TARGETED CATIONIC POLY(D,L-LACTIC-CO GLYCOLIC ACID) NANOPARTICLES FOR GENE DELIVERY TO CULTURED CELLS

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Abstract: We developed a new targeted cationic nanoparticulate system composed of poly(D,L-lactic-co-glycolic acid) (PLGA), 1,2-dioleoyl-3-(trimethylammonium) propane (DOTAP) and asialofetuin (AF), and found it to be a highly effective formulation for gene delivery to liver tumor cells. The nanoparticles (NP) were prepared by a modified solvent evaporation process that used two protocols in order to encapsulate (NP1 particles) or adsorb (NP2 particles) plasmid DNA. The final particles are in the nanoscale range. pDNA loaded in PLGA/DOTAP/AF particles with high loading efficiency showed a positive surface charge. Targeted asialofetuin-nanoparticles (AF-NP) carrying genes encoding for luciferase and interleukin-12 (IL-12) resulted in increased transfection efficiencies compared to free DNA and to plain (non-targeted) systems, even in the presence of 60% fetal bovine serum (FBS). The results of transfections performed on HeLa cells, defective in asialoglycoprotein receptors (ASGPr-), confirmed the receptor-mediated endocytosis mechanism. In summary, this is the first time that asialoglycoprotein receptor targeting by PLGA/DOTAP/DNA nanoparticles carrying the therapeutic gene IL-12 has been shown to be efficient in gene delivery to liver cancer cells in the presence of a very high concentration of serum, and this could be a potential system for in vivo application.

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Abbreviations used: AF – asialofetuin; ASGPr – asialoglycoprotein receptor; DMEM-HG – Dulbecco’s modified Eagle’s medium-high glucose; DOTAP – 1,2-dioleoyl-3-(trimethylammonium) propane; FBS – fetal bovine serum; IL-12 – interleukin-12; NP – nanoparticle; PLGA – poly(D,L-lactic-co-glycolic acid); SEM – scanning electron microscopy
Key words: Poly(D,L-lactic-co-glycolic acid) (PLGA), 1,2-dioleoyl-3-(trimethylammonium) propane (DOTAP), Asialofetuin, Targeted gene delivery, Pharmaceutical nanotechnology

INTRODUCTION

A primary objective of current gene therapy research is the development of suitable delivery vehicles for efficient and cell type-specific uptake of therapeutic genes. The two approaches for gene delivery are viral and non-viral systems. Although very efficient at transfection, there are still safety issues with viral gene delivery systems for human use. Furthermore, due to their inherent immunogenicity, they are problematic when repeated doses with the same carrier are required. Non-viral delivery systems are generally synthetic agents that are much safer, but their transfection efficiency in vivo is limited [1, 2]. Therefore, it is important to develop methods that would increase their transfection activity and efficiency. Poly(D,L-lactide-co-glycolide) (PLGA) polymers, which are biocompatible and biodegradable, have been approved by the FDA for certain human clinical uses; however, PLGA anionic particles do not efficiently transfect non-phagocytic cells. Thus, using cationically modified PLGA particles [3-5] that can bind and condense negatively charged plasmids is an interesting alternative. In recent years, significant effort has been put into developing nanotechnology for drug delivery, since it offers a suitable means of delivering small molecular weight drugs, as well as macromolecules such as proteins, peptides and genes, by either localized or targeted delivery to the tissue of interest [6]. The nanometer size range of these delivery systems has advantages for drug delivery. Thanks to their sub-cellular and sub-micron size, nanoparticles can penetrate deep into tissues through fine capillaries and cross the fenestrations present in the epithelial lining (e.g. in the liver), and are generally taken up efficiently by cells [7]. Nanoparticles have other advantages such as high stability and the ability to target specific tissues or organs by adsorption or coating with ligand materials at the surface. There are two types of nanoparticle system carrying DNA: DNA entrapment systems [8, 9]; and surface binding systems [10, 11]. DNA entrapment systems, which encapsulate the DNA, are more common. Surface binding systems utilize the ionic interaction between cationic molecules and the anionic DNA. It is known that free DNA is highly susceptible to DNase I degradation. The anionic compounds present in serum often complex with positively charged transfection reagents, resulting in decreased transfection efficiency, which represents a serious limitation for their use in vivo. PLGA was reported to protect pDNA from digestion by DNase I in vitro [12-14]. One of the possible reasons for the relatively low level of gene expression often obtained with non-viral vectors is the inefficient entry of the system into the cells. Targeted delivery of therapeutic agents to specific tissues was made
feasible via different developments, such as the discovery of specific receptors that are either overexpressed or expressed only in specific tissues. This strategy results in a higher bioavailability of the therapeutic agent at its site of action, and at the same time yields reduced side effects. Specific targeting to liver cells was achieved using ligands that bind the asialoglycoprotein receptor (ASGPr) [15-19], which is uniquely present on hepatocytes in large numbers with high affinity binding [20]. Asialofetuin (AF), a glycoprotein with triantennary galactose terminal sugar chains, is known as an excellent ligand molecule selectively recognized by ASGPr [21]. Based on these observations and our previous work, this study aimed to develop and evaluate targeted cationic PLGA nanoparticles to enhance gene delivery to liver cancer cells.

MATERIALS AND METHODS

Materials
Poly(D,L-lactic-co-glycolic acid) (PLGA) (50:50) and Resomer® RG502H (Mw 12 000 Da, with acidic termination) were purchased from Boehringer Ingelheim (Germany), and the lipid 1,2-dioleoyl-3-(trimethylammonium) propane (DOTAP) from Avanti Polar Lipids (Alabaster, AL, USA). The plasmid pCMV-Luc (VR-1216) encoding luciferase was obtained from Clontech (Palo Alto, CA, USA) and pCMV IL-12, encoding for interleukin 12, was provided by the laboratory of Dr. Cheng Qian (CIMA, University of Navarra). The plasmids were amplified in E. coli, isolated, and purified using a QIAGEN Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany). The purity was confirmed by 0.8% agarose gel electrophoresis followed by ethidium bromide staining, and the DNA concentration was measured by UV absorption at 260 nm. Polyvinyl alcohol (PVA, 87-89% hydrolyzed, Mw 13 000-23 000), Trizma® hydrochloride, asialofetuin (AF) type I, HEPES and D (+)-glucose were obtained from Sigma-Aldrich (Madrid, Spain). Alamar blue dye was purchased from Accumed International Companies (Westlake, OH, USA), and DNAseI and ethidium bromide from GibcoBRL Life Technologies (Barcelona, Spain).

Cell culture
HepG2 and HeLa cells (American Type Culture Collection, MD, USA) were maintained at 37°C under 5% CO2 in Dulbecco’s modified Eagle’s medium-high glucose (DMEM-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). The cells were passaged 1:3 by trypsinization twice a week.

Preparation of the cationic nanoparticles
The cationic nanoparticles were prepared using a modified solvent evaporation process. In the NP1 particles, the DNA was encapsulated into the cationic PLGA-DOTAP nanoparticles thusly. One hundred twenty microliters of 30 mM
Tris-HCl (pH 8.5) containing 200 μg of plasmid DNA was emulsified in 650 μl of chloroform containing 100 mg of PLGA and 15 mg of DOTAP 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 (PVA) and emulsified using an Ultra-Turrax (T 20 b, Ika Labortechnik, Germany) at 13 500 rpm 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 the chloroform had completely evaporated. The nanoparticles were collected by ultracentrifugation (40 000 g, 20 min) at room temperature, washed 3 times with distilled water, freeze-dried and stored at -20°C until use.

The DNA in the NP2 particles was adsorbed on the cationic surface of a mixture of anionic PLGA particles and cationic DOTAP liposomes. Anionic PLGA particles were prepared via a modified solvent evaporation process. One hundred twenty microliters of 30 mM Tris-HCl (pH 8.5) was emulsified in 500 μl of chloroform containing 100 mg of PLGA using the microtip probe sonicator set at level 20 for 10 s. The resulting primary emulsion was added to 2 ml of 9% (w/v) polyvinyl alcohol (PVA) and emulsified using the sonicator at level 20 for 10 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 the chloroform had completely evaporated. DOTAP liposomes were prepared by drying a chloroform solution of DOTAP (25 mg/ml) by rotary evaporation under reduced pressure. Then the lipid film was hydrated with 1 ml of 10 mM HEPES, 10% (w/v) glucose buffer, pH 7.4. The resulting multilamellar vesicles were extruded five times through polycarbonate membranes with 100 nm pore diameter using a Liposofast device (Avestin, Toronto, Canada) to obtain a uniform size distribution. The final NP2 particles were prepared by mixing DOTAP liposomes, anionic PLGA nanoparticles and pDNA in the proportion: 5 μl of liposomes: 0.5 mg of PLGA-nanoparticles: 1 μg of DNA. The charge ratio (+/-) DOTAP/PLGA is approximately 2-4.

The targeted nanoparticles were prepared by adding 1 μg of AF per μg of DNA (based on our previous studies). The ligand was added just before use of the particles, by adding it to the NP1 suspension or by adding the asialofetuin to the mixture of anionic PLGA-NP and DOTAP liposomes, before adding the DNA in the case of NP2. For the stability studies, NP were lyophilized and stored at -20°C for one year.

**Particle size and zeta potential measurements**

Nanoparticle size and zeta potential were determined by laser diffractionometry using a Zetasizer Nano Series (Malvern Instruments, UK) after resuspension of NP in 1 mM HEPES, 1% (w/v) glucose, pH 7.4. All the measurements were performed in triplicate for 60 seconds at 1000 Hz, and an electric current of 3 mA with zero field correction.
DNA loading of cationic particles

The amount of DNA loaded in the nanoparticles was estimated by a fluorimetric assay (PicoGreen® dsDNA Quantitation Kit, Molecular Probes, USA). The encapsulation efficiency of pDNA into NP1 was determined indirectly. 10 ml of a freshly prepared nanoparticle suspension was centrifuged at 40 000 g for 20 min. The amount of free pDNA in the supernatant was determined with Picogreen®. The percentage of DNA encapsulated was calculated by subtracting the amount of unencapsulated pDNA from the initial amount of pDNA present. The encapsulation efficiency was defined as the amount of DNA encapsulated in the nanoparticles relative to the initial amount of DNA used (encapsulated DNA x 100/initial DNA). Lyophilisation and storage did not affect the DNA loading efficiency.

The loading level of the DNA on NP2 was determined by diluting 3 mg of particles in 200 µl of TE buffer (Tris-HCl 10 mM, EDTA 1 mM, pH 7.5). The supernatant was collected by centrifugation (10 000 rpm, 10 min) and the non-adsorbed DNA was measured by Picogreen®. pDNA loading on the cationic nanoparticles was calculated by subtracting the pDNA content in the supernatant from the initial concentration of pDNA added. The determinations were done in triplicate.

Morphology studies by SEM

The shape and morphology of the nanoparticles were assessed by scanning electron microscopy (SEM). A monolayer of freeze-dried particles was mounted on an aluminium stub using double-sided carbon tape. The sample was coated with 8-nm molecular gold film using a sputter coater (Emitech K550 Equipment, UK), and the SEM photographs were taken with a Zeiss DSM 940A microscope.

DNase I protection assays

The protection of pDNA inside NP1 particles was demonstrated in our previous study [14]. To test whether PLGA nanoparticles can protect adsorbed plasmid DNA in NP2 from nuclease digestion, 15 mg of microspheres were suspended in 1 ml of TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) containing 1 unit of DNase I (Invitrogen Life Technologies, UK) per µg of DNA. The suspension was incubated for 30 min at 37°C in a water bath. After that, 5.7 µl of 0.5 M EDTA was added to stop the reaction. Afterwards, 10 µl of TBE 1X and 50 UI of heparin were added to the samples and incubated for 1 h at room temperature to separate the DNA from the particles. The nanoparticles were collected by centrifugation (14 000 rpm, 10 min) and the pDNA was analyzed by agarose gel electrophoresis, running the gel for 2 h at 80 mV, and visualised under UV illumination after ethidium bromide staining using a camera Gel (doc 2000, Bio-Rad, USA).
Cell culture transfection
For transfection, 3 x 10^5 HepG2 or HeLa cells were seeded in 1 ml of medium in 48-well culture plates (10 mm well diameter, Iwaki, Japan) 24 h before the addition of the complexes, and used at approximately 80% confluence. The cells were washed twice with DMEM without antibiotics, then 0.3 ml of fetal bovine serum (FBS) or DMEM and 0.2 ml of complexes containing 1 μg of DNA and 1 μg of AF were added gently to each well. After a 4-h incubation in 60% FBS or DMEM (at 37ºC in 5% CO₂), the medium was replaced, and the cells were further incubated for 48 h in medium containing 10% FBS.

In vitro transfection activity
After 48 h, the cells were washed with phosphate buffered saline (PBS) and lysed using 100 μl of reporter lysis buffer (Promega) at room temperature for 10 min, followed by two alternating freeze-thaw cycles. The cell lysate was centrifuged for 2 min at 12,000 g to pellet the debris. Twenty microliters of the supernatant was assayed for total luciferase activity using the luciferase assay reagent (Promega), according to the manufacturer’s protocol. A luminometer (Sirius-2, Berthold Detection Systems, Innogenetics, Diagnóstica y Terapéutica, S.A., Barcelona, Spain) was used to measure luciferase activity. The protein content of the lysates was measured by the DC Protein Assay reagent (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as the standard. The data was expressed as ng of luciferase (based on a standard curve for luciferase activity) per milligram of protein. In vitro IL-12 levels were determined with an ELISA kit for murine IL-12 p70 (BD OptEIA ELISA sets, Pharmingen, San Diego, CA) following the manufacturer’s instructions.

Cell viability assay
Cell viability was quantified in a modified Alamar Blue Assay. Briefly, 1 ml of 10% (v/v) Alamar Blue dye in DMEM-HG supplemented with 10% (v/v) FBS medium was added to each well 48 h after transfection. After 2.5 h of incubation at 37ºC, 200 μl of the supernatant was assayed by measuring the absorbance at 570 and 600 nm. Cell viability (as a percentage of control cells) was calculated according to the formula (A_570 - A_600) of treated cells x 100/(A_570 - A_600) of control cells.

RESULTS
Physicochemical characterization of cationic PLGA-DOTAP-pDNA formulations
Nanoparticles (NP1 and NP2) were prepared by a modified solvent evaporation process with two different protocols, as indicated in the Materials and Methods section. They were characterized in terms of particle size, zeta potential and DNA loading before and after lyophilization, and after one year of storage. The nanoparticle sizes ranged from 425 ± 20 to 671 ± 129 nm, demonstrating a unimodal size distribution (polydispersity index < 0.25). The particles prepared
via protocol 2 (NP2) were smaller than the particles prepared via protocol 1 (NP1), with the sizes respectively ranging in value from 425 to 463, and 580 to 671 nm (Tab. 1). There was little disparity in the size of the NP before and after lyophilization, and no aggregation was observed even after one year of storage. The formulations showed a surface positive charge, because of the inclusion of the cationic lipid DOTAP in the preparation process, in contrast to the surface charge of the PLGA nanoparticles without DOTAP, which was negative. The zeta potential of the NP2 particles was smaller than that of NP1 (14 ± 2 vs 37 ± 2 mV before lyophilization), as can be seen in Tab. 1. Lyophilization and storage did not affect the the zeta potential of the particles.

Tab. 1. The size and zeta potential of NP1 and NP2 particles before (A) and after lyophilization (B) and after one year of storage (C). The results are expressed as the mean ± S.D. of three experiments.

| Particles | Size [nm]   | Zeta potential [mV] |
|-----------|-------------|---------------------|
|           | A  | B   | C  | A  | B   | C  |
| NP1       | 620 ± 228 | 671 ± 129 | 580 ± 162 | 37 ± 2 | 47 ± 5 | 31 ± 3 |
| NP2       | 425 ± 20  | 472 ± 70  | 463 ± 49  | 14 ± 2 | 16 ± 7 | 14 ± 3 |

Fig. 1. Scanning electron micrograph of NP1 and NP2 particles (3000 x, 5 mm, 20 kV, left) and closer view (right).

The loading efficiencies of the PLGA-DOTAP nanoparticles were respectively 95 ± 5% and 99 ± 4% for NP1 and NP2. They showed spherical although slightly irregular shapes, as can be seen in the SEM-micrographs (Fig. 1). They
were individualized, smooth, and homogeneously distributed without evidence of collapsed particles. The size measured by photon correlation spectroscopy was confirmed by scanning electron microscopy.

**Protection from DNase I**

The extent of protection of the plasmid DNA from enzymatic degradation by DNase I was investigated via agarose gel electrophoresis (Fig. 2). It can be seen that naked DNA was degraded within the first 5 min of incubation with the enzyme, as confirmed by the disappearance of the DNA bands in the gel. By contrast, the PLGA-DOTAP nanoparticles protected the plasmid against degradation over a 30-min time period. We demonstrated that pDNA is protected inside NP1 particles in our previous study [14].

![Fig. 2. Agarose gel electrophoresis of plasmid DNA. Lane 1 corresponds to the control, non-treated pDNA; lane 2 to naked pDNA after incubation with DNase I for 5 min; and lane 3 to NP1 or NP2 particles after incubation with DNase I for 30 min. Only relevant fragment is shown.](image)

**In vitro transfection by non-targeted nanoparticles**

The gene expression levels in HepG2 cells in the absence or presence of serum following transfection with non-targeted nanoparticles are given in Fig. 3. The cells were transfected with the pCMV-luciferase plasmid either alone, or encapsulated in (NP1) or adsorbed (NP2) on PLGA-DOTAP nanoparticles. The presence of serum resulted in increased transfection efficiencies in both kinds of nanoparticle. 1.5- and 8-fold increases were respectively observed for NP1 and NP2 particles when serum was present. When the plasmid was encapsulated in PLGA-DOTAP nanoparticles (NP1), the levels of transfection were higher than in the case of the NP2 particles, on which the plasmid is only adsorbed. These increases were respectively 15 and 3 times in the absence or presence of serum. The highest level of transfection was obtained with NP1 particles in the presence of serum. In the case of naked DNA or PLGA nanoparticles without DOTAP, luciferase activity was practically absent. The cell viability following transfection was assessed to evaluate whether NP were toxic to HepG2 and HeLa cells in the absence or presence of serum. A viability of at least 80% was observed in all the transfected wells (Fig. 4A and B). The relative cytotoxicity was also assessed by the total amount of extractable cellular proteins in the cell lysate per well, and the results were confirmed using the Alamar Blue assay.
Fig. 3. The effect of serum on gene expression in HepG2 cells transfected with the cationic nanoparticles NP1 and NP2. The data is the mean ± S.D. of measurements from three wells, and is representative of three independent experiments.

Fig. 4. The viability of HepG2 (A) and HeLa cells (B) after transfection with NP1 and NP2 particles in the absence or presence of serum. The data is the mean ± S.D. of the measurements for three wells, and is representative of three independent experiments.

Targeted asialofetuin-PLGA-DOTAP nanoparticles
In order to prepare nanoparticles targeted to HepG2 cells, the ligand asialofetuin (AF) was included in the formulations. NP1 and NP2 nanoparticles containing AF had respective particle sizes of 594 ± 51 and 604 ± 51 nm, and respective positive surface charges of 38 ± 8 and 20 ± 9 mV. Thus the NP1 particle size did not change, but there was an approximately 200-nm change detected for NP2 formulations. The zeta potential did not change significantly (p > 0.05) when the ligand was added.

In order to investigate the effect of AF on the transfection activity in HepG2 cells, plain and AF-nanoparticles were evaluated in the presence of a reporter (pCMV-Luc) and a therapeutic gene (pCMVIL-12). As shown in Fig. 5A and B, nanoparticles containing the ligand always had higher values of expression than plain (non-targeted) ones. In the case of NP1, the presence of asialofetuin
increased the luciferase and IL-12 expression 1.4- and 2-fold, respectively. For NP2 particles, the increases were respectively 1.7- and 2-fold. In these targeted systems, NP1 formulations also showed higher values of gene expression than NP2 particles with both plasmids. The presence of the ligand did not change the viability of the transfected cells, which was also 80%.

Fig. 5. Gene expression in HepG2 cells due to plain and AF-NP1 and AF-NP2 particles transfected in the presence of 60% FBS and containing pCMVLuc (A) or pCMV-IL12 (B) plasmids. The data is the mean ± S.D. of measurements from three wells and is representative of three independent experiments.

Fig. 6. Asialoglycoprotein receptor specificity in gene delivery. The transfection activity of plain and AF-NP1 in HepG2 and HeLa cells in the presence of 60% FBS. The data is the mean ± S.D. of measurements from three wells and is representative of three independent experiments.

To make sure that the intracellular delivery of AF-NP occurred in an AF receptor-mediated specific manner, comparative cellular uptakes were estimated using two different cell lines, HepG2 (ASGPr+) and HeLa (ASGPr-). Fig. 6 shows an increase in gene expression only in HepG2 cells, while the AF-NP transfection of HeLa cells did not yield increased transfection efficiency. These results suggest that targeted intracellular delivery of AF-NP took place via an AF-receptor-mediated endocytosis mechanism.
DISCUSSION

Much attention has been focused on biocompatible, biodegradable polymers such as PLGA for the encapsulation of proteins and peptides [22] and for genes [23-26]. However, the levels of gene expression obtained using this polymer are very low, especially in non-phagocytic cells [27-29]. It should also be considered that, although PLGA controlled release polymeric systems offer the advantage of sustained pDNA activity, in some cases, a rapid release of DNA is desirable. For example, it is known that the ability of DNA to induce an immune response in vivo will decrease as a function of the time of release. For this reason, in this study, we used a PLGA of low molecular weight (Mw 12,000 Da) and with terminal acidic groups (RG502H), which is known as a hydrophilic polymer, and which presents a faster DNA release compared to other polymers.

Usually, cationic molecules are required to maximize the binding with pDNA in terms of efficient gene delivery. Therefore, the preparation of cationic nanoparticles under optimum conditions is important in terms of the physicochemical properties of these systems. The cationic lipid, 1,2-dioleoyl-3-trimethyl ammonium-propane (DOTAP) was included in our formulation because of its stable association and ability to complex with plasmid DNA. The effect of its addition to the nanoparticles was evaluated. Other authors have formulated PLGA particles with different cationic agents, such as polyethylenimine (PEI), polylysine (PLL), cetyltrimethylammonium bromide (CTAB), and dimethyldioctadecylammonium bromide (DDAB) [3-5, 30, 31], but the transfections were performed in the absence or presence of very low concentrations of serum, and low gene expression levels were obtained.

We studied two processes for the formation of cationic nanoparticles via different modified double emulsion methods, which lead to particles with the DNA encapsulated (NP1) or adsorbed (NP2). Following the preparation of the cationic nanoparticles, they were characterized in terms of size, zeta potential, loading efficiency and morphology, in order to be afterwards evaluated for in vitro transfection. All the pDNA-loaded PLGA-DOTAP particles were found to be in the nanorange (Tab. 1). The smaller size of NP2 compared to NP1 is explained by the different protocol used to prepare NP2, in which a higher agitation (sonication) is used to prepare the emulsions. The double sonication employed in NP2 formation gave a smaller particle size, probably because smaller drops are formed. Also, the stable particle size obtained suggests that the preparation technique is reproducible and results in the formulation of uniform particles. This is an important factor in relation to the prolonged storage of a dispersion system.

It is important to note that the zeta potential is an important particle characteristic as it can also influence particle stability. More pronounced zeta potential values, being positive or negative, tend to stabilize the particle suspension. The electrostatic repulsion between particles with the same electric charge prevents aggregation. In NP1 and NP2, the positive surface charge of all
the particles is the result of the orientation of DOTAP on the surface, since PLGA nanoparticles have a negative surface charge. This indicates that DOTAP is stably associated with the surface of the nanoparticles. In NP1, almost all the pDNA was entrapped in the nanoparticle; in NP2, the zeta potential following adsorption of DNA showed a reduction in positive charge due to the adsorption of the negatively charged DNA (Tab. 1). On the other hand, lyophilization and storage did not significantly affect (p > 0.05) the size and zeta potential. The nanoparticles showed respective DNA association efficiencies of 95 and 99%. This high and reproducible loading efficiency correlates with efficient surface adsorption that prevents material loss and suggests that there is an excess positive charge from the DOTAP over the phosphate groups of the DNA. Related to the morphology of the particles, the presence of the cationic lipid means that the surface of the particles, although spherical, is more irregular (Fig. 1). Although the adsorption of DNA could be also a reason, the morphology of the NP1 and NP2 particles did not confirm this hypothesis. It is also possible that some isolated liposomes are present. 

As shown in Fig. 3, cationic nanoparticles with DNA encapsulated (NP1) or adsorbed to their surface (NP2) are able to efficiently transfect HepG2 cells in vitro in the presence of 60% FBS; however, no gene expression was detected with naked plasmid DNA. The positively charged particle surface can facilitate adherence to negatively charged cellular membranes, inducing and increasing intracellular uptake. The presence of DOTAP may contribute to endosomal disruption and increased cytoplasmic or nuclear localization. It should also be considered that the protection of plasmid DNA from nucleases is one of the crucial factors for efficient gene delivery in vivo as well as in vitro [32, 33]. In our particles, the protection of adsorbed pDNA in NP2 from DNase I can be due to the fact that the plasmid on the surface of the particles is complexed with DOTAP, and not exposed to the enzyme. This protective effect can also contribute to the efficient transfection. NP1 particles showed higher levels of transfection than NP2. This could possibly be explained by a different arrangement of the cationic molecule in the PLGA polymer matrix. In NP2 particles, the presence of adsorbed lipid can hinder the interaction with cells leading to less efficient complexes. Moreover, it can be related to the bigger size in NP1 particles, which sediment more rapidly and are in contact with the surface for a longer time. Also, the different structures influence the binding process and consequently the internalization of the particles.

The inhibition of gene transfer by serum is considered to be one of the limitations to their in vivo applications. Because of that, one important element in emulating in vivo conditions is the use of high concentrations of serum for transfection experiments in vitro. Most of the studies in the literature have been carried out in the presence of a low concentration of serum, a condition that is far from that found in vivo. It is interesting to note that in our case, the presence of serum increased the transfection levels. The reason for this increase is not clear at present. Some authors state that in the presence of serum, it is possible
that the particles form small aggregates with serum proteins which could sink to the bottom of culture plates and enhance transfection. However, this is not the explanation here, given that in our nanoparticles, no aggregation or increased sized was observed in the presence of serum. Other authors stated that the bigger particle size in the presence of serum decreased transfection [34, 35]. The lower zeta potential due to the binding to anionic serum proteins can also contribute to this enhancement [36-38], but here, the charge was positive enough that the particles were not converted to negatively charged particles in the presence of serum, and the systems became serum-resistant.

Another fact to be taken into account is that the transfection efficiency of a polymeric carrier is profoundly influenced by the cytotoxicity of the cationic polymers to be used [39], due to electrostatic interactions with the negatively charged glycocalix of the cellular surface [40]. As shown in Fig. 4A and B, PLGA/DOTAP nanoparticles did not affect the viability of HepG2 or HeLa cells. The differences in transfection activity between the formulations cannot be explained by the different toxicities, since cell viability was similar in all cases.

Finally, and in order to improve transfection efficiency, targeted cationic nanoparticles were prepared in the presence of the ligand asialofetuin. The systems always mediate higher gene expression compared to plain (non-targeted) particles in the presence of serum (Fig. 5). AF-particles can interact with the cell surface receptors. The association of asialofetuin facilitates the internalization of the complex due to the ability of the ligand to stimulate endocytosis [41]. Although the enhancement of transfection activity with AF is modest, it should be enough to show a higher therapeutic effect when the gene for a cytokine like IL-12 is delivered. As is shown in Fig. 6, the transfection activity of targeted systems in HeLa Cells (defective in ASGPr) decreased compared to that obtained in HepG2 cells. It is possible that the presence of the ligand inhibits the interaction with ASGPr-defective cells, and consequently the internalization of the complex is decreased. The same behaviour was also observed by Arangoa et al. [15]. This observation supports the role of ASGPr receptors in the mechanism of transfection by AF-nanoparticles.

In summary, the novel PLGA/DOTAP/AF nanoparticles developed in this study have improved transfection activity in liver cancer cells in the presence of 60% serum. Although various methods have been used for the preparation of PLGA-ligand-DNA complexes, covalent binding is usually needed, and sometimes insoluble aggregates are formed unless the concentration of the polycation or DNA is minimized. However, the NP1 and NP2 particles in this study were prepared by simple mixing of the ligand, without having problems of aggregation. It can be also concluded that the encapsulation of DNA in NP1 particles seems to be a more efficient method for the preparation of these particles. Although previous studies have shown that the association of AF with different systems enhances transfection, this is the first demonstration of the efficacy of the combination of the PLGA/DOTAP/AF/DNA system encoding a very potent antitumor cytokine (IL-12). Based on these results, these vectors
could have significant potential for applications in nucleic acid-based immunotherapy. Actually, the formulations are being evaluated for their in vivo ability to serve as DNA carriers for the purpose of cancer therapy. In preliminary in vivo studies, it is observed that the presence of the ligand AF increases transfection efficiency in the liver. A 20-fold increase is detected in the levels of gene expression in the lung compared to the liver.

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