European Journal of Pharmaceutical Sciences 41 (2010) 644–649

Contents lists available at ScienceDirect

European Journal of Pharmaceutical Sciences

journal homepage: www.elsevier.com/locate/ejps

PLGA:poloxamer blend micro- and nanoparticles as controlled release systems for synthetic proangiogenic factors

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ARTICLE INFO

Article history:
Received 6 May 2010
Received in revised form
14 September 2010
Accepted 15 September 2010
Available online 30 September 2010

Keywords:
PLGA
Poloxamer
Microparticles
Nanoparticles
Synthetic proangiogenic molecule
Tissue engineering
Angiogenesis

ABSTRACT

Tissue engineering is one of the most promising research areas in bioregenerative medicine. However, the restoration of biological functionalities by implanting bioartificially engineered tissues is still highly limited because of their lack of vascular networks. The use of proangiogenic molecules delivered from a controlled release device is a promising strategy to induce tissue vascularization. Indeed, the controlled release system can enhance the therapeutic effect *in vivo* of many short half-life drugs, while circumventing the need for repeated administrations. In this work, PLGA:poloxamer blend based micro- and nanoparticles have been developed for the sustained delivery of a recently developed synthetic proangiogenic compound: SHA-2-22. Drug-loaded PLGA:poloxamer blend microparticles were prepared by an oil-in-oil solvent extraction/evaporation technique. Drug-loaded PLGA:poloxamer nanoparticles were prepared by a modified solvent diffusion technique. These drug carriers were characterized with regard to their physicochemical properties, morphology, drug encapsulation efficiency and release kinetics *in vitro*. The results show that by adjusting the formulation conditions, it is possible to obtain PLGA:poloxamer micro- and nanoparticles with very high drug loadings, and with the capacity to release the active compound in a controlled way for up to one month. *In vitro* cell assays performed in an endothelial cell model confirmed the bioactivity of SHA-22-2 encapsulated in PLGA:poloxamer microparticles.

1. Introduction

The development of functional vascular connexions is the principal limitation restricting our access to clinically relevant engineered tissues (Kelm et al., 2005; Soker et al., 2000). To address this problem, the induction of angiogenesis has been widely studied and several growth factors such as VEGF, PDGF or FGF have been identified as regulators of this process (Nomi et al., 2002; Boontheekul and Mooney, 2003; Cao et al., 2005). Unfortunately, the pharmacological effect of these growth factors has important limitations *in vivo*, related to their very short physiological half-life and to the possibility of undesirable side effects at distant sites (Babensee et al., 2000). To overcome these problems, new proangiogenic factors, mostly peptides or peptidomimetic derivatives, have been synthesized (Horváth et al., 2008a). These molecules usually present better pharmacokinetics parameters than natural growth factors, although typically, their effect is still too short-lived for tissue engineering applications. Additionally, most of these molecules distribute rather freely within the body, a process that might lead to potential side effects. Therefore, the maintenance of prolonged and localized concentrations of synthetic proangiogenic drugs is a limitation that remains to be solved (Boontheekul and Mooney, 2003).

One of the approaches for improving the efficacy of these molecules, while minimizing their side effects, non-enzymatic and enzymatic degradation, is the regional implantation of controlled release systems capable of maintaining appropriate drug levels in a specific tissue for long time periods (Tayalia and Mooney, 2009). Poly(lactic-glycolic acid) (PLGA) based delivery systems have already demonstrated their potential for proangiogenic factor delivery (Richardson et al., 2001; Elisseeff et al., 2001; Borselli et al., 2010). Indeed, PLGA microparticles and nanoparticles are one of the most interesting technologies for growth factor delivery, given the important knowledge available with regard to their delivery properties and the extended documentation on their safety (Csaba et al., 2005; Kang et al., 2008; Mundargi et al., 2008; Schoenhammer et

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doi:10.1016/j.ejps.2010.09.008

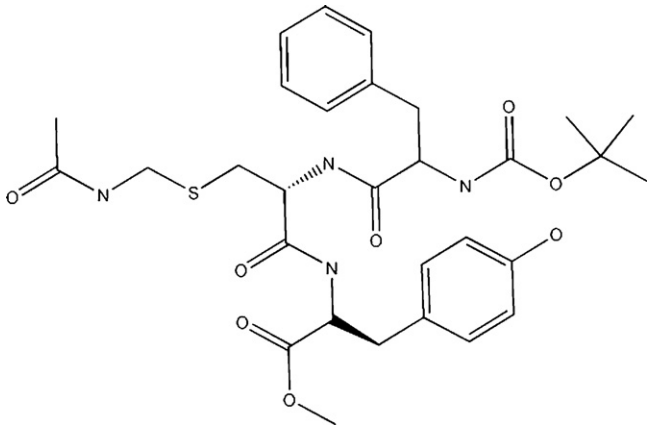

Fig. 1. SHA-2-22 structure.

al., 2010). However, PLGA delivery systems still face some limitations as controlled release systems. First, it has been reported that encapsulation techniques used for their preparation often create mechanical, thermal and chemical conditions which may degrade the molecule to be encapsulated (Sánchez et al., 1999; Wei et al., 2007). Furthermore, it has also been observed that the accumulation of PLGA degradation products inside the particles, during the release process, may cause the inactivation of the encapsulated drug, both due to its interaction with the resulting PLGA oligomers, and also because of the resulting acidic microenvironment inside the matrices (Tobio et al., 1999; Wischke and Schwendeman, 2008). The use of protective excipients such as polaxamers or poloxamines prevents undesired interactions between the drug and the PLGA degradation products, as it has been previously verified for peptides, i.e. insulin, and for pDNA encapsulated in PLGA:poloxamer nanoparticles. (Csaba et al., 2004, 2005, 2006; Santander-Ortega et al., 2006, 2009).

From this previous knowledge, in this work, we have aimed at designing micro- and nanoparticles based on PLGA:poloxamer blends to be used as carriers for the delivery of a recently developed proangiogenic compound, SHA-2-22 (Fig. 1). SHA-2-22 is a new proangiogenic drug candidate that presumably acts through the stabilization of the hypoxia inducing factor (HIF) (Horváth et al., 2006, 2008a,b).

The developed micro- and nanoparticles were characterized for size, morphology, production yield, encapsulation efficiency, actual loading and release kinetics in PBS pH 7.4.

2. Materials and methods

2.1. Materials

The polymer poly (D,L-lactide-co-glycolide) 50:50 Resomer® RG 503 (PLGA) was purchased from Boehringer Ingelheim (Germany). Poloxamer 188, Pluronic® F68, was obtained from Sigma Aldrich (Spain). SHA-2-22 was synthesized by the Cooperative Research Centre, Semmelweis University of Budapest, Hungary (Horváth et al., 2008a). Immortalized bovine capillary endothelial cells (BCE 4T GRT) were provided by Dr. Y. Cao, Microbiology and Tumor Biology Centre, Karolinska Institute, Stockholm, Sweden. XTT reagent was obtained from Roche, Switzerland. Rat tail collagen was obtained from Roche, Switzerland. Eagle's MEM with Earle's BSS, L-glutamine and sodium pyruvate and fetal calf serum were obtained from ATCC (US). The penicillin/streptomycin stock solution was obtained from PAA (US). All other solvents and chemicals used were HPLC grade or better. Ultrapure water obtained from a Milli-Q® water purification system (Millipore Iberica S.A.U., Madrid, Spain) was used throughout the study.

2.2. Encapsulation of SHA-2-22 into PLGA:poloxamer microparticles

PLGA:poloxamer (10:1 in mass ratio) blend microparticles encapsulating SHA-2-22 were prepared by an oil-in-oil (O_1/O_2) solvent extraction/evaporation technique as previously described (Sánchez et al., 1999). Three different formulations were studied, henceforth named Micro A, Micro B and Micro C. Each formulation differed on either their internal phase or their drug loading. For Micro A formulation, 2 mg of SHA-2-22 and 10 mg of poloxamer were dissolved in water, incubated for 1 h and freeze-dried; the resulting powder was then added to 1 ml of acetonitrile (ACN) containing 100 mg of PLGA. Micro B and Micro C were prepared just by dissolving poloxamer 188 (10 mg) and SHA-2-22 (2 mg for Micro B, 5 mg for Micro C formulation) in 1 ml of acetonitrile containing 100 mg of PLGA. For all formulations, the acetonitrile solution was added dropwise to 80 ml of cottonseed oil containing soybean lecithin (0.5% w/v) under agitation at 700 rpm (Janke & Kunkel/IKA-WERK RW 20 DZM, Germany). The obtained microparticles were stirred for 30 min, collected by filtration, washed with petroleum ether and dried.

2.3. Encapsulation of SHA-2-22 into PLGA:poloxamer nanoparticles

PLGA:poloxamer nanoparticles (1:1 PLGA:poloxamer mass ratio) with 2% SHA-2-22 theoretical loading were prepared by a modified solvent diffusion technique as previously described (Csaba et al., 2004). Concretely, 400 μg of SHA-2-22, 20 mg of PLGA and 20 mg of poloxamer were dissolved in 1.4 ml of ACN. The solution was added to 12.5 ml of Milli-Q water:ethanol (EtOH) (1:1), under moderate magnetic stirring, leading to immediate polymer precipitation in the form of nanoparticles. The formulation was diluted by adding 12.5 ml of Milli-Q water, keeping magnetic stirring for 10 min. Afterwards, the solvents were evaporated under vacuum at 30 °C, reducing the final nanoparticle suspension volume to 5 ml. The nanoparticles were characterized for size, polydispersity, ζ potential, actual loading, yield of production and release kinetics in solution.

2.4. Morphological appearance

Microparticle shape and morphology were analysed by Scanning Electron Microscopy (SEM) (SEM, JSM-T220A, Leica S440, Germany). The samples were stuck on a metal stub and coated with gold under vacuum. The morphological examination of the nanoparticles was carried out by transmission electron microscopy (TEM, CM12 Philips, The Netherlands). The samples were stained with 2% (w/v) phosphotungstic acid, and placed on copper grids with Formvar® films for transmission electron microscopy (TEM) analysis.

2.5. Determination of particle size and ζ potential

The microparticle size was evaluated by optical microscopy, measuring the size of 300 microparticles (Olympus B071, Spain). Measurement of nanoparticle size and ζ potential, were performed by photon correlation spectroscopy and laser Doppler anemometry respectively (Zetasizer® 3000HS, Malvern Instruments, UK). For particle size analysis, each sample was diluted to the appropriate concentration with filtered distilled water. Each analysis was

carried out at 25 °C with an angle of detection of 90°. For the determination of the ζ potential, samples were diluted with KCl 1 mM and placed in an electrophoretic cell in which an electric potential of ±150 mV was established.

2.6. Determination of SHA-2-22 encapsulation efficiency

Microparticle and nanoparticle actual loading was determined by an extraction technique. One mg of microparticles or 3.2 mg of nanoparticles were dissolved in 1 ml of ACN. Then, PLGA was precipitated by mixing the sample with ethanol (1:1 v:v); the amount of SHA-2-22 encapsulated was evaluated by measuring its concentration in the ACN/EtOH phase through reverse phase HPLC-UV (1200 Systems, Agilent, USA).

The HPLC analysis conditions were: Column: Phenomenex Luna 5 μm C18, 4.6 mm × 10 mm; mobile phase: (A) 0.1% TFA in water, (B) 10% phase A/90% ACN, gradient: 0–60% B in 20 min, flow rate: 1 ml/min; UV detection: 214 nm. The goodness of the fit concentration–response was verified in the concentration range 2–100 μg/ml ($r^2 > 0.999$).

2.7. In vitro release of SHA-2-22 from PLGA:poloxamer micro- and nanoparticles

The microparticles release kinetics was evaluated by suspending 5 mg of microparticles in 2 ml of PBS pH 7.4. The samples were incubated at 37 °C under static conditions. At scheduled time points, the microparticles were centrifuged at 3000 × *g* for 10 min at 5 °C (Beckman Avanti™ 30, Beckman, Spain). The supernatant was discarded and the microparticle pellet was dissolved in 1 ml of ACN. The PLGA was precipitated from this solution by adding 1 ml of EtOH. This ACN/EtOH solution was filtrated by 0.22 μm pore size filters and analysed by HPLC to quantify the amount of SHA-2-22 that remained in the formulation.

Nanoparticle release studies were performed by suspending 2 mg/ml of nanoparticles in PBS pH 7.4 at 37 °C and in static conditions. At scheduled time points, nanoparticle suspensions were centrifuged in two cycles, first 6000 × *g* for 20 min at 15 °C, and second 10,000 × *g* for 20 min at 15 °C. The supernatant was discarded and nanoparticles were dissolved in 1 ml of ACN. The PLGA was precipitated from this solution by adding 1 ml of EtOH. This ACN/EtOH solution was filtrated by 0.22 μm pore size filters and analysed by HPLC to quantify the amount of SHA-2-22 that remained in the formulation.

2.8. In vitro bioactivity of microencapsulated SHA-2-22

To quantify the stimulation of BCE cell proliferation by microencapsulated SHA-2-22, a collagenized 24-well plate was inoculated with 10^4 BCE cells per well using the following medium composition: Eagle's MEM with Earle's BSS, 2 mM L-glutamine, 1 mM sodium pyruvate, supplemented with 10 vol% fetal calf serum and 1 vol% of a penicillin/streptomycin stock solution. After 24 h the medium was exchanged with the following formulations: (a) plain medium, as above; (b) medium supplemented with 1 ng/ml bFGF; (c) microparticle suspension (Micro C) in plain medium, total SHA-2-22 concentration 75 μM, corresponding to 25 μM released SHA-2-22 within 3 days according to the *in vitro* release profile; and (d) microparticle suspension as above plus 1 ng/ml of free bFGF. After three additional days, all wells were washed and subjected to a XTT test according to the manufacturer's recommendations. Optical densities were measured at 420 nm (assay product of viable cells) and 600 nm (internal reference) with a Spectra Max M2 plate reader (Molecular Devices, US). Measured optical densities ($n = 6$) were referenced to the control group (a) and expressed as arithmetic mean ± standard deviation.

Table 1
SHA-2-22 loaded microparticle formulations.

Formulation[a]	Drug physical form	Theoretical loading
Micro A	SHA-2-22/poloxamer freeze-dried mixture	2%
Micro B	SHA-2-22 powder	2%
Micro C	SHA-2-22 powder	5%

The formulations differed in the physical form in which SHA-2-22 was encapsulated and in the drug theoretical loading.
[a] The composition of the formulations was always identical: PLGA (100 mg), poloxamer (10 mg).

3. Results and discussion

Our main objective was to develop a new technology to induce the vascularization of engineered and ischemic tissues. More concretely, the purpose of this work is the development of micro- and nanoparticles based on PLGA:poloxamer blends, to be used as controlled release devices for a new proangiogenic compound, SHA-2-22. The ultimate goal is to generate a delivery system that, upon implantation, will ensure the localized and prolonged availability of the drug in the targeted tissue.

3.1. Preparation and physicochemical characterization of micro- and nanoparticles

During the preparation of the microparticles, the use of organic solvents, strong agitation and exposure of the drug to water/oil interfaces may cause protein denaturation or aggregation. The O_1/O_2 solvent evaporation technique is one of the most convenient for the encapsulation of peptides or peptidomimetics within PLGA microparticles because choosing an external phase for which the drug shows low affinity can enhance drug encapsulation efficiency. Moreover, through the use of protective excipients that incorporate into the polymeric matrix such as poloxamers, we can reduce unwanted drug–polymer interactions, resulting in reduced drug degradation (Blanco and Alonso, 1998; Tobio et al., 1999; Bilati et al., 2005). In the current work, SHA-2-22 was incorporated into the inner phase of the O_1/O_2 emulsion in two different forms: as a freeze-dried powder consisting of a homogeneous mixture of the drug and poloxamer (Micro A), or as a pure drug powder (Micro B and Micro C). The differences between these three formulations are indicated in Table 1.

The physicochemical properties of these microparticles and their capacity to encapsulate the compound SHA-2-22 are summarized in Table 2. Microparticle size was around 20 μm, similar to that of PLGA:poloxamer microparticles previously prepared by our group (Sánchez et al., 1999; Tobio et al., 1999). The particle formation process is very efficient, with yields of production above 96% for the three formulations. Furthermore, SHA-2-22 could be encapsulated very efficiently in PLGA:poloxamer microparticles, reaching encapsulation efficiency values around 80%. Cotton seed

Table 2
Properties of PLGA:poloxamer microparticles containing SHA-2-22.

Formulation	Size (mm)	Yield of production (%)	Encapsulation efficiency (%)	SHA-2-22 actual loading (%)
Micro A	20 ± 7	96 ± 1.5	72 ± 6	1.51 ± 0.11
Micro B	19 ± 5	100 ± 0.7	83 ± 4	1.79 ± 0.09
Micro C	21 ± 9	100 ± 1.0	85 ± 7	4.35 ± 0.37

Table 3
Physicochemical properties of PLGA/Poloxamer nanoparticles containing SHA-2-22.

Formulation	Size (nm)	PDI	Yield of production (%)	ζ Potential (mV)	Actual loading (%)	Encapsulation efficiency (%)
Nano	185 ± 24	0.116	80 ± 6	−14 ± 3	1.26 ± 0.06	51 ± 3

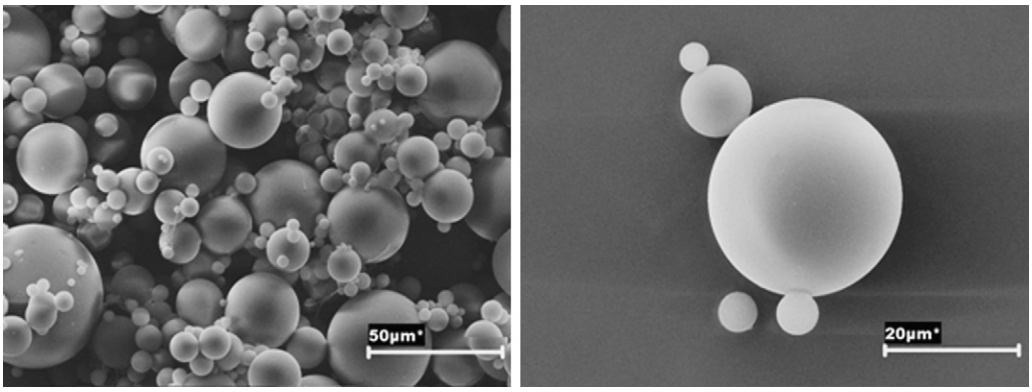

Fig. 2. Scanning electron micrograph of SHA-2-22-loaded PLGA:poloxamer microparticles.

oil, used as continuous phase, may prevent undesirable drug diffusion from the inner phase allowing the achievement of these high encapsulation efficiencies (Carrasquillo et al., 2001). Encapsulation efficiency was determined by the extraction technique previously described in Section 2.6. The drug extracted was analysed by HPLC, and for all formulations, the obtained chromatograms were identical to those of non-encapsulated SHA-2-22, suggesting that SHA-2-22 preserves its integrity during the encapsulation process. This stabilizing effect could be related to the presence of poloxamer 188 into the blend (Sánchez et al., 1999; Tobio et al., 1999; d'Angelo et al., 2010).

PLGA:poloxamer nanoparticles, were prepared by a emulsification-solvent diffusion technique, as previously described by our group (Csaba et al., 2004). This technique does not require the use of high energy sources, thus providing very mild conditions for the encapsulation of delicate drugs (Csaba et al., 2004, 2005). The physicochemical properties of these nanoparticles and their capacity to encapsulate the compound SHA-2-22 are summarized in Table 3. The mean size of the nanoparticles is slightly below 200 nm and the particle size distribution is monodisperse, with a polydispersity index (PDI) of 0.12. The ζ potential is negative, which is typical of the presence of PLGA terminal carboxylic residues. However, the value of this negative charge is much lower than that of classical PLGA nanoparticles, which has been attributed to the presence of a fraction of poloxamer on the surface of the nanoparticles (Csaba et al., 2004; d'Angelo et al., 2010; Santander-Ortega et al., 2006, 2010). Similar to microparticles, nanoparticle formation process is quite efficient, being the yield of production 80%. The drug, SHA-2-22, could be encapsulated in the nanoparticles with an encapsulation efficiency around 50%. This lower encapsulation efficiency value, as compared to that of the microparticles formulations, could be explained by the higher affinity of SHA-2-22 for the external oily phase used for nanoparticle preparation. For all formulations, actual loading values were in the range expected taking into account the theoretical loading (2% or 5% for microparticles, 2% for nanoparticles) and their respective encapsulation efficiencies.

The morphological appearance of the microparticles was examined by SEM. The images shown in Fig. 2 indicate that the microparticles have a spherical shape and a smooth surface. The same appearance was observed for all formulations independently of the physical form of the incorporated drug and its loading. These results agree with those previously described for other PLGA:poloxamer based compositions (Blanco and Alonso, 1998).

The morphological appearance of the nanoparticles was examined by TEM. The images shown in Fig. 3 indicate that the

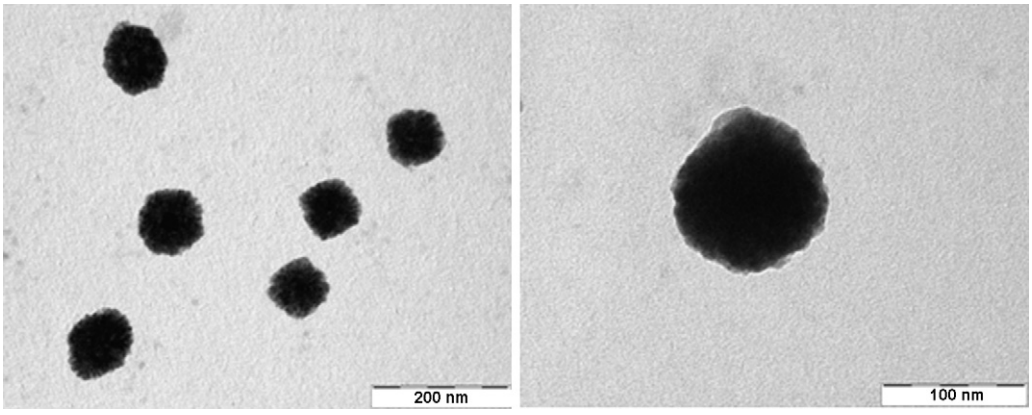

Fig. 3. Transmission electron micrograph of SHA-2-22-loaded PLGA:poloxamer nanoparticles.

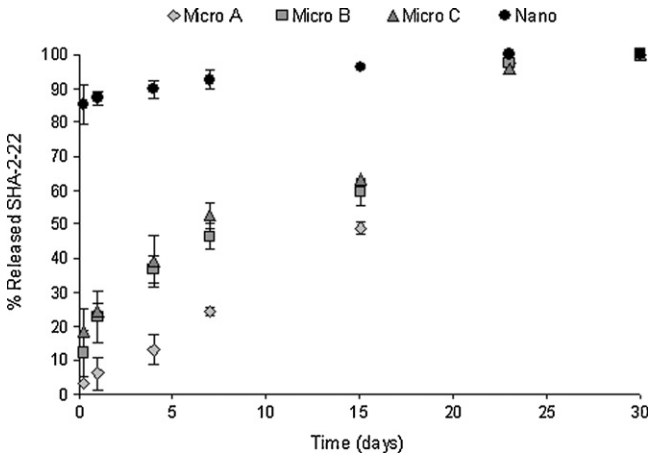

Fig. 4. SHA-2-22 release from PLGA:poloxamer micro- and nanoparticles in PBS at 37 °C. Values represent means ± S.D., $n = 3$.

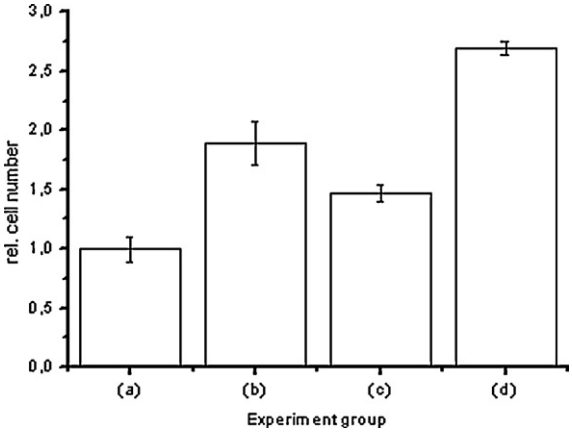

Fig. 5. Relative cell numbers evaluated by an XTT test after BCE cell incubation in: (a) plain medium (negative control); (b) medium supplemented with 1 ng/ml free bFGF (positive control); (c) Micro C microparticle suspension in plain medium (75 μM SHA-2-22 concentration); (d) microparticle suspension as above plus 1 ng/ml free bFGF.

nanoparticles have a spherical shape and form a homogeneous population. Particle size measured by TEM was within the range suggested by PCS.

3.2. In vitro release of SHA-2-22 from micro- and nanoparticles

The release profile of SHA-2-22 from PLGA:poloxamer micro- and nanoparticles in PBS (pH 7.4) at 37 °C, under static conditions is displayed in Fig. 4. SHA-2-22 is released from the nanoparticle system in a very uncontrolled fashion (Fig. 4). Indeed, a very high burst effect is observed (85%), which could be attributed to the diffusion of the drug towards the surface of the nanoparticles during the encapsulation process, resulting in the deposition of a large fraction of drug on the nanoparticle surface. Following the initial burst there is a constant and slow release for 15 days. This second phase should correspond to the release of the drug that was well incorporated within the polymer matrix. For this encapsulated drug, release might occur mainly by diffusion, limited by polymer degradation and particles erosion (Csaba et al., 2005; Sánchez et al., 2003).

The microparticle formulations show an initial SHA-2-22 release between 5% and 20%, depending on the formulation. This burst release is interpreted as the result of SHA-2-22 deposition on the surface of the microparticles. Interestingly, the Micro A formulation exhibits a constant release profile, with a minimum initial release. In contrast, the release from Micro B and Micro C formulations show three release phases. In this profile, an initial release phase, probably associated to desorption of drug molecules, is followed by a second phase of moderate release. After more than two weeks, the rest of the encapsulated drug releases suddenly (3rd phase). We hypothesize that these release profiles result from a fraction of the drug that is encapsulated deep in the polymeric matrix. The release of this fraction lags for many days until polymer degradation is sufficient to allow drug hydration and diffusion from the matrix (Csaba et al., 2005; Sánchez et al., 2003).

Micro A and Micro B formulations (Fig. 4) differ on the physical form of SHA-2-22 when incorporated to the inner phase of the O_1/O_2 emulsion. These different physical forms were (i) SHA-2-22 dissolved as a freeze-dried powder consisting on a homogeneous mixture of this drug and poloxamer (Micro A), or (ii) SHA-2-22 dissolved directly in the acetonitile solution (Micro B). The initial release of SHA-2-22 from Micro A is only 5%, being 10% for Micro B. This and other significant differences in drug release observed between Micro A and Micro B formulations, particularly at early time points (see Fig. 4), suggest us that the physical form is an important parameter modifying drug encapsulation and release. Our interpretation is that poloxamer might be forming micelles with SHA-2-22 in aqueous solution, and that these structures might be preserved during the freeze-drying/acetonitrile dissolution process. This would lead to the encapsulation of the drug forming micellar nanostructures with poloxamer, and ultimately to a different pattern of drug/poloxamer dispersion in the polymeric matrices.

The main difference between the release profiles of formulations Micro B and Micro C (Fig. 4) is localized in the initial release. After 6 h almost 20% of SHA-2-22 was released from Micro C formulation, but only 10% from Micro B formulation. These two formulations were prepared exactly in the same way, except for their drug loading (2% for Micro B and 5% for Micro C). This indicates that the burst release of the formulation increases together with SHA-2-22 loading, a result that is in agreement with other works reporting a relation between drug loading and burst effect (Zhang and Gao, 2007). Irrespective of the formulation, total SHA-2-22 release occurs after 25 days, a sustained release kinetics that can be interesting from a tissue engineering perspective.

Another important consideration is that SHA-2-22 encapsulation in PLGA:poloxamer microparticles endows the drug with enhanced stability in simulated physiological media. We found that SHA-2-22 degrades in PBS pH 7.4 (37 °C) following a monoexponential decay kinetic with a half-life of 2.45 days. On the other hand, it was possible to detect microencapsulated SHA-2-22 by HPLC analysis almost one month after the start of its incubation. Clearly, encapsulation of SHA-2-22 in an inert polymeric matrix is beneficial for its stability and sustained effect *in vivo*. Moreover, these results indicate that by adjusting the particles size, the physical form of the drug during encapsulation and the drug loading, it is possible to obtain different release kinetics to suit different therapeutic purposes.

3.3. In vitro bioactivity of microencapsulated SHA-2-22

From previous studies it was known that 25 μM SHA-2-22 had a significant effect on the proliferation of Karposi Sarcoma cells (Horváth et al., 2006, 2008b). For this experiment, we adjusted the dose of SHA-2-22-loaded microparticles to ensure that the amount of drug released corresponded approximately to 25 μM. Our positive control experiment (Fig. 5, column "b") confirmed that BCE cells react to mitogens involved in endothelial cell recruitment by

producing almost a 200% increase in BCE cell numbers relative to the negative control (Fig. 5, column "a"). SHA-2-22 encapsulated in the microparticles was also able to induce a mitogenic effect (Fig. 5, column "c"), although this was lower than the positive control. However, SHA-2-22 might turn the table *in vivo* as this molecule should present relevant biopharmaceutical advantages compared to bFGF, mainly a longer physiological half-life. It is also interesting to note that bFGF and SHA-2-22 have a synergistic mitogenic effect *in vitro* (Fig. 5, column "d"), which might reflect different but complementary proangiogenic signalling pathways.

4. Conclusions

A new proangiogenic compound, SHA-2-22, has been successfully encapsulated in PLGA:poloxamer blend-based microparticles. The release profile of these formulations can be modulated depending on several technological parameters. For microparticles where SHA-2-22 can be incorporated together with poloxamer as lyophilized powder, a zero order release profile can be achieved for 25 days. Cell culture confirmed the bioactivity of microencapsulated SHA-2-22, indicating the interest of this controlled release system for proangiogenic treatments in tissue engineering and for ischemia-related diseases.

Acknowledgments

This work is sponsored by the 6th Framework Program of the European Community within the priority 3-NMP (VASCUPLUG NMP3-CT-2005-013811). MGF acknowledges an Isidro Parga Pondal Contract from Xunta de Galicia.

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