up to three months. Concerning *in vivo* phagocytic uptake of microparticles, a moderate level of macrophage-mediated phagocytosis of PLGA microparticles was observed in the heart tissue. In particular, this result helps us to understand better the heart tissue response to a polymeric delivery system in the context of biomaterial research for cardiac regeneration.

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References

[1] M. Simons, B.H. Annex, R.J. Laham, N. Kleiman, T. Henry, H. Dauerman, J.E. Udelson, E.V. Gervino, M. Pike, M.J. Whitehouse, T. Moon, N.A. Chronos, Pharmacological treatment of coronary artery disease with recombinant fibroblaft growth factor-2. Double-blind, randomized, controlled clinical trial, Circulation, 105 (2002) 788-793.

[2] T.D. Henry, B.H. Annex, G.R.A. McKendall, M. A., J.J. Lopez, F.J. Giordano, P.K. Shah, J.T. Willerson, R.L. Benza, D.S. Berman, C.M. Gibson, A. Bajamonde, A.C.

Rundle, J. Fine, E.R. McCluskey, The VIVA Trial. Vascular Endothelial Growth Factor in Ischemia for Vascular Angiogenesis, Circulation, 107 (2003) 1359-1365.
[3] C.R. Ozawa, A. Banfi, e. al, Microenvironmental VEGF concentration, not total dose, determines a threshold between normal and aberrant angiogenesis., J. Clin. Invest., 113 (2004) 516-527.
[4] M. Hariawala, J.R. Horowitz, D. Esakof, D.D. Sheriff, D.H. Walter, G.M. Chaudhry, V. Desai, B. Keyt, J.M. Isner, J.F. Symes, VEGF improves myocardial bood flow but produces EDRF-mediated hypotension in porcine hearts, J. Surg. Res., 63 (1996) 77-82.
[5] J. Wu, F. Zeng, X.P. Huang, J.C. Chung, F. Konecny, R.D. Weisel, R.K. Li, Infarct stabilization and cardiac repair with a VEGF-conjugated, injectable hydrogel, Biomaterials, 32 (2011) 579-586.
[6] R.C. Scott, J.M. Rosano, Z. Ivanov, B. Wang, P.L. Chong, A.C. Issekutz, D.L. Crabbe, M.F. Kiani, Targeting VEGF-encapsulated immunoliposomes to MI heart improves vascularity and cardiac function, FASEB J., 23 (2009) 3361-3367
[7] F.R. Formiga, B. Pelacho, E. Garbayo, G. Abizanda, J.J. Gavira, T. Simon-Yarza, M. Mazo, T. Tamayo, C. Jauquicoa, C. Ortiz-de-Solorzano, F. Prósper, M.J. Blanco-Prieto, Sustained release of VEGF through PLGA microparticles improves vasculogenesis and tissue remodeling in an acute myocardial ischemia-reperfusion model, J. Control. Release, 147 (2010) 30-37.
[8] K.S. Oh, J.Y. Song, S.J. Yoon, Y. Park, D. Kim, S.H. Yuk, Temperature-induced gel formation of core/shell nanoparticles for the regeneration of ischemic heart, J. Control. Release, 146 (2010) 207-211.
[9] X. Hao, E.A. Silva, A. Månsson-Broberg, K.H. Grinnemo, A.J. Siddiqui, G. Dellgren, E. Wärdell, L.A. Brodin, D.J. Mooney, C. Sylvén, Angiogenic effects of sequential release of VEGF-A165 and PDGF-BB with alginate hydrogels after myocardial infarction, Cardiovasc. Res., 75 (2007) 178-185.
[10] F.B. Engel, P.C. Hsieh, R.T. Lee, M.T. Keating, FGF1/p38 MAP kinase inhibitor therapy induces cardiomyocyte mitosis, reduces scarring, and rescues function after myocardial infarction, Proc. Natl. Acad. Sci. USA, 103 (2006) 15546-15551.
[11] J.C. Garbern, E. Minami, P.S. Stayton, C.E. Murry, Delivery of basic fibroblast growth factor with a pH-responsive, injectable hydrogel to improve angiogenesis in infarcted myocardium, Biomaterials, 32 (2011) 2407-2416.
[12] H. Wang, X. Zhang, Y. Li, Y. Ma, Y. Zhang, Z. Liu, J. Zhou, Q. Lin, Y. Wang, C. Duan, C. Wang, Improved myocardial performance in infarcted rat heart by co-injection of basic fibroblast growth factor with temperature-responsive chitosan hydrogel, J. Heart Lung Transplant., 29 (2010) 881-887.
[13] G. Crotts, T.G. Park, Protein delivery from poly(1actic-co-glycolic acid) biodegradable microspheres: release kinetics and stability issues, J. Microencapsul., 15 (1998) 699-713.
[14] W. Jiang, R.K. Gupta, M.C. Deshpande, S.P. Schwendeman, Biodegradable poly(lactic-co-glycolic acid) microparticles for injectable delivery of vaccine antigens, Adv. Drug Deliv. Rev., 57 (2005) 391-410.
[15] R.A. Jain, The manufacturing techniques of various drug loaded biodegradable poly(lactide-co-glycolide) (PLGA) devices, Biomaterials, 21 (2000) 2475-2490.
[16] E. Garbayo, E. Ansorena, J.L. Lanciego, M.S. Aymerich, M.J. Blanco-Prieto, Sustained release of bioactive glycosylated glial cell-line derived neurotrophic factor from biodegradable polymeric microspheres, Eur. J. Pharm. Biopharm., 69 (2008) 844-851.

[17] G. Garcıa del Barrio, F.J. Novo, J.M. Irache, Loading of plasmid DNA into PLGA microparticles using TROMS (Total Recirculation One-Machine System): evaluation of its integrity and controlled release properties, J. Control. Release, 86 (2003) 123-130.
[18] S.K. Sahoo, J. Panyam, S. Prabha, V. Labhasetwar, Residual polyvinyl alcohol associated with poly(D,L-lactide-co-glycolide) nanoparticles affects their physical properties and cellular uptake, J. Control. Release, 82 (2002) 105-114.
[19] S. Freitas, H.P. Merkle, B. Gander, Microencapsulation by solvent extraction/evaporation: reviewing the state of the art of microsphere preparation process technology, J. Control. Release, 102 (2005) 313-332.
[20] C. Thomasin, P. Johansen, R. Alder, R. Bemsel, G. Hottinger, H. Altorfer, A.D. Wright, E. Wehrli, H.P. Merkle, B. Gander, A contribution to overcoming the problem of residual solvents in biodegradable microspheres prepared by coacervation, Eur. J. Pharm. Biopharm., 42 (1996) 16- 24.
[21] K. Bowey, R.J. Neufeld, Systemic and mucosal delivery of drugs within polymeric microparticles produced by spray drying, BioDrugs, 24 (2010) 359-377.
[22] T.W. King, C.W. Patrick, Development and in vitro characterization of vascular endothelial growth factor (VEGF)-loaded poly(DL-lactic-co-glycolic acid)/poly(ethylene glycol) microspheres using a solid encapsulation/single emulsion/solvent extraction technique, J. Biomed. Mater. Res., 51 (2000) 383-390.
[23] S. Freitas, A. Walz, H.P. Merkle, B. Gander, Solvent extraction employing a static micromixer: a simple, robust and versatile technology for the microencapsulation of proteins, J. Microencapsul., 20 (2003) 67-85.
[24] A. Naylor, A.L. Lewis, L. Ilium, Supercritical fluid-mediated methods to encapsulate drugs: recent advances and new opportunities, Ther. Deliv., 2 (2011) 1551-1565.
[25] S. Cohen, T. Yoshioka, M. Lucarelli, L.H. Hwang, R. Langer, Controlled delivery systems for proteins based on poly(lactic/glycolic acid) microspheres, Pharm. Res., 8 (1991) 713-720.
[26] E. Garbayo, C.N. Montero-Menei, E. Ansorena, J.L. Lanciego, M.S. Aymerich, M.J. Blanco-Prieto, Effective GDNF brain delivery using microspheres--a promising strategy for Parkinson's disease, J. Control. Release, 135 (2009) 119-126.
[27] X. Cao, M.S. Schoichet, Delivering neuroactive molecules from biodegradable microspheres for application in central nervous system disorders, Biomaterials, 20 (1999) 329-339.
[28] M.F. Zambaux, F. Bonneaux, R. Gref, P. Maincent, E. Dellacherie, M.J. Alonso, P. Labrude, C. Vigneron, Influence of experimental parameters on the characteristics of poly (lactic acid) nanoparticles prepared by a double emulsion method, J. Control. Release, 50 (1998) 31-40.
[29] Z. Liu, H. Wang, Y. Wang, Q. Lin, A. Yao, F. Cao, D. Li, J. Zhou, C. Duan, Z. Du, Y. Wang, C. Wang, The influence of chitosan hydrogel on stem cell engraftment, survival and homing in the ischemic myocardial microenvironment, Biomaterials, 33 (2012) 3093-3106.
[30] R.P.H. Ahmed, K.H. Haider, J. Shujia, M.R. Afzal, M. Ashraf, Sonic hedgehog gene gelivery to the rodent heart promotes angiogenesis via iNOS/Netrin-1/PKC pathway, PLoS one, 5 (2010) e8576.
[31] V.G. Lara, M.L. Gallardo, M.E.M. Hernandez, M.A.M. Ruiz, Ondansetron: design and development of oral pharmaceutical suspensions, Pharmazie, 64 (2009) 90-93.
[32] Z.Y. Cai, P. Galettis, Y. Lu, D.L. Morris, M.H. Pourgholami, Pharmacokinetics of Albendazole in New Zealand White Rabbits: Oral versus Intraperitoneal Administration, Anticancer Res., 27 (2007) 417-422.

[33] A. Battler, M. Scheinowitz, A. Bor, D. Hasdai, Z. Vered, E. Di Segni, Intracoronary injection of basic fibroblast growth factor enhances angiogenesis in infarcted swine myocardium, J. Am. Coll. Cardiol., 22 (1993) 2001-2006.
[34] M. Arras, H. Mollnau, R. Strasser, R. Wenz, W.D. Ito, J. Schaper, W. Schaper, The delivery of angiogenic factors to the heart by microsphere therapy, Nat. Biotechnol., 16 (1998) 159-162.
[35] J.M. Anderson, M.S. Shive, Biodegradation and biocompatibility of PLA and PLGA microspheres, Adv. Drug Deliv. Rev., 28 (1997) 5-24.
[36] K.H. Wu, X.M. Mo, Z.C. Han, B. Zhou, Stem cell engraftment and survival in the ischemic heart, Ann. Thoracic Surg., 92 (2011) 1917-1925.
[37] J.V. Terrovitis, R.R. Smith, E. Marbán, Assessment and optimization of cell engraftment after transplantation into the heart, Circ. Res., 106 (2010) 479-494.
[38] J.C. Sy, G. Seshadri, S.C. Yang, M. Brown, T. Oh, S. Dikalov, N. Murthy, M.E. Davis, Sustained release of a p38-inhibitor from non-inflammatory microspheres inhibits cardiac dysfunction, Nat. Mater., 7 (2008) 863-868.
[39] A.J. Gomes, C.N. Lunardi, F.H. Caetano, L.O. Lunardi, A.E.H. Machado, Phagocytosis of PLGA microparticles in rat peritoneal exudate cells: a time-dependent study, Microsc. Microanal., 12 (2006) 399-405.
[40] Y. Yang, N. Bajaj, P. Xu, K. Ohn, M.D. Tsifansky, Y. Yeo, Development of highly porous large PLGA microparticles for pulmonary drug delivery, Biomaterials, 30 (2009) 1947–1953.
[41] N.G. Frangogiannis, C.W. Smith, M.L. Entman, The inflammatory response in myocardial infarction, Cardiovasc. Res., 53 (2002) 31-47.
[42] A. Deten, H.C. Volz, W. Briest, H.G. Zimmer, Cardiac cytokine expression is upregulated in the acute phase after myocardial infarction. Experimental studies in rats, Cardiovasc. Res., 55 (2002) 329-340.
[43] K. Ono, A. Matsumori, T. Shioi, Y. Furukawa, S. Sasayama, Cytokine gene expression after myocardial infarction in rat hearts: possible implication in left ventricular remodeling, Circulation, 98 (1998) 149-156.
[44] T. Harel-Adar, T.B. Mordechai, Y. Amsalem, M.S. Feinberg, J. Leor, S. Cohen, Modulation of cardiac macrophages by phosphatidylserine-presenting liposomes improves infarct repair, Proc. Natl. Acad. Sci. USA, 108 (2011) 1827-1832.
[45] M. Zeisser-Labouebe, N. Lange, R. Gurny, F. Delie, Hypericin loaded nanoparticles for the photodynamic treatment of ovarian cancer, Int. J. Pharm., 326 (2006) 174-181.

Figure Captions

Fig. 1. (A) Fluorescence image of representative 5 μm rhodamine loaded microparticles. Scale bars: 10 μm. (B) Scanning electron microscopy of representative 5 μm PLGA microparticles prepared using TROMS. Scale bars: 8 μm.

Fig. 2. Macroscopic view of the heart following 5 μm-sized PLGA microparticle implantation. Four days after LAD coronary artery occlusion, rats were anesthetized with isoflurane and ventilated. The chest was held opened by a retractor. Microparticles were injected into the infarct zone through a 29G needle while the heart was beating. Note the presence of the microparticles in the beating heart demonstrating that microparticles were not rapidly washed out from the infarcted myocardium.

Fig. 3. Histological evaluation of myocardial tissue reaction 4 days after microparticle administration in hematoxylin-eosin stained sections. Microparticles with a diameter of 2 μm (A, B) and 30 μm (C, D) are clearly visualized at high magnification (B, D indicated by arrows). Scale bars: 20 μm (A, C) and 100 μm (B, D).

Fig 4. Biodegradation and tissue retention of 5 μm-sized fluorescent microparticles intramyocardially implanted in the infarcted heart 4 days after LAD coronary artery occlusion. Representative images of confocal microscopy (A, D) and hematoxylin eosin staining (B, C, E, F) of the peri-infarted area 1 and 3 months after PLGA microparticle (A, B, D, E) or resuspension media (control) (C, F) injection. The administration of microparticles was well tolerated by the tissue and no differences on tissue inflammation were found between the administration of medium or microparticles. Note that microparticles were still present in the peri-infarted area 3 months after the injection. Scale bars: 50 μm.

Fig. 5. *In vivo* phagocytic uptake of 5 μm-sized PLGA microparticles**.** Representative pictures of CD68 (green) macrophage immunofluorescence of heart sections 1 week (A), 1 month (B) and 3 months (C) after intramyocardial administration of microparticles labeled with rhodamine (red). Nuclear staining was performed with TOPRO-3 (blue). Scale bars: 20 μm. Quantification of microparticle phagocytosis was determined as the ratio between rhodamine-loaded microparticles internalized into CD68-positive macrophage and the total number of microparticles detected in each section (D). ***$P<0.001$, **$P<0.01$.

Graphical abstract. The administration of 5 μm-sized microparticles was well tolerated by the infarcted myocardium and no differences on tissue inflammation were found between the administration of medium or microparticles. Representative images of confocal microscopy (A, D) and hematoxylin eosin staining (B, C, E, F) of the peri-infarted area 1 and 3 months after PLGA microparticle (A, B, D, E) or resuspension media (control) (C, F) injection. CD68 immunolabeling revealed 31%, 47% and below 4% microparticle uptake by macrophages one week (G), one month (H) and three months (I) after injection respectively ($P<0.001$).