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Research paper

Versatility of biodegradable poly(D,L-lactic-co-glycolic acid) microspheres for plasmid DNA delivery

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Abstract

In this study, we have optimized different formulations of DNA encapsulated into PLGA microspheres by correlating the protocol of preparation and the molecular weight and composition of the polymer, with the main characteristics of these systems in order to design an efficient non-viral gene delivery vector. For that, we prepared poly(D,L-lactic-co-glycolic acid) (PLGA) microparticles with an optimized water–oil–water double emulsion process, by using several types of polymers (RG502, RG503, RG504, RG502H and RG752), and characterized in terms of size, zeta potential, encapsulation efficiency (EE%), morphology, DNA conformation, release kinetics, plasmid integrity and erosion. The size of the particles ranged between 0.7 and 5.7 μm depending on the protocol of formulation and the molecular mass of the polymer used. The microspheres prepared by using in their formulation polymers of high molecular weight (RG503 and RG504) were bigger in size than in the case of using a lower molecular weight polymer (RG502). The EE (%) of plasmid DNA increased with increasing the molecular mass of the polymer and by using the most hydrophilic polymer RG502H, which contains terminal acidic groups in its structure. The plasmid could be encapsulated without compromising its structural and functional integrity. Also a protective effect of PLGA on endonuclease digestion is observed. Plasmid DNA release from microspheres composed of low molecular weight or hydrophilic polymers, like RG502H, was faster than from particles containing high molecular weight or hydrophobic polymers. These PLGA microspheres could be an alternative to the viral vectors used in gene therapy, given that may be used to deliver genes and other bioactive molecules, either very rapidly or in a controlled manner.

Keywords: Poly(D,L-lactic-co-glycolic acid) (PLGA); Microparticles; Gene delivery; Biodegradable polymers; Plasmid DNA; Microencapsulation

1. Introduction

Gene therapy is a form of molecular medicine that has the potential to influence significantly human health in this century. It promises to provide new treatments for a large number of inherited and acquired diseases. However, to achieve this goal, gene therapy requires technologies capable of gene transfer into a wide variety of cells, tissues and organs without causing cytotoxicity. Despite that naked DNA has been used successfully when injected locally, it is highly prone to tissue clearance and totally inefficient for systemic delivery. Because of that, it is important to design appropriate DNA carriers, which protect the plasmid from degradation and allow efficient gene delivery.

The molecular form of the DNA has been reported to affect the efficiency with which the pDNA will transfect cells. Linear pDNA is much less efficient in transfection than open circle or supercoiled DNA, and there is little difference between the supercoiled and open circle forms [1,2]. Therefore, it is desirable to have the plasmid DNA encapsulation conditions optimized for minimal effect on the supercoiled DNA topology. In this respect, it is important

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to note that the double emulsion technique commonly used for DNA microencapsulation utilizes shear forces typically thought to be harmful to the structural integrity and expression capacity of plasmid DNA. This effect has been studied by different authors [3–5].

There are two main groups of vectors used in gene delivery: viral and non-viral vectors. Toxicity and immunogenicity concerns associated with viral vectors have led to an active interest in non-viral systems for gene delivery [6,7], such as liposomes, cationic blok copolymers, polymer complexes and polymeric micro- and nanoparticles, because they are relatively safe and are easy to formulate [8,9]. Specifically, much attention has been focused on biocompatible, biodegradable polymers, such as PLGA, for the encapsulation of genes [10–15]. Also, chitosan [16,17] and gelatin [18] have been used to encapsulate pDNA. On the other hand, the composition and molecular weight of the polymers not only determines the release pattern of encapsulated material, but it is suspected to also considerably affect the hydrophobicity of the resulting microspheres and therefore may influence the behavior [12,19]. It should be also considered that, although controlled release polymeric systems offer the advantage of sustained pDNA activity [20], in some cases a rapid release of DNA is of interest. For example, it is known that the ability of pDNA to induce an immune response in vivo will decrease as a function of the time of release.

Taking all this into account, the aim of this study was to optimize different formulations of PLGA/DNA microspheres to be used as an efficient non-viral gene delivery system, by correlating the properties of the microparticles with their capabilities to encapsulate and release DNA. For that, microspheres were prepared and characterized in the presence of different PLGA polymers (RG502, RG503, RG504, RG502H and RG752), by using two protocols of formulation.

2. Materials and methods

2.1. Materials

Poly(D,L-lactic-co-glycolic acid) (PLGA) polymers of different molecular weight and co-polymerization rate (lactic:glycolic), Resomer® RG502 (M_w 12,000 Da, 50:50), Resomer® RG503 (M_w 34,000 Da, 50:50), Resomer® RG504 (M_w 65,000 Da, 50:50), Resomer® RG502H (M_w 12,000 Da, 50:50) and Resomer® RG752 (M_w 12,000 Da, 75:25), were purchased from Boehringer Ingelheim (Germany). The plasmid pCMVLuc (BioServe Biotechnologies, USA) encoding for luciferase and the lipids 1,2-dioleoyl-3-(trimethylammonium) propane (DOTAP) and cholesterol (CHOL) (Avanti Polar Lipids, Alabaster, AL, USA) were used in the transfection studies. Polyvinyl alcohol (PVA, 87–89% hydrolyzed, M_w 13,000–23,000), *N*-(2-hydroxyethyl) piperazine-*N*′-[2-ethanesulfonic acid] (HEPES) and Trizma hydrochloride were obtained from Sigma–Aldrich (Madrid, Spain).

2.2. Cell culture

HepG2 cells (American Type Culture Collection, MD, USA) were maintained at 37 °C under 5% CO_2 in Dulbecco's modified Eagle's medium-high glucose (DME-HG) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), penicillin (10,000 U/mL) and streptomycin (10,000 μg/mL) (Invitrogen Life Technologies, UK). Cells were passaged 1:3 by trypsinization twice a week. For transfection 3×10^5 HepG2 cells were seeded in 1 mL of medium in 48-well culture plates (Iwaki Microplate 48-well, Japan) 24 h before addition of the plasmid and used at approximately 80% confluence.

2.3. Preparation of microparticles

PLGA microparticles containing pCMVLuc were prepared by a water–oil–water emulsion solvent evaporation method. For that, 120 μL of 30 mM Tris–HCl (pH 8.5) containing 200 μg of plasmid DNA was emulsified in 500 μL of chloroform containing 100 mg of PLGA using a microtip probe sonicator (Microson XL 2000, Misonix Incorporated, USA) set at level 20 for 5 s. The resulting primary emulsion was added to 2 mL of 9% (w/v) polyvinyl alcohol and emulsified using an Ultra-Turrax (T 20 b, Ika Labortechnik, Germany) at 13,500 rpm (protocol 1) or at 27,000 rpm (protocol 2) for 30 s. The resultant double emulsion was added dropwise into 8 mL of 9% (w/v) PVA and agitated using a magnetic stirrer for 3 h at room temperature, until complete evaporation of chloroform. The microparticles were collected by ultracentrifugation (40,000 g, 10 min) at room temperature, washed three times with distilled water, freeze-dried and stored at −20 °C until use.

2.4. Particle size and zeta potential measurements

Microparticles size and zeta potential were determinated by laser diffractometry using a Zetasizer Nano Series (Malvern Instruments, UK) after resuspension in distilled water and 1 mM HEPES, 1% (w/v) glucose buffer (pH 7.4), respectively. All measurements were performed in triplicate.

2.5. Encapsulation efficiency

The amount of DNA loaded in the microparticles was estimated by a fluorimetric assay (PicoGreen dsDNA Quantitation Kit, Molecular Probes, USA). For that, the DNA was extracted from the particles by dissolving 2.5 mg of samples in 1 mL of chloroform. The resulting solution was diluted in 1 mL of TE buffer (Tris–HCl 10 mM, EDTA 1 mM, pH 7.5) and agitated using a magnetic stirrer for 3 h at room temperature. The extracted DNA was collected by centrifugation (8000*g*, 2 min) and stored at −20 °C. The encapsulation efficiency was defined as the amount of DNA recovered from the microparticles

relative to the initial amount of DNA used (recovered DNA × 100/initial DNA).

2.6. Morphology and in vitro degradation studies by SEM

The shape and morphology of the microparticles were assessed by scanning electron microscopy (SEM). A monolayer of freeze-dried microparticles was mounted on an aluminium stub using double-sided carbon tape. The sample was coated with 9 nm molecular gold film using a sputter coater (Emitech K550 Equipment, UK) and SEM photographs made with a Zeiss DSM 940 A microscope. For the degradation studies, samples of microparticles were taken at different times from the "in vitro release assay" medium and analyzed.

2.7. Structural integrity of the encapsulated pDNA

The integrity of the encapsulated plasmid was analyzed by agarose gel electrophoresis. For this, 10 mg of microparticles was dissolved in 1 mL of chloroform and diluted in 250 mL TE buffer (Tris–HCl 10 mM, EDTA 1 mM, pH 7.5). The samples were agitated using a magnetic stirrer for 3 h at room temperature. The extracted DNA was collected by centrifugation (8000*g*, 2 min) and stored at −20 °C. Samples of control and recovered pDNA were applied to a 0.8% agarose gel in TBE buffer (100 mM Tris, 90 mM boric acid, 1 mM EDTA, pH 8.4). Adequate separation of the bands for supercoiled and open circular pDNA was obtained by running the gel for 2 h at 80 mV, 140 mA and visualised under UV illumination after ethidium bromide staining, using a camera Gel (doc 2000, Bio-Rad, USA).

2.8. Plasmid DNA functional integrity

Biological functionality of plasmid was assessed by transfection of HepG2 cells. Encapsulated DNA was extracted from microparticles as described above, and complexed with DOTAP/CHOL (1:0.9 molar ratio) liposomes at a lipid/DNA (+/−) charge ratio of 5/1. For transfection, HepG2 cells were seeded in medium and incubated for 24 h at 37 °C in 5% CO_2. After this time, the medium was removed and the cells were washed with phosphate-buffered saline (PBS). Then, 0.3 mL of DME-HG and 0.2 mL of complexes containing 1 μg of DNA were gently added to each well. After 4 h incubation (at 37 °C in 5% CO_2) the medium was replaced with DME-HG containing 10% FBS and antibiotics, and the cells were further incubated for 48 h. After that, cells were washed with PBS and lysed using 100 μL of reporter lysis buffer (Promega, Madison, WI, USA) at room temperature for 10 min, followed by alternating freeze–thaw cycles. The cell lysate was centrifuged for 2 min at 12,000*g* to pellet debris. A 20 μL amount of supernatant was assayed for total luciferase activity using the Luciferase Assay Kit (Promega, USA) according to the manufacturer's protocol and a luminometer (Sirius-2, Berthold Detection Systems, Innogenetics, Spain). The protein content of the lysates was measured by the DC protein Assay Reagent (Bio-Rad, Hercules, CA, USA). The data were expressed as nanograms of luciferase per milligram of protein.

2.9. Protection from DNase I

To test whether PLGA microparticles can protect encapsulated plasmid DNA from nucleases digestion, 15 mg of microspheres were suspended in 1 mL of TE (10 mM Tris–HCl, 1 mM EDTA, pH 7.5) containing 1 U of DNAse I (Invitrogen Life Technologies, UK) per microgram of DNA. The suspension was incubated for 4 h at 37 °C in a water bath. After digestion, the microparticles were collected by centrifugation (14,000 rpm, 10 min) and the pDNA was extracted and analyzed by agarose gel electrophoresis as described above.

2.10. In vitro release of pDNA

Release kinetics experiments were carried out in vitro in TE buffer (Tris–HCl 10 mM, EDTA 1 mM, pH 7.4) at 37 °C. For that, 10 mg of microparticles were suspended in 1 mL of buffer in microcentrifuge tubes and shaken in a water bath at 37 °C. At predetermined time intervals, the tubes were centrifuged (14,000 rpm, 10 min) and the supernatants collected for analysis. Microparticles were immediately dispersed in fresh buffer and placed back in the incubator. The concentration of the released DNA was evaluated by using the PicoGreen fluorimetric assay.

3. Results

3.1. Physical characterization of microparticles

DNA-loaded microparticles by using polymers of different composition and molecular weights (RG502, RG503, RG504, RG752 and RG502H) were formulated and characterized for physical properties, such as particle size and surface charge. The influence of the method of formulation, by changing the homogenization rate in the preparation of the second emulsion, as described in Section 2, was also analyzed.

As shown in Table 1, particles prepared by protocol 2 (high homogenization rate) resulted to be smaller in size than particles prepared by protocol 1 (low homogenization rate), ranging in values between 3.4–0.7 and 5.7–1.6 μm, respectively. The particles were uniform in size as indicated by the low polydispersity index and do not aggregate under physiologic conditions. On the other hand, microspheres prepared by using in their formulation polymers of high molecular weight (RG503 and RG504) were bigger than in the case of using a lower molecular weight polymer (RG502). This effect was observed independently of the protocol of formulation. The size obtained in particles formulated with the highest molecular weight polymer

Table 1
Size, zeta potential and encapsulation efficiency of microparticles prepared by two different protocols, by changing the homogenization rate in the preparation of the second emulsion, as described in Section 2

Polymer	Size (μm)	Zeta potential (mV)	EE (%)
502-1	2.1 ± 0.4	-27 ± 4	24 ± 1
502-2	0.9 ± 0.1	-26 ± 5	10 ± 3
503-1	3.2 ± 0.5	-28 ± 3	49 ± 2
503-2	2.5 ± 0.2	-29 ± 6	38 ± 2
504-1	5.7 ± 0.6	-24 ± 2	41 ± 3
504-2	3.4 ± 0.6	-25 ± 4	39 ± 3
752-1	3.0 ± 0.6	-29 ± 1	3 ± 1
752-2	1.0 ± 0.1	-26 ± 5	3 ± 1
502H-1	1.6 ± 0.2	-23 ± 4	48 ± 1
502H-2	0.7 ± 0.1	-26 ± 5	39 ± 5

Protocol 1 (low homogenization rate), protocol 2 (high homogenization rate). Size and zeta potential were measured after resuspension of the particles in water and HEPES glucose buffer, respectively. The EE (%) was determined as the amount of DNA recovered from the particles relative to the initial amount of DNA used. All measurements were performed in triplicate.

(RG504) was 5.7 and 3.4 μm by protocols 1 and 2, respectively, while by using the polymer with the lowest molecular weight (RG502) the values were 2.1 and 0.9 μm. No significant differences were observed in the diameter of particles prepared with polymers of different co-polymerization rate lactic:glycolic (RG502 versus RG752) or polymers with or without terminal acidic groups (RG502 versus RG502H). The PLGA microspheres containing plasmid DNA were similar in size to the control microspheres without plasmid.

No differences in the surface charge of microparticles were detected, independently of the protocol of formulation or composition and molecular weight of the polymer used. All the particles were negatively charged with an average value of the zeta potential around -26 ± 2 mV (Table 1).

3.2. Encapsulation efficiency

The encapsulation efficiency (EE%) of plasmid in the PLGA microparticles was a function of the protocol of formulation, as well as of the composition and molecular weight of the polymer used. Particles prepared with protocol 1 presented, in general, a higher EE (%) than those prepared by protocol 2. As can be observed in Table 1, microspheres formulated with the polymer RG502 showed an EE (%) of 24% and 10% by using the methods 1 and 2, respectively. In the case of using the polymer RG503, values of 49% and 38% in the EE (%) were observed. At the same time, higher EE (%) was obtained when the polymers RG504 and RG503 of high molecular weight were used to prepare the particles, compared to using the polymer RG502 of low molecular weight, leading to values of 41 ± 3, 49 ± 2 and 24 ± 1, respectively, in particles prepared by protocol 1. A small EE (%) was detected when particles were prepared with the polymer RG752, with a co-polymerization rate lactic to glycolic of 75:25, while higher EE (%) was achieved with the more hydrophilic polymer RG502H, which contains terminal acidic groups in its structure. A 48% versus 24% of EE (%) was obtained by using the polymers RG502H and RG502, respectively, in particles prepared by protocol 1.

3.3. Morphological studies

Fig. 1 shows representative images of the external structure of microparticles prepared from RG502, RG752 and RG502H containing pDNA or buffer, obtained using

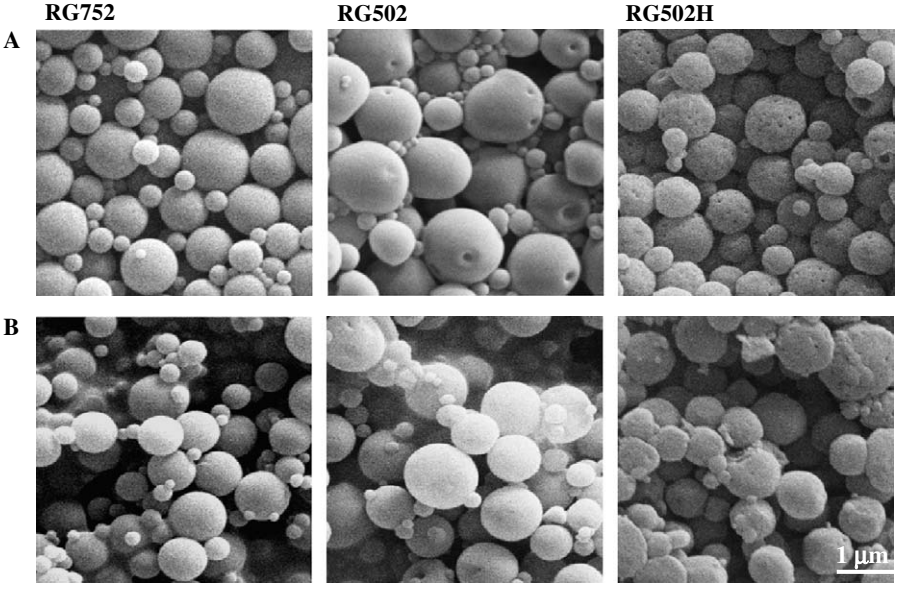

Fig. 1. Scanning electron micrographs of PLGA microparticles without plasmid (A) or encapsulated DNA (B), with RG752, RG502 and RG502H. Particles were prepared with protocol 1 (low homogenization rate).

SEM. All the microspheres are spherical, individualised and homogeneously distributed without evidence of collapsed particles. A significant difference is observed in the morphology of the particles prepared with the polymer RG502H, which were highly porous and rough, compared to particles prepared with the polymer RG752 or RG502, which are smooth and without porosity. The appearance of the microspheres prepared with or without DNA was indistinguishable. Particle size observed was similar to the measurements obtained by dynamic light scattering.

3.4. Structural and functional integrity

Plasmid DNA extracted from PLGA microparticles was analyzed for structural integrity by agarose gel electrophoresis by comparison with non-treated control DNA and with plasmid treated under the same conditions used to prepare the particles. The original pDNA was predominantly supercoiled although a small amount of open circular pDNA was visible. In Fig. 2 can be observed that naked DNA treated under the same conditions used to prepare the particles is completely degraded (lane 2). However, extracted DNA encapsulated in PLGA microspheres is protected independently of the protocol of formulation and the composition and molecular weight of the polymer used (lanes 3–12). Compared to untreated pDNA, there was a decrease in the proportion of supercoiled DNA, whereas the proportion of open circular DNA increased. It is important to note that always remains a visible amount of supercoiled, and that a small smear of degradation is also detected. The restriction endonuclease digestion patterns of extracted plasmid were exactly the same as that of the control plasmid DNA (data not shown).

The functional integrity of DNA extracted from the microspheres was also studied by a transfection assay using a human hepatocarcinoma cell line (HepG2), after complexation of DNA with the cationic liposomes DOTAP/CHOL. The results showed that plasmid DNA was able to transfect this cell line similar to the control plasmid (Fig. 3). The luciferase activity of the lysates of cells transfected with plasmid extracted from PLGA microparticles and unencapsulated control DNA was comparable (10.08 ng luciferase/mg protein and 10.35 ng luciferase/mg protein).

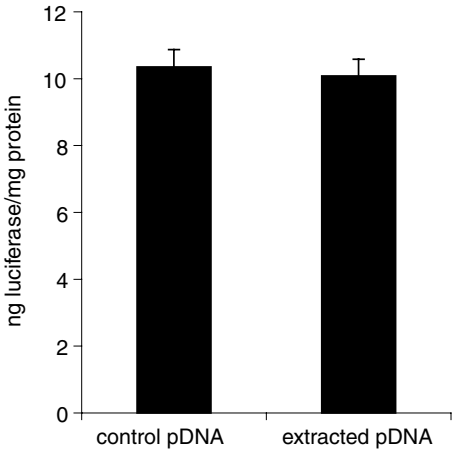

Fig. 3. Luciferase activity in HepG2 cells. The extracted DNA from microparticles RG503 prepared with protocol 1 was complexed with DOTAP/CHOL (1:0.9 molar ratio) liposomes at a lipid/DNA (+/−) charge ratio of 5/1. For transfection, 0.3 mL of DME-HG and 0.2 mL of complexes containing 1 μg of DNA were added to each well. The data represent means ± SD and are representative of three independent experiments.

3.5. Protection from DNAse I

To test whether PLGA microparticles can protect encapsulated plasmid DNA from nucleases digestion, the microspheres were exposed to DNAse I. Fig. 4 shows that naked plasmid DNA (lane 2) was completely digested within 5 min of incubation, while DNA encapsulated in PLGA microparticles by using different polymers (RG502,

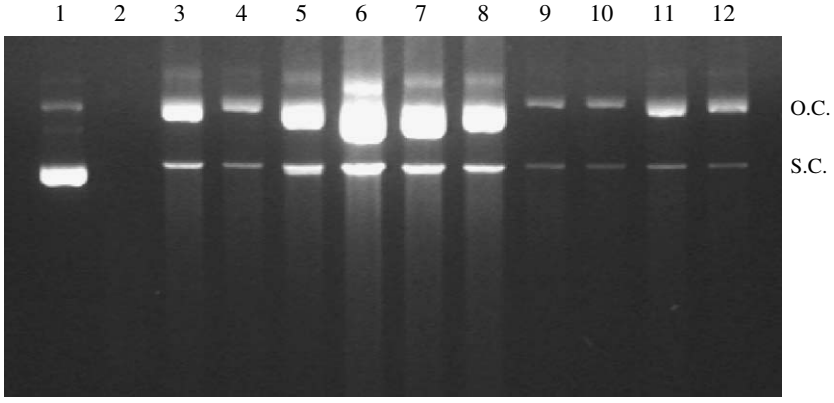

Fig. 2. Agarose gel electrophoresis of pDNA extracted from particles. Lane 1 corresponds to control non-treated pDNA, lane 2 to naked pDNA after subject to the preparation process, and lanes 3–12 correspond to pDNA encapsulated in PLGA particles 502-1, 502-2, 503-1, 503-2, 504-1, 504-2, 752-1, 752-2, 502H-1 and 502H-2, respectively. Samples of control and recovered DNA were applied to a 0.8% agarose gel in TBE buffer (100 mM Tris, 90 mM boric acid, 1 mM EDTA, pH 8.4).

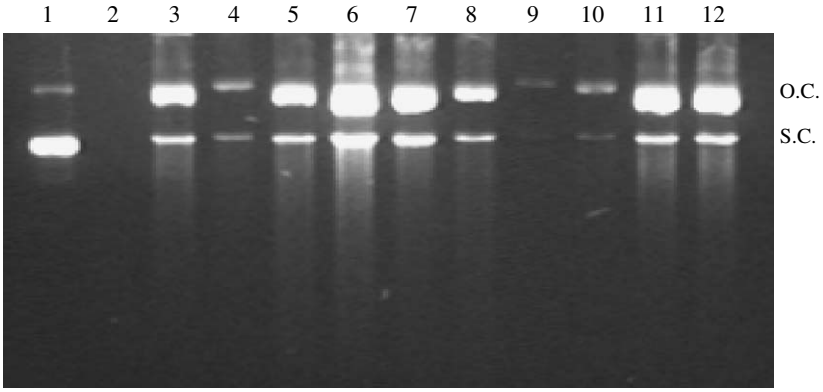

Fig. 4. Agarose gel electrophoresis of plasmid DNA extracted from particles after treatment with DNase I. Lane 1 corresponds to control non-treated pDNA, lane 2 to naked pDNA after incubation with DNase I (1 U/μg DNA) for 5 min, and lanes 3–12 correspond to pDNA encapsulated in PLGA particles 502-1, 502-2, 503-1, 503-2, 504-1, 504-2, 752-1, 752-2, 502H-1 and 502H-2, respectively, after incubation with DNase I for 4 h.

RG503, RG504, and RG502H) (lanes 3–8, 11 and 12) remained intact in the presence of DNAse I for up to 4 h of incubation, independently of the protocol of formulation and the composition and molecular mass of the polymer. In the case of RG752 microparticles (lanes 9 and 10), a very small amount of DNA is detected, due to the low EE (%) and to the surface adsorption of the plasmid in the particles, which leads to the degradation of the pDNA.

At the same time, Fig. 5 shows the integrity of the plasmid extracted from RG502H particles at different times of incubation with DNAse I. Plasmid presented the same configuration after 5, 15, 30, 60 min and 4 h of incubation as the DNA extracted from the particles in the absence of the enzyme (lanes 4–8 versus lane 3). It can be also observed that naked plasmid is degraded after 5 min (lane 2). It is interesting to note that plasmid DNA encapsulated in microparticles prepared with the polymer RG502H is protected after 4 h (lane 8), however, the simple mixture of DNA and empty microspheres did not protect it from degradation (lane 9), which shows the efficiency of the encapsulation process against the degradatory effect of the enzyme. As shown in Fig. 2, an appreciable amount of supercoiled DNA always remained although a decrease in the amount is observed, while the open circular form increases.

3.6. In vitro pDNA release from microparticles

The in vitro cumulative release profiles of plasmid DNA from PLGA microspheres, expressed as percentage of the encapsulated DNA versus time, are shown in Fig. 6. Formulations prepared with the polymers RG502, RG503, RG504 and RG502H released the DNA in a biphasic way, characterized by an initial and short release period (burst effect) followed by a longer period, in which the DNA is released in a sustained manner. The second phase release in RG502 particles starts earlier in time, compared to RG503 and RG504 particles, finishing at the same time after 28 days, and showing the effect of the molecular

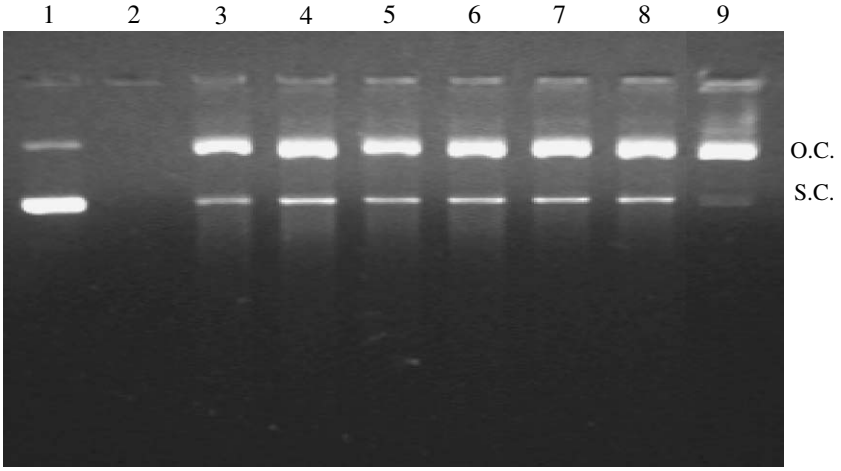

Fig. 5. Agarose gel electrophoresis of pDNA extracted from particles after treatment with DNase I (1 U/μg DNA). Lane 1 corresponds to control non-treated pDNA, lane 2 to naked pDNA after incubation with DNase I for 5 min, lane 3 to non-treated pDNA extracted from 502H-1 particles, lanes 4–8 to pDNA extracted from 502H-1 particles after incubation with DNase I for 5 min, 15 min, 30 min, 1 h and 4 h, respectively, and lane 9 corresponds to naked pDNA in the presence of empty 502H-1 particles after 4 h of incubation.

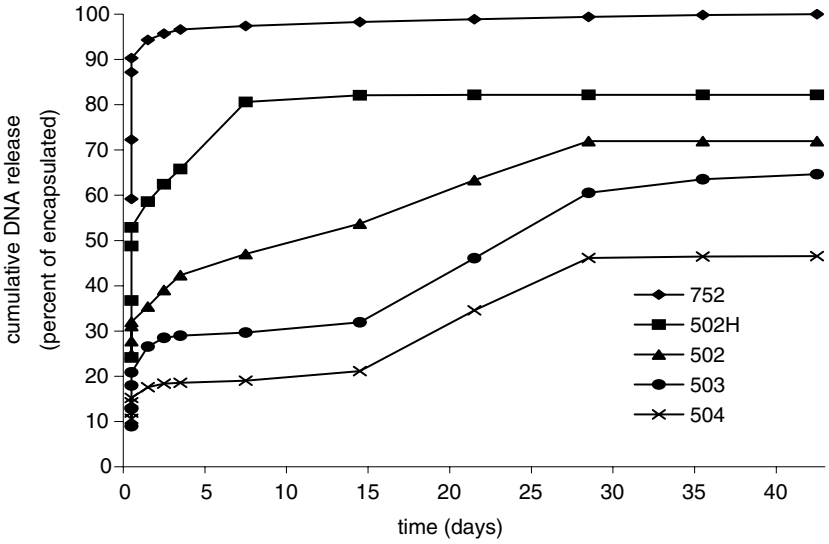

Fig. 6. In vitro release profiles of pDNA from the PLGA microparticles (prepared with protocol 1) differing in molecular mass, co-polymerization rate and presence of acidic terminations of the polymer. Experiments were carried out in TE buffer (Tris–HCl 10 mM, EDTA 1 mM, pH 7.4) at 37 °C. The concentration of the released DNA was evaluated by using the Picogreen fluorimetric assay.

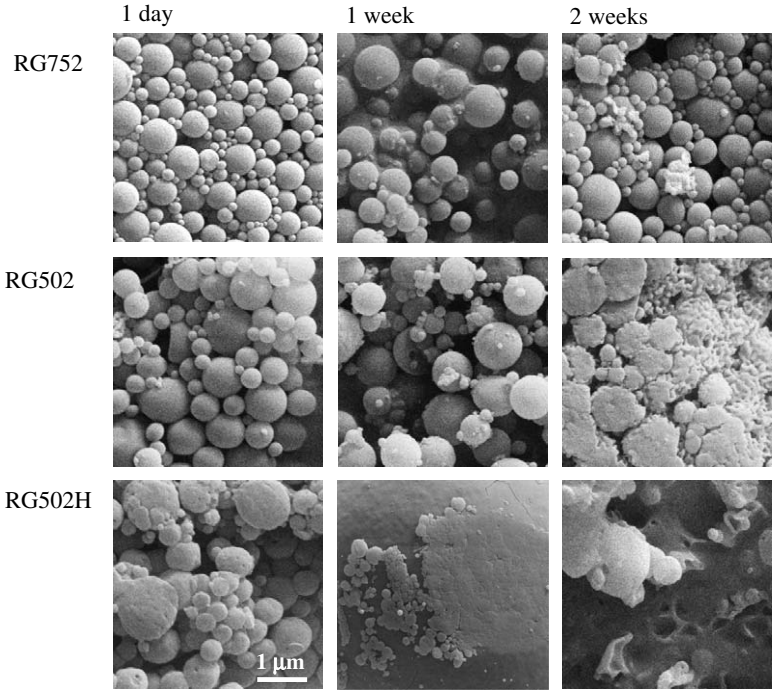

Fig. 7. Scanning electron micrographs of PLGA particles prepared with RG752-1, RG502-1 and RG502H-1, incubated in TE buffer for 1 day, 1 week and 2 weeks at 37 °C. Samples of microparticles were taken at different times from the "in vitro release assay" medium and analyzed.

weight of the polymer used. In RG502H microspheres this second phase release starts and finishes earlier compared to RG502 particles, which shows the influence of the presence of acidic groups of the polymer on the release. On the other hand, RG752 particles released the small quantity of DNA associated in one single phase. The low amount associated to this particles is released almost totally (90%) during the burst time in 8 h. However, the initial burst release values for particles prepared with the polymers RG502, RG503, RG504 and RG502H resulted to be, respectively, 32%, 21%, 15% and 53%, showing that by decreasing the molecular mass of the polymer the DNA released increased, and that microspheres prepared with the more hydrophilic polymer RG502H released also more plasmid than RG502 particles (53% of DNA versus 15%).

The final DNA release from the microparticles formulated using the polymers RG752, RG502, RG503, RG504 and RG502H, after 28 days, was 100%, 72%, 65%, 47% and 82%, respectively, showing that by increasing the molecular mass of the polymer slowed the rate of plasmid

DNA release, and that microspheres composed of hydrophilic polymers like RG502H released DNA faster.

3.7. In vitro degradation of microspheres by SEM

Fig. 7 displays representative SEM micrographs of RG752, RG502 and RG502H microspheres during various stages of in vitro release studies, showing that after one day all the particles showed an intact surface. After one week, PLGA752 and PLGA502 microparticles remained intact, which is related to the slow kinetic, while RG502H microspheres degraded very rapidly, which corresponds with the fast release rate. After two weeks, RG502 and RG502H particles were completely eroded, while RG752 microspheres still remained intact.

4. Discussion

In this study, we have investigated the feasibility of DNA encapsulation and release in PLGA microparticles, formulated with two protocols in the presence of different PLGA polymers (RG502, RG503, RG504, RG502H and RG752). Following preparation of the PLGA–DNA microspheres, by using a double emulsion technique, the particles were characterized in terms of size, zeta potential, encapsulation efficiency, morphology, DNA conformation, release kinetics, integrity and erosion.

As shown in Table 1, the diameter of the microspheres was dependent on the mixing speed of the homogenizer used to prepare the formulations, and was also affected by the molecular weight of the polymer used. Higher speed of homogenization, on protocol 2, leads to a smaller particle size in the formulations, probably because smaller drops in the second emulsion, while preparing the particles, are formed. On the other hand, by decreasing the molecular mass of the polymer a smaller size is also detected. The different co-monomer ratio lactic:glycolic (75:25 versus 50:50), as well as the presence of terminal acidic groups in the polymer used in the formulation, did not have influence in the particle size. On the other hand, neither the protocol of formulation nor the molecular weight and composition of the polymer showed effect on the surface charge of the microspheres, being all of them negatively charged, as indicated by the zeta potential values.

Our results showed a higher EE (%) in microparticles prepared with the protocol 1 compared to the ones prepared with the protocol 2. This can be related to the bigger size obtained for microspheres prepared by the first protocol, which avoids DNA escape to the external water phase during preparation. On the other hand, the molecular mass of the PLGA had a direct effect on the percentage of encapsulated DNA, in the way that by increasing the molecular weight of the polymer increased the encapsulation efficiency. An increase in the viscosity occurs when the molecular mass of the polymer increases, which influences the stability of the primary w/o emulsion and therefore the encapsulation. A lower diffusion of DNA from the particles during the formulation step is also obtained. Some authors have studied this effect [13,19,21]. The lower EE (%) observed for RG752 particles can be due to the hydrophobicity of the polymer. Similar effect was observed by Chen et al. [22] when comparing the EE (%) in RG502 and RG752 particles of approximately 60 μm. The use of RG502H polymer increased DNA EE (%) significantly compared to particles prepared with the polymer of similar molecular weight but without acidic terminal groups (RG502), since the EE (%) in microspheres 502H was approximately two times higher than in RG502 microparticles (Table 1). This effect is due to the higher hydrophilicity of the RG502H polymer.

Although fabrication conditions such as sonication, lyophilization and change of pH were reported to decrease the supercoiled content of DNA [5,11,20,23], in our case the integrity of plasmid before encapsulation and recovered from different batches of microparticles was demonstrated (Fig. 2). The DNA extracted from the microspheres showed supercoiled and some open circle form, independently of the protocol of formulation and molecular mass and composition of the polymer. These results suggest protective effects of PLGA on shearing of plasmid during high energy sonication, although a decrease in the proportion of supercoiled and an increase of the open circular form were observed. However, when plasmid DNA was sonicated under the double emulsion conditions without PLGA, the DNA was completely degraded. This is important, given that supercoiled plasmid is believed to be the most efficient form in terms of transfection efficiency in vitro and in vivo [24]. Also it should be taken into account that the energy used in this study in the encapsulation process was a compromise between that required to produce microparticles of a suitable size and that which damaged the DNA.

On the other hand, plasmid DNA extracted from PLGA microspheres was able to transfect HepG2 cells similar to the control plasmid, as evidenced by the luciferase activity of the cell lysate (Fig. 3), which demonstrated that the fabrication conditions used in our study, despite the decreased supercoiled content, did not significantly affect the in vitro bioactivity of the encapsulated DNA. Transfection efficiency of particles in suspension was also tested in HepG2 cells, getting very low values of gene expression, which is in accordance with the results described by Panyam et al. [15].

It is known that free pDNA is highly susceptible to DNAse I degradation as is shown in Fig. 4 (lane 2). However, the plasmid encapsulated into PLGA microspheres resulted to be protected from digestion by the enzyme after 4 h of incubation. The presence of a remaining supercoiled form of the plasmid shows the protective effect. The encapsulation process contributed to this fact, which can be deducted from the agarose gel, where it is shown that the simple mixture of DNA and RG502H empty microspheres did not protect the plasmid (lane 9 in Fig. 5). In the RG752 microparticles the degradatory effect of the enzyme is more visible, given that the small amount of loaded DNA in the particles is adsorbed on the surface, being more exposed to

the DNAse I. The in vitro release kinetics of these RG752 particles support also this hypothesis.

Microparticles prepared in the presence of the polymers RG502, RG503, RG504 and RG502H presented a typical biphasic profile, composed of an initial burst release, related to the drug adsorbed to the carrier surface, which causes immediate release of DNA and a second release phase related to the remainder DNA entrapped in the matrix-type particle [25], which is liberated due to the gradual degradation of the polymer [26,27] (Fig. 6). There is a delay in the second release of RG503 and RG504 versus RG502 particles. This can be due to the bigger size of these particles (3.2 and 5.7 μm versus 2.1 μm), which leads to a decrease in the surface area of the big particles compared to the small ones, giving a shorter contact with the release media. On the other hand, a monophasic profile is shown in RG752 particles, which showed a burst effect of about 90% release after 8 h, and points out that the encapsulated DNA was apparently accumulated in the external layers of the microspheres and is liberated immediately.

The percentage of plasmid DNA released is related to the molecular mass of PLGA used to prepare the microspheres (Fig. 6). The lower the molecular weight of the polymer, the more rapidly the polymer degrades [28] and consequently a faster DNA release is detected. Also the total release of DNA increased as the molecular mass decreased. The microparticles composed of hydrophilic polymers like RG502H released DNA faster during the second release phase, given that the hydration and degradation of the polymer occurs more rapidly. This is in accordance with the porosity detected in the RG502H particles by morphological studies (Fig. 1), and also with the erosion and degradation observed by SEM in these particles (Fig. 7).

The different erosion observed in RG502, RG752 and RG502 microspheres is attributed to the increased content of the hydrophilic monomer, glycolic acid, in RG502 versus RG752, and to the presence of acidic terminations in RG502H versus RG502. The polymer degradation was also tested by measuring the pH during the release process. The presence of the degradation products of PLGA (glycolic and lactic acid) leads to a decrease in the pH, especially in RG502H particles (from 5.5 to 3.5 after 3 weeks). In the case of RG752 microspheres no change in the pH was observed, due to the no degradation of the polymer after 6 weeks.

In summary, in this work we have studied the encapsulation of DNA in biodegradable PLGA microspheres without loss of its structural and functional integrity. Our results show that the protocol of formulation as well as the molecular weight and composition of the polymer used in the preparation of the particles are important determinants for the size, the encapsulation and the release behavior of these systems. These microspheres have several advantages, like that they can be used to deliver plasmid DNA in a fast way and also at a controlled rate, which is important because in some cases the use of rapid release formulations of microparticles, such as some of those described in this paper, is interesting in order to maximise the pDNA released in a biologically active form. Moreover, their use can be applied to different plasmids and there is no size limitation on the size of the encapsulated gene; the formulation of the microparticles is simple and final preparations are stable without signs of aggregation. Further experiments are currently in progress in order to improve gene delivery in vitro and in vivo.

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