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Nanoparticles Based on PLGA:Poloxamer Blends for the Delivery of Proangiogenic Growth Factors

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Abstract: New blood vessel formation is a critical requirement for treating many vascular and ischemia related diseases, as well as for many tissue engineering applications. Angiogenesis and vasculogenesis, in fact, represent crucial processes for the functional regeneration of complex tissues through tissue engineering strategies. Several growth factors (GFs) and signaling molecules involved in blood vessels formation have been identified, but their application to the clinical setting is still strongly limited by their extremely short half-life in the body. To overcome these limitations, we have developed a new injectable controlled release device based on polymeric nanoparticles for the delivery of two natural proangiogenic GFs: platelet derived growth factor (PDGF-BB) and fibroblast growth factor (FGF-2). The nanoparticle system was prepared by a modified solvent diffusion technique, encapsulating the GF both in presence and in the absence of two stabilizing agents: bovine serum albumin (BSA) and heparin sodium salt (Hp). The developed nanocarriers were characterized for morphology, size, encapsulation efficiency, release kinetics in vitro and GF activity in cell cultures. The results have indicated that the coencapsulation of stabilizing agents can preserve the GF active structure and, in addition, increase their encapsulation efficiency into nanoparticles. Through this optimization process, we were able to raise the encapsulation efficiency of FGF-2 to 63%, and that of PDGF-BB to 87%. These PLGA:poloxamer blend nanoparticles loaded with GFs were able to release PDGF-BB and FGF-2 in a sustained fashion for more than a month. This work also confirms other positive features of PLGA:poloxamer nanoparticles. Namely, they are able to maintain their stability in simulated biological medium, and they are also nontoxic to cell culture models. Incubation of nanoparticles loaded with FGF-2 or PDGF-BB with endothelial cell culture models has confirmed that GFs are released in a bioactive form. Altogether, these results underline the interest of PLGA:poloxamer nanoparticles for the controlled delivery of GFs and substantiate their potential for the treatment of ischemic diseases and for tissue engineering applications.

Keywords: PLGA:poloxamer blend; nanoparticles; PDGF; FGF; angiogenesis

Introduction

Tissue engineering is one of the most promising research areas in bioregenerative medicine. In broad terms, tissue engineering aims to develop biological substitutes able to restore, maintain, or improve tissue function or even a whole organ.[1] In recent years, many milestones have been realized through tissue engineering strategies; the most important ones from a clinical perspective are related to the regeneration of small defects, such as in bone,[2] to the regeneration of flat

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tissues (e.g., skin),[3] and to engineering nonvascularized tissues (e.g., cartilage).[4] There is a specific reason for these engineered grafts to have reached this level of success: they do not require vascularization. Indeed, tissue engineers are still unable to design functional vascularized tissues, and therefore, the design of large tissue constructs is difficultly achieved due to the inadequate diffusion of nutrients to the cells.[5]

Blood vessel sprouting from pre-existing ones, angiogenesis, is a very complex phenomenon where an extensive range of coordinated signals are involved. Several growth factors (GFs) and other signaling molecules are now known to have a role in blood vessel sprouting.[6] Among growth factors, the families of platelet derived growth factor (PDGF) and fibroblast growth factor (FGF) are being currently investigated for their potential role in angiogenesis. PDGF-BB, a protein isoform of the PDGF family, is a potent mitogen and chemotactic factor for vascular smooth muscle cells, but it lacks significant effects on endothelial cells. Because of this biased effect PDGF-BB administration results in a very fast vascular network regression.[7] On the other hand, FGF-2 is a potent proangiogenic factor that preferentially acts on endothelial cells, lacking significant effect on vascular smooth muscle cells. Again, this biased effect results in quick vascular network regression when FGF-2 is withdrawn.[8] Recently, it has been shown that combinations of PDGF-BB and FGF-2 result in a synergic proangiogenic activity and in the formation of stable blood vessels.[9]

Although the potential of the PDGF-BB/FGF-2 combination as a pharmaceutical therapy has been shown in *in vivo* models,[9] it is broadly accepted that the clinical activity of growth factors can be markedly improved through drug delivery strategies.[10] The reason behind the inability of growth factors to fully realize their biological activity in the preclinical/clinical setting is related to their extremely short half-life in the body, which results in ephemeral bioactivity.[5] Moreover, integration of growth factors in drug delivery devices brings additional benefits such as improved stability of pharmaceutical product upon storage,[11,12] and the possibility of generating sustained concentration gradients of growth factors that will enhance their chemotactic activity.[13−15] GF controlled release from particulate delivery devices has been widely reported. Frequently, these delivery systems were based on natural polymers, such as gelatin microparticles and alginate beads, that have been employed for the delivery of different GFs: FGF-2,[16] TGF-β1,[17] VEGF.[18] Particulate systems based on these polymers are able to encapsulate GFs with minimum loss of bioactivity of the encapsulated molecule. However, these polymers lack the capacity to provide long-term release properties, particularly when formed as nanoparticles. These limitations are fully overcomed by particulate systems based on biodegradable polyesters (i.e., PLA, PLGA), which have been successfully employed for the delivery of bFGF,[19] BMP-2[20,21] and IGF/TGF combinations.[22] Nevertheless, polyester-based particles also show some limitations regarding their capacity to preserve the stability of the encapsulated molecules and their biological activity.[23,24]

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PLGA:poloxamer blend micro- and nanoparticles were first designed in our group as a technology intended to improve the characteristics of conventional PLGA particles.[25−30] More specifically, PLGA:poloxamer nanoparticles were able to encapsulate delicate therapeutic molecules with minimum loss in their biological activity.[27−30] Additionally, these carriers also prevented the degradation of encapsulated molecules during the release phase,[26,27] a stability problem that is often observed for PLGA carriers as a consequence of the accumulation of acidic oligomers in the PLGA matrix upon its degradation.[31]Previous studies have indicated that PLGA:poloxamer mass ratios of 1:1 result in high and efficient incorporation of poloxamer in the nanoparticle matrix, and successful protection of the encapsulated molecule[27,28]

Considering our background knowledge in the use of PLGA:poloxamer particles for peptide, protein and DNA delivery,[25−30] in this work we have optimized this technology for its application to proangiogenic GFs. In particular, we focus in the design of delivery systems for PDGF-BB and FGF-2 for their application in ischemic-related diseases and in tissue engineering. As injectability by several parenteral routes would also be a beneficial feature of this formulation, we have selected to encapsulate these GFs in nanometric carriers rather than in micrometric ones. Moreover, nanometric size allows loading these particles onto polymeric scaffolds by simple adsorption,[32] resulting in growth factor-activated scaffolds. This represents an alternative application of these systems of relevance in tissue engineering.

Materials and Methods

Materials. Poly (D,L-lactide-*co*-glycolide) (PLGA) 50:50 Resomer RG 503 (inherent viscosity 0.32 − 0.44 dL/g) was purchased from Boehringer Ingelheim (Ingelheim, Germany). Poloxamer 188 (Pluronic F68), bovine serum albumin (BSA), heparin sodium salt (Hp), the liquid substrate 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid), fetal bovine serum (FBS), L-glutamine, MTT reagent, DMSO and phosphate buffer solution (PBS) were obtained from Sigma Aldrich (USA). Platelet derived growth factor (PDGF-BB), fibroblast growth factor (FGF-2) and their relative ELISA assay kits were purchased from PeproTech EC Ltd. (U.K.). Primary bovine capillary endothelial (BCE) 4T GRT cells were provided by Dr. Y. Cao, Microbiology and Tumor Biology Center, Karolinska Institutet, Stockholm, Sweden. C3a cells, a subclone of the Hep G2 line, were obtained from ATCC. Eagle's MEM with Earle's BSS and 2 mM L-glutamine and 1 mM sodium pyruvate and fetal calf serum were obtained from ATCC (USA). Penicillin/streptomycin stock solution was obtained from PAA (USA). The XTT reagent and collagen from rat tail were obtained from Roche (Switzerland). All other reagents were analytical grade or better. Ultrapure water was used throughout the study.

Methods. *Preparation of PDGF-BB and FGF-2 Loaded PLGA:Poloxamer Blend Nanoparticles.* PLGA:poloxamer blend based nanoparticles with a PLGA:poloxamer mass ratio of 1:1 were prepared by a modified solvent diffusion technique as previously described:[27] an aqueous solution (100 μL) containing 2 μg of the GF (FGF-2 or PDGF-BB) was emulsified by vortex mixing (2400 min^{-1}, Heidolph, Germany) with 2 mL of PLGA (2% w/v) and poloxamer (2% w/v) solution in methylene chloride. The resulting emulsion was quickly added to 12.5 mL of ethanol, leading to an immediate precipitation of the polymer in the form of nanoparticles. Immediately, this suspension was diluted with 12.5 mL of ultrapure water, and stirred at room temperature

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in a fume hood for 10 min. Afterwards, the organic solvents were eliminated through evaporation under vacuum at 30 °C (Rotavapor, Heidolph VV 2000, Germany). Finally, the nanoparticle suspension was isolated by centrifugation at 8000 rcf for 1 h at 5 °C. The nanoparticle pellet was resuspended in ultrapure water. To optimize the GF encapsulation, Hp (2 μg) and BSA (200 μg) were added as stabilizing agents and included in the 100 μL inner water phase during the preparation step.

Physicochemical Characterization of PLGA:Poloxamer Nanoparticles. PLGA:poloxamer blend nanoparticles were characterized for particle size and ζ-potential by photon correlation spectroscopy (PCS) and laser Doppler anemometry respectively (Zetasizer 3000HS Malvern Instruments, U.K.). For particle size analysis, each sample was diluted to the appropriate concentration with ultrapure water and analyzed at 25 °C with an angle of detection of 90°. For ζ-potential determination, the samples were diluted with 1 mM KCl and placed in an electrophoretic cell in which a potential of $\pm$150 mV was established.

The nanoparticle morphology was evaluated by transmission electron microscopy (TEM) (CM 12 Philips, Eindhoven, The Netherlands) using 10 μL of nanoparticle aqueous suspension (3 mg of nanoparticles/mL) stained with a 2% (w/v) phosphotungstic acid solution.

Growth Factor Encapsulation Efficiency. Nanoparticles were loaded with PDGF-BB or FGF-2 (0.01% w/w theoretical loading). The actual amount of PDGF-BB and FGF-2 encapsulated in the nanoparticles was calculated after alkaline hydrolysis of 1 mg of nanoparticles in 1 mL of 0.05 N NaOH. This hydrolytic process was maintained for 1 h under moderate magnetic stirring at room temperature to achieve the complete nanoparticle degradation. The samples obtained were opportunely diluted and analyzed by ELISA assay. Color development was monitored with an ELISA plate reader (Wallac 1420 Victor 3, PerkinElmer Instruments, USA) at 405 nm, with wavelength correction set at 650 nm.

Nanoparticle Stability in Cell Culture Medium. The stability of PLGA:poloxamer nanoparticles loaded with BSA and Hp was tested by incubation in cell culture medium (Eagle's MEM with Earle's BSS, 2 mM L-Glutamin, 1 mM sodium pyruvate with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin). Nanoparticle suspensions were diluted in this cell medium to a concentration range measurable by PCS. Then, the nanoparticle suspensions were incubated for 48 h under static condition at 37 °C. At set time points, nanoparticle size was analyzed by PCS.

Nanoparticle Cytotoxicity. Nanoparticle cytotoxicity assays were carried out on a hepatic cell line (C3a), by a cell viability assay (XTT) based on mitochondrial activity. For this assay, blank nanoparticles prepared in aseptic conditions were diluted in cell medium to five different concentrations: 1 mg/mL, 0.25 mg/mL, 0.5 mg/mL, 1 mg/mL and 1.5 mg/mL. By using suitable stock solutions the concentrations of the remaining components were kept constant.

Falcon Microtest Primaria, flat bottom, surface modified PS plates were collagenized as follows: Collagen from rat tail was dissolved in 0.2% sterile acetic acid to a concentration of 2 mg/mL. To coat all wells of the described 96-well plates 150 μL of the collagen stock solution was diluted with 10 mL of sterile water and dispensed into the wells. After 1 day incubation at 5 °C and two rinsing steps with phosphate buffered saline, the plates were used for cell culture. C3a cells were plated and maintained in Eagle's MEM with Earle's BSS and 2 mM L-glutamine and 1 mM sodium pyruvate supplemented with 10 vol % fetal calf serum and 1 vol % of a penicillin/streptomycin stock solution.

The cytotoxicity assay was conducted as follows: Two collagenized 96-well plates were seeded with 40×10^3 cells per well. On the first day of cell culture, one plate was used for testing the cytotoxicity of nanoparticles in nonconfluent cells while the second plate was kept in culture. When the second plate reached confluence (approximately 160×10^3 cells/well) a second cytotoxicity test was run in that plate. For the tests, cell media were exchanged for nanoparticle suspensions at different concentrations. After 24 h of incubation, the nanoparticle suspensions were exchanged for fresh medium supplemented with XTT reagent according to the manufacturer's recommendation. After 1 h of incubation time (30 min in the case of confluent cell layers) optical densities were measured (Spectra Max M2 plate reader, Molecular Devices, USA) at 420 nm (assay product of viable cells) and 600 nm (internal reference).

Freeze-Drying of PLGA:Poloxamer Nanoparticles. A freeze-drying method was optimized for PLGA:poloxamer nanoparticles containing BSA/Hp and for the same formulations containing PDGF-BB or FGF-2. To prepare the formulations for the freeze-drying process, nanoparticles were suspended in a cryoprotectant aqueous solution: trehalose at 5% (w/v). Nanoparticle concentration in the final suspension was always 2 mg/mL (w/v). Afterward, the obtained suspension was frozen at -80 °C and freeze-dried for 48 h (Labconco, Kansas City, MO). The freeze-dried nanoparticles were reconstituted with 1 mL of ultrapure water and analyzed by PCS to evaluate the particle size and polydispersity index (PI) after the freeze-drying process.

In Vitro GF Release Kinetics from PLGA:Poloxamer Nanoparticles. GF release kinetics were studied by an indirect method, i.e. by measuring the amount of GF that remained encapsulated in the nanoparticles. The release kinetics of PDGF-BB and FGF-2 from PLGA:poloxamer nanoparticles were studied upon dilution of the suspensions to 1 mg/mL in PBS pH 7.4. These diluted suspensions were incubated at 37 °C under static conditions. At set time points, release samples were collected and centrifuged at 8000 rcf for 1 h at 5 °C to isolate the nanoparticles from released GFs. Then, the isolated nanoparticles were degraded with 500 μL of 0.05 N NaOH; this hydrolytic process was maintained under moderate magnetic stirring for 1 h at room temperature, to achieve the complete polymer degradation. The alkaline GF solutions were opportunely diluted and analyzed by ELISA assay to quantify the amount of GF that is not released. The amount of

released GF was calculated from the difference between the total amount of encapsulated GF and the amount of nonreleased GF for each time point.

Biological Effect of GF-Loaded PLGA:Poloxamer Nanoparticles on BCE Cells. The proangiogenic activity of GF-loaded nanoparticles was tested on primary bovine capillary endothelial cells (BCE) 4T GRT. The BCE cells were continuously grown on gelatinized cell culture dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2.5% fetal bovine serum (FBS), 4 mM L-glutamine, and antibiotic solution. The immortalized BCE cells in medium containing 2.5% serum (FBS) were plated (2.5×10^4 cell/well) into a 24-well plate previously covered with a collagen gel layer. One day after inoculation, cells were treated with medium containing (i) soluble FGF-2, (ii) soluble PDGF-BB, (iii) nanopaticles containing BSA and Hp (control NPs), (iv) nanoparticles containing BSA, Hp and FGF-2 (FGF NPs) or (v) nanoparticles containing BSA, Hp and PDGF-BB (PDGF NPs). The amount of soluble GF used in the experiment was 8 ng/mL, while the amount of nanoparticles used was equivalent to a total GF amount of 2 ng/mL, 4 ng/mL or 8 ng/mL. As negative controls, cells were treated with cell medium or with control NPs. After 24 and 48 h, the cell viability was evaluated through MTT test, which was performed according to the manufacturer's instructions. The result was a colorimetric signal proportional to the cell number, which is expressed as a percentage of the untreated cell number. Each data point represents the mean ± standard deviation on eight samples.

Statistical Analysis. Statistical analysis was performed by applying one-way ANOVA tests and Bonferroni post-hoc contrasts ($p < 0.01$).

Results and Discussion

In this work, we disclose a new nanometric delivery platform for two proangiogenic growth factors (GFs) that have recently shown great potential when used in combination: FGF-2 and PDGF-BB.[9] To further improve the therapeutic activity of these molecules, we propose a nanometric carrier capable of integrating these substances in a solid matrix, and to release them over prolonged periods of time. Through this device, growth factors that degrade in physiological conditions in a few minutes can produce sustained effects for prolonged periods of time with a minimum loss of bioactivity.[27] To optimize the encapsulation process and preserve the bioactivity of the encapsulated molecule, two stabilizing agents were encapsulated in association with the GFs: BSA and Hp. Moreover, because of their small size, PLGA:poloxamer nanoparticles are also readily injectable and, thus, they do not bring up additional drug administration issues as compared to solutions of the growth factors. In the following lines, specific formulation strategies to adapt PLGA:poloxamer nanoparticles to cytokine administration are discussed.

GF Encapsulation in PLGA:Poloxamer Nanoparticles. PDGF-BB is a homodimer constituted by two identical chains of 218 amino acid residues, which results from an alternative splicing of the gene of the PDGF family.[33] This GF is a potent proangiogenic factor and is characterized by a molecular weight of 24.3 kDa and an isoelectric point of 10.5. On the other hand, FGF-2 is an isoform of the FGF family, constituted by a single chain of 154 total amino acid residues, with a lower molecular weight (17.2 kDa) and an isoelectric point of 9.6.[34] These two GFs share some characteristics, such as the presence of a Hp binding domain responsible for Hp−GF complex formation. The formation of the Hp−GF complex can stabilize the growth factor tridimensional structure and promote their biological activity.[35,36] Taking this information into account, we studied the encapsulation of both GFs forming a Hp−GF complex. Additionally, we have also tested the coencapsulation of BSA, incorporated as second stabilizing agent during the encapsulation process.[37] Because of its amphiphilic properties, BSA localizes at the water/oil interface, in the first emulsion step during nanoparticle preparation, protecting the growth factor (sacrificial lamb approach).[37]

Table 1. Size, Polydispersity Index (PI) and ζ-Potential of PLGA:Poloxamer Blend Based Nanoparticle Encapsulating Different Molecules[a]

encapsulated molecules	size (nm)	PI	ζ-potential (mV)	EE (%)
Hp	147 ± 1	0.128−0.156	−37.1 ± 5.4	
Hp, PDGF-BB	158 ± 1	0.129−0.157	−27.0 ± 1.9	35 ± 2
BSA, Hp	150 ± 1	0.136−0.150	−23.6 ± 5.3	
BSA, Hp, PDGF-BB	154 ± 1	0.118−0.142	−25.2 ± 1.1	87 ± 2

[a] Encapsulation efficiency (EE) was evaluated for PDGF-BB. Values represent means ± standard deviation ($n = 3$), except for PI, expressed as range.

To investigate which is the best strategy for GF encapsulation in PLGA:poloxamer nanoparticles, PDGF-BB was encapsulated in nanoparticles as a complex with Hp and alternatively it was encapsulated with both stabilizers Hp and BSA. The physicochemical properties of the nanoparticles and the PDGF-BB encapsulation efficiency are displayed in Table 1. For comparison, the same nanoparticles

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Table 2. Size, Polydispersity Index (PI), ζ-Potential, Yield of Production, Encapsulation Efficiency (EE), and Actual Loading (% w/w) of Different PLGA:Poloxamer Formulations[a]

formulation	encapsulated molecules	size (nm)	PI	ζ-potential (mV)	yield of production (%)	EE (%)	actual loading (%)
blank NPs		148 ± 1	0.118−0.142	−23.2 ± 7.5	77 ± 2		
control NPs	BSA, Hp	150 ± 1	0.136−0.150	−23.6 ± 5.3	72 ± 6		
PDGF NPs	BSA, Hp, PDGF-BB	154 ± 1	0.118−0.142	−25.2 ± 1.1	73 ± 2	87 ± 2	0.012 ± 0.001
FGF NPs	BSA, Hp, FGF-2	165 ± 1	0.113−0.171	−26.2 ± 1.4	73 ± 2	63 ± 2	0.008 ± 0.001

[a] Values represent means ± standard deviation ($n = 3$), except for PI, expressed as range.

including the stabilizers (i.e., Hp or Hp/BSA) but without PDGF-BB were also characterized.

The properties of the PLGA:poloxamer nanoparticles prepared by the solvent diffusion method, and encapsulating Hp or Hp/BSA, were similar to those previously reported.[27] PLGA:poloxamer blend based nanoparticles show a mean diameter below 200 nm, an unimodal particle population (polydispersity index <0.15), and a negative ζ-potential. The nanoparticles presented similar characteristics independently of the GF and stabilizers encapsulated (Table 1). As an exception, the ζ-potential values of PLGA:poloxamer nanoparticles encapsulating only Hp showed a significant increase compared to other formulations. In fact, Hp-loaded nanoparticles are characterized by a ζ-potential of −37 mV, while all other analyzed formulations show ζ-potential values around −25 mV. This higher surface negative charge of the Hp-loaded nanoparticles could suggest that part of the encapsulated Hp is anchored onto the nanoparticle surface, enhancing its anionic nature. When PDGF-BB is encapsulated in the presence of Hp, the negative ζ-potential value is significantly reduced. This result is suggesting that Hp interacts with PDGF-BB through its Hp binding domain, effectively masking the anionic nature of Hp.[35] Coencapsulation of BSA as a second stabilizer also reduces the absolute value of the ζ-potential, which hints at partial accumulation of this protein on the nanoparticle surface (Table 1).

PDGF-BB encapsulation efficiency was strongly influenced by the particle formulation conditions (Table 1). In fact, PLGA:poloxamer nanoparticles encapsulated PDGF-BB complexed with Hp with low efficiency (35%). By employing BSA as second stabilizing agent, it was possible to increase PDGF-BB encapsulation efficiency up to 87%; this is more than a 50% increase in encapsulated GF (Table 1). This increase is probably due to the amphiphilic properties of BSA, which can reduce the exposure of the GF to the water/oil interface during the particle preparation step,[37,38] reducing possible losses of GF bioactivity during this procedure.

Taking into account the previous study performed with PDGF-BB loaded nanoparticles, FGF-2 was encapsulated directly in the presence of both stabilizing agents, BSA and Hp. Table 2 displays the resulting PLGA:poloxamer nanoparticle formulations for both PDGF-BB and FGF-2 and their characteristics. For comparison, blank nanoparticles and nanoparticles encapsulating the stabilizers Hp/BSA are also shown. Henceforth, the formulations will be referred as blank, control, PDGF and FGF nanoparticles (for details, see Table 2). As shown in Table 2, FGF nanoparticles present overall physicochemical properties that are very similar to those of PDGF nanoparticles. However, FGF-2 encapsulation efficiency is lower than that of PDGF-BB (65% vs 87%). This difference could be connected to the differences in molecular weight of the two GFs (i.e., 24 kDa for PDGF-BB and 18 kDa for FGF-2) that result in faster diffusion of FGF-2 to the external phase during the encapsulation step.[28] It is also worth mentioning that encapsulated GFs were determined by a direct method, and quantified by ELISA assay. Therefore, our results guarantee that at least the stated amount of antigenically active GF is associated to the nanoparticles.

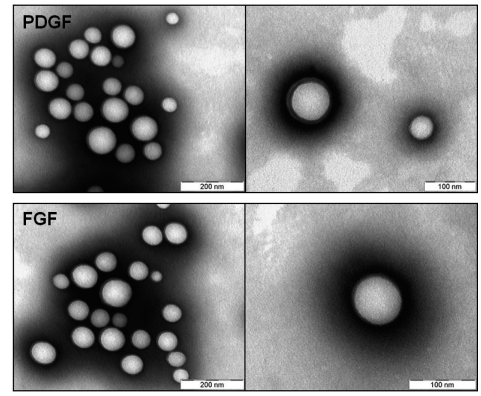

Figure 1. TEM images of PLGA:poloxamer blend based nanoparticles containing BSA/Hp/PDGF-BB (PDGF nanoparticles), and BSA/Hp/FGF-2 (FGF nanoparticles).

PDGF and FGF nanoparticles were also characterized for morphology by transmission electron microscopy (TEM) (Figure 1). As indicate in Figure 1, the GF-loaded nanoparticles show a spherical and regular morphology, which is in agreement with the particles usually obtained by this preparation technique.[27] Additionally, TEM images confirmed their homogeneus particle size distribution, as already suggested by PCS measurements (Table 2). TEM micrographs show that PLGA:poloxamer blend NPs are swathed by a loose shell, typically observed for PLGA:poloxamer nanoparticles, and that has been attributed to the presence of a fraction of the poloxamer on the surface of the nanostructure.[39]

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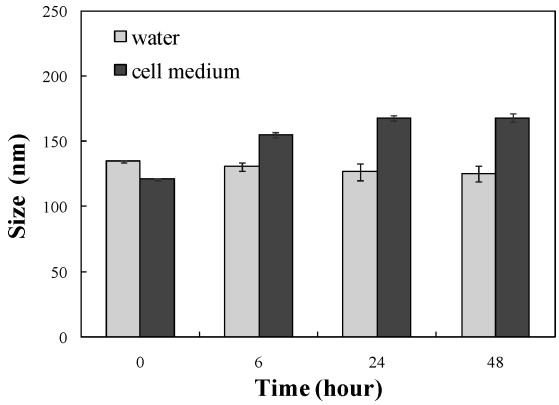

Figure 2. Size of blank PLGA:poloxamer based nanoparticles suspended in cell culture medium and incubated at 37 °C. The nanoparticle size was measured at different set time point until 48 h. Nanoparticle suspension in water at 37 °C was used as control. Bars represent means ± standard deviation ($n = 3$).

Nanoparticle Stability in Cell Culture Medium. The stability of nanoparticles in biological media is critical for the performance of the formulation in the *in vivo* setting. In particular, it is essential that the nanoparticles maintain their size and polydispersity index under physiological conditions, to guarantee their possible administration as an injectable formulation. Furthermore, an aggregated formulation will probably release the GFs with different kinetics than a stable nanoparticle suspension. Therefore, having formulations that do not aggregate is important to ensure that release kinetics achieved *in vitro* will resemble the *in vivo* setting and possible GF release during cell culture experiments.

To test for particle stability, blank nanoparticles were incubated in cell culture medium at 37 °C, and particle size was analyzed by PCS at different incubation times.

As observed in Figure 2, nanoparticles incubated in cell medium for 48 h show only slight increases in particle size. This particle size increase could result from extremely slow aggregation processes due to the ionic strength of the medium, or from the attachment of protein molecules present in the cell medium onto the nanoparticle surface. This increase in nanoparticle size seems to stabilize at 165 nm, as no further increases in particle size occurred between 24 and 48 h. Particle size of blank PLGA:poloxamer nanoparticles suspended in water for 48 h at 37 °C was nearly constant (130 nm), indicating that the small size increase observed in the cell medium is related to the composition of the cell medium.[40] In conclusion, the particle size of PLGA:poloxamer nanoparticles remained fairly constant in water and in cell medium, suggesting that this formulation will maintain its granulometric properties in cell culture experiments and after *in vivo* administration.

Nanoparticle Cytotoxicity. Considering the potential use of this formulation for parenteral administration in drug delivery and tissue engineering applications, we also aimed at characterizing the toxicity profile of our nanocarriers. To this purpose, PLGA:poloxamer blend nanoparticles were tested after incubation with a hepatic model cell line (C3a). Cytotoxicity was measured by a metabolic activity assay (XTT), producing a soluble dye and thereby avoiding the need of cell lysis prior to colorimetric reading. The cells were incubated in the presence of the nanoparticles at different concentrations and at 37 °C for 24 h. The cytotoxicity studies were performed using subconfluent as well as confluent cell layers.

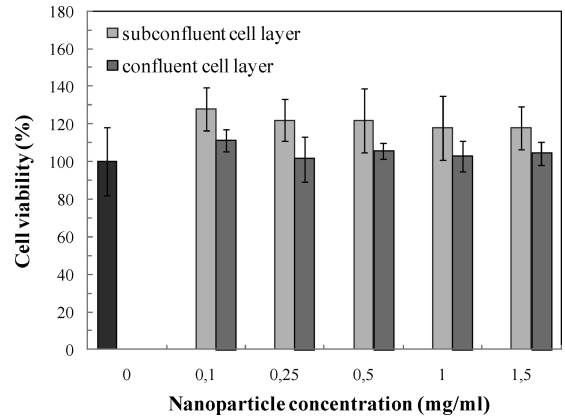

Figure 3. Cell viability measured by a metabolic activity assay (XTT) in C3a cell in the presence of increasing concentrations of PLGA:poloxamer blend nanoparticles. The nanoparticle suspensions in cell medium were incubated with the cells for 24 h at 37 °C. Two different assays were performed, one on subconfluent cell layers (light shaded bars) and the other on confluent cell layers (medium shaded bars). Bars represent means ± standard deviation ($n = 5$).

As shown in Figure 3, cell viability is close to the 100% for the range of particle concentrations tested, and in some cases, the readouts from the metabolic activity assays were even higher than the control. Similar paradoxical readouts of increased cell viability had been obtained previously with the MCF-7 breast cancer cell line,[27] and were attributed to the presence of poloxamer in the nanoparticle matrix. In fact, it is known that poloxamers can influence mitochondrial respiration,[41] therefore inducing small increase in the readout of cell respiratory assays (e.g., XTT) that are uncorrelated to real increases in viability.[42,43]

Freeze-Drying of PLGA:Poloxamer Nanoparticles. Freeze-drying of a nanoparticle formulation is a crucial step to achieve long-term storage of delicate molecules in

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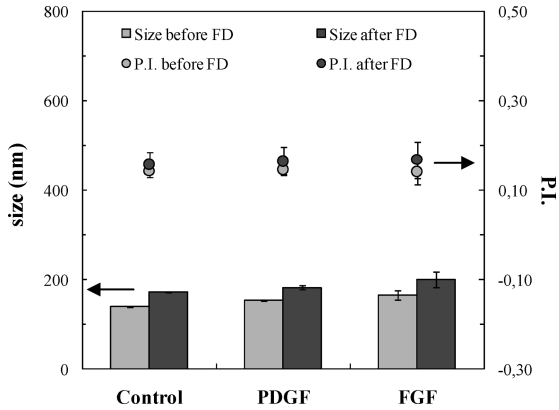

Figure 4. Average size and polydispersity index (PI) of PLGA:poloxamer nanoparticles before and after freeze-drying (FD) in the presence of trehalose 5% (w/v). The formulations tested were control, PDGF and FGF nanoparticles. Bars represent means ± standard deviation ($n = 3$).

nanoparticle form. Indeed, it is known that many molecules that degrade quickly at room conditions can markedly improve their stability when encapsulated in solid matrices and freeze-dried.[44,45] For this reason, freeze-dried preparations are often required for nanoparticle formulations aimed at industrial development. For this reason, we have developed a technique to freeze-dry GF-loaded PLGA:poloxamer nanoparticles, maintaining almost unmodified the nanoparticle characteristics. To achieve this, nanoparticle suspensions at a concentration of 2 mg/mL (w/v), were freeze-dried in the presence of a cryoprotectant (i.e., trehalose 5% w/v).

Our results show that, with the optimized conditions described in the Methods section, it is possible to freeze-dry PLGA:poloxamer nanoparticles encapsulating GFs, and resuspend them with a minimal loss in their granulometric properties. Indeed, resuspended nanoparticles show particle sizes still below 200 nm and polydispersity index about 0.15 (Figure 4). These results suggest that a small size and a unimodal particle size distribution are maintained during the freeze-drying process. Even more importantly, bioactivity assays have shown that no loss in GF potency can be observed when formulations are stored for 6 months at 4 °C (see further in the manuscript).

GF Release Profile from PLGA:Poloxamer Nanoparticles. The major aim of our drug delivery approach is to integrate PDGF-BB and FGF-2 in drug delivery devices capable of providing a sustained release. It is known that FGF-2 and PDGF-BB have extremely short physiological half-lives,[33,34] and therefore, both molecules would greatly benefit from a technology that allows the creation of a prolonged proangiogenic stimulus. Additionally, by achieving controlled release of these GFs, we could create a concentration gradient of these chemotactants, which will also be beneficial for stimulating angiongenesis.

Drug release profiles provided by PLGA-based drug delivery systems are typically characterized by three phases: (i) an initial drug release associated to the diffusion of the encapsulated molecule localized near the particle surface; (ii) a lag period in which the drug is released very slowly; (iii) a controlled release phase associated with polymer matrix degradation.[46,47] The lag phase corresponds to the time of polymer matrix hydration and the generation of water channels, which represent the beginning of the erosion phase. Nevertheless, the presence of poloxamer modifies the hydration property of the PLGA-based matrix, facilitating water infiltration and promoting the formation of channels.[48] Consequently, PLGA:poloxamer based particles show faster onset of second and third phases of drug release. In the present case, the release profiles of PDGF and FGF nanoparticles (Figure 5) were very similar: an initial burst release around 40%, followed by a sustained GF release for 30 days. These results are in agreement with the release kinetics previously found in PLGA:poloxamer blend nanoparticles encapsulating DNA and insulin;[27,28] however, in those cases, the burst release was slightly smaller. On the other hand, similar profiles were observed in PLGA:poloxamer nanoparticles encapsulating BSA.[48] Taking into account the presence of relatively high BSA/GF ratios (70:1 BSA:GF ratio), it seems reasonable that BSA could strongly influence GF release.

Our release data confirms the presence of relevant fractions of antigenically active GFs for more than two weeks at 37 °C in PBS. Considering the controlled release capabilities of our formulations, we expect that FGF and PDGF nanoparticles will present higher preclinical and clinical efficacy than the drugs in solution, as it has already been observed with other noninjectable formulations.[49−51]

Biological Activity of Microencapsulated GFs. A critical issue in the characterization of microencapsulated drug is

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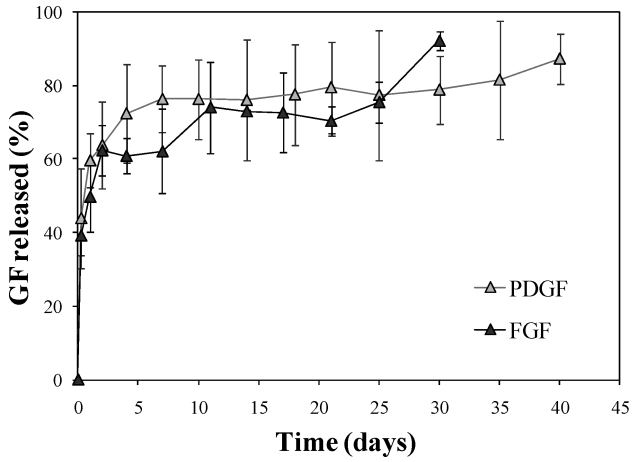

Figure 5. GF release profiles from PLGA:poloxamer blend nanoparticles: PDGF-BB (gray △) and FGF-2 (black △).The nanoparticles were incubated in PBS (pH 7.4) at 37 °C under static conditions. Data points represent means ± standard deviation ($n = 3$).

the evaluation of its bioactivity. For this purpose, proliferation studies in the presence of FGF NPs and PDGF NPs were carried out in BCE cells. Noteworthy, for experiments involving nanoparticles (i.e., control NPs, FGF NPs and PDGF NPs), cells were exposed to resuspended formulations that had been stored at 4 °C for 6 months in freeze-dried form. Untreated cells, cells treated with soluble GFs and nanoparticles containing only the stabilizers (control NPs) were used as controls. As shown in Figure 6, the activity of FGF and PDGF was preserved when the GFs were encapsulated in PLGA:poloxamer NPs. For example, after 24 h of incubation in the presence of FGF NPs (Figure 6A), cell viability increased by 40%. After 48 h of incubation (Figure 6A), cells treated with FGF NPs showed a 300% increase in relative cell viability ($p < 0.001$), which was equal to that seen with FGF in solution. Moreover, in the range tested, the concentration of FGF used did not significantly influence cell response.

On the other hand, a slight but significant dose dependent effect could be observed at corresponding PDGF doses of 2 ng/mL and 4 ng/mL. After 24 h (Figure 6B), PDGF NPs induced a 20% enhancement in relative cell viability at PDGF concentrations of 2 ng/mL and 30% at 4 ng/mL. After 48 h (Figure 6B), this proliferative effect was reduced, but significant differences ($p < 0.001$) between controls and

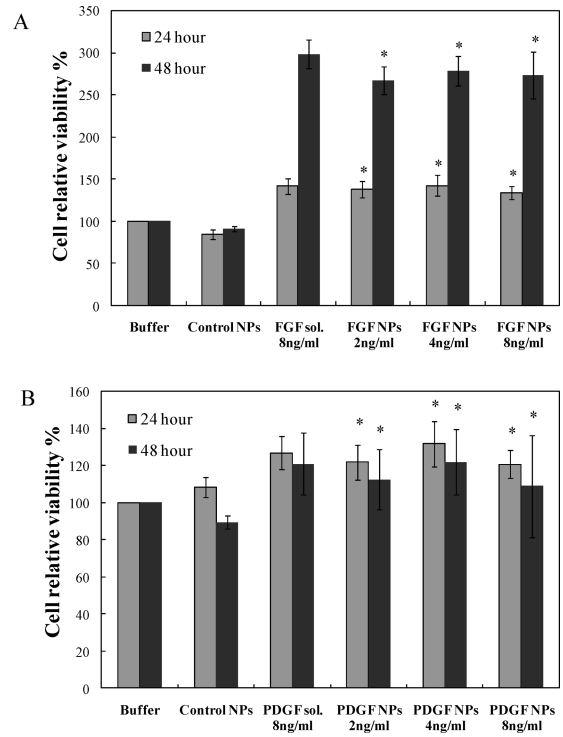

Figure 6. Biological activity of GFs loaded in PLGA:poloxamer NPs tested on BCE cells. Relative cell viability was tested for formulations loaded with (A) FGF-2 and (B) PDGF-BB and for appropriate controls. Cell viability was tested after 24 and 48 h of incubation with the samples. Data are represented as means ± standard deviation ($n = 8$). *Statistically different from buffer and control NPs ($p < 0.001$).

PDGF NPs and FGF NPs were maintained. PDGF-BB pro-proliferative activity after 48 h is significantly lower than FGF's ($p < 0.001$). This represents a reasonable result considering the secondary role of PDGF in endothelial cell proliferation if compared to FGF.[9]

PLGA:poloxamer nanoparticles were designed to provide a sustained release of GFs in active form, warranting a continuous proangiogenic stimulus under *in vivo* conditions. Thus, the exposure of cells to the nanoparticle suspension is probably too brief to ensure that the entire dose of the GFs is released. Indeed, *in vitro* release studies suggest that at most 65% of the GF dose will be available to the cells during this bioactivity test (see Figure 5). Despite this, microencapsulated GFs presented similar bioactivity *in vitro* than soluble GFs. This result might be explained by (i) the complex dose/response relations of GFs and/or (ii) a bioactivity increase in the GFs resulting from their sustained availability to the cells in comparison to soluble GFs. In any case, the result underlines the capacity of this formulation to preserve the GFs bioactivity, even when stored for prolonged periods of time in freeze-dried form.

Conclusions

Nanoparticles composed of PLGA:poloxamer blends have been studied as drug delivery devices for PDGF-BB and

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FGF-2. Formulation conditions, such as the presence of stabilizing agents, were crucial to optimize the nanoencapsulation of these GFs. Under these optimized conditions, nanocarriers achieved high or very high encapsulation efficiencies for both FGF-2 and PDGF-BB, and were able to release GFs in controlled fashion. PLGA:poloxamer nanoparticles are characterized by excellent pharmaceutical properties: they are stable in simulated biological fluids, they are nontoxic, and they can be efficiently freeze-dried for long-term storage. Furthermore, the nanosystems are able to preserve the biological activity of the encapsulated GFs, as demonstrated by the *in vitro* cellular assay. Taking into account all these properties, PLGA:poloxamer-based nanoparticles can be considered very promising drug delivery devices for PDGF-BB and FGF-2, and in more broad terms for GF delivery in tissue engineering and regenerative medicine applications.

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