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Research paper

Serum-resistant lipopolyplexes for gene delivery to liver tumour cells

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Abstract

In this study, an efficient non-viral gene transfer system has been developed by employing polyethylenimine (PEI 800, 25 and 22 kDa) and DOTAP and cholesterol (Chol) as lipids (lipopolyplex), at three different lipid/DNA molar ratios (2/1, 5/1 and 17/1) by using five different protocols of formulation. Condensation assays revealed that PEI of 800, 25 and 22 kDa were very effective in condensing plasmid DNA, leading to a complete condensation at N/P ratios above 4. Addition of DOTAP/Chol liposomes did not further condense DNA. Increasing the molar ratio lipid/DNA in the complex resulted in higher positive values of the ζ-potential, while the particle size increased in some protocols, but not in others. High molecular weight PEI (800 kDa) used in the formulation of lipopolyplexes lead to a bigger particle size, compared to that obtained with smaller PEI species, whether branched (25 kDa) or linear (22 kDa). These vectors were also highly effective in protecting DNA from attack by DNAse I. Transfection activity was maximal by using protocols 3 and 4 and a lipid/DNA molar ratio of 17/1. These complexes showed high efficiency in gene delivery of DNA to liver cancer cells, even in the presence of high concentration of serum (60% FBS). On the other hand, complexes formed with linear PEI (22 kDa) were more effective than lipopolyplexes containing branched PEI (800 or 25 kDa). The complexes resulted to be much more efficient than conventional lipoplexes (cationic lipid and DNA) and polyplexes (cationic polymer and DNA). The same behaviour was observed for complexes prepared in the presence of the therapeutic gene pCMVIL-12. Toxicity assays revealed a viability higher than 80% in all cases, independently of the protocol, molar ratio (lipid/DNA), molecular weight and type of PEI.

Keywords: Gene delivery; Cationic liposomes; Polyethylenimine; Lipopolyplexes; Non-viral vectors; Cancer gene therapy

1. Introduction

Gene therapy focuses on the therapeutic use of genes delivered to cells and promises considerable advances in the treatment of several important diseases. Although remarkable progress has been made in identifying target structures for cancer gene therapy, actual therapy is mainly hampered by the lack of a safe and efficient delivery system. Thus, one of the primary objectives of gene therapy is the development of efficient, non-toxic gene carriers that can effectively deliver foreign genetic material into specific cell types, including cancerous cells.

The two main gene carrier systems that have been utilized in gene therapy are viral vectors and non-viral delivery systems. Viral vectors, including retroviruses, adenoviruses and adeno-associated viruses, have a high efficiency of gene delivery. Due to serious safety risks with viral vectors that have become apparent in the last few years, however, their utility is being re-appraised. Furthermore, the addition of targeting ligands on the surface of viral vectors to transfect specific cell types is problematic. Because of these concerns, non-viral vectors are emerging as a viable alternative. Non-viral systems show significantly lower safety risks, they are capable of carrying large molecules and can be produced in large quantities easily and inexpensively. The major disadvantage of these

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non-viral-vectors is their low transfection efficiency, especially in the presence of serum in vivo.

Different strategies including particle bombardment [1], ultrasound transfection [2] or the application of naked DNA [3] have been used as gene delivery systems. Their applicability, however, is restricted to specific circumstances. Cationic polymers and lipids are by far the most widely used vectors in non-viral gene and oligonucleotide delivery. Some liposome formulations are not satisfactory due to their low transfection efficiency and cytotoxicity [4,5]. Polycationic polymers are able to compact DNA, which is an advantage in gene transfer [6,7]). Polyethylenimine (PEI) has been used successfully for in vitro and in vivo gene delivery [8,9]. It plays several roles in the process of transfection, such as condensing and protecting DNA, binding to the cell surface, triggering endocytosis and releasing DNA/lipid complexes from endosomes due to the ''proton-sponge'' effect [10]. It has the ability to enter the nucleus [11] and to accelerate gene entry into the nucleus from the cytosol [12]. Although cationic liposomes can deliver DNA into the cytosol through the endosomal pathway, the entry of the DNA into the nucleus is very inefficient [13,14]. Lipopolyplexes (i.e. a ternary complex of cationic liposomes, cationic polymer and DNA) represent a second generation of non-viral gene delivery vectors that can improve gene transfer compared to the first generation cationic liposome–DNA complexes (lipoplexes) [6,15–20].

Because of our interest in gene therapy of hepatocellular carcinoma, we have examined the ability of various lipopolyplexes to deliver genes into liver cancer cells. We evaluated different parameters such as the protocol of preparation, the lipid/DNA molar ratio, and the molecular weight and type of PEI, to optimize the formulation to achieve high transfection activity. Our hypothesis was that the association of PEI with cationic liposomes (lipopolyplexes) would increase luciferase expression compared to lipoplexes (cationic lipid and DNA) and polyplexes (cationic polymer and DNA) alone.

2. Materials and methods

2.1. Materials

The cationic lipid 1,2-dioleoyl-3-(trimethylammonium) propane (DOTAP) and cholesterol (Chol) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Polyethylenimine 800 (MW 800 kDa, branched) was purchased from Fluka (Steinheim, Germany), polyethylenimine 25 (MW 25 kDa, branched) from Aldrich (Madrid, Spain) and linear PEI (MW 22 kDa, ExGen® 500) from Quimigranel (Madrid, Spain). The plasmids, pCMVLuc (VR-1216) (6934 bp) (Clontech, Palo Alto, CA, USA) and pCMV100-IL-12 (5500 bps) (kindly provided by Dr. Chen Qian, University of Navarra) encoding luciferase and interleukin-12 (IL-12), respectively, were used for carrying out the transfection experiments. The following materials were used for DNAse I protection assays: agarose D-1 (Pronadisa, Madrid, Spain), Tris-boric acid–EDTA Buffer (10× TBE Buffer) (Invitrogen, Barcelona, Spain), DNAse I and ethidium bromide (Gibco BRL, Barcelona, Spain). To release DNA from the complexes sodium dodecyl sulphate (SDS) and NaCl were used (Roig Farma, Barcelona, Spain); heparin sodium salt and ethylenediaminetetraacetic acid (EDTA) were supplied by Sigma (Madrid, Spain). Alamar blue dye was purchased from Accumed International Companies (Westlake, OH, USA).

2.2. Cell culture

HepG2 human hepatoblastoma cells (American Type Culture Collection, Rockville, MD, USA) were maintained at 37 °C under 5% CO_2 in Dulbecco's modified Eagle's medium-high glucose (DMEM), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 μg/ml) and L-glutamine (4 mM) (Gibco BRL Life Technologies). Cells were passaged 1:10 by trypsinization once a week.

2.3. Preparation of lipopolyplexes

Lipopolyplexes were prepared with plasmid DNA and B-PEI (branched, 800 or 25 kDa) or L-PEI (linear, 22 kDa) at a N/P ratio of 4. The N/P ratio of the nitrogen atoms of PEI to DNA phosphates describes the amount of polymer used for polyplex formation. Different amounts of lipids were added in order to prepare complexes at molar ratios of total lipid/DNA of 2/1, 5/1 and 17/1. The final DNA concentration in the lipopolyplexes was 25 μg/ml in 3 ml of total volume.

Lipopolyplexes were formulated by five different strategies: *Strategy 1* was to dry a chloroform solution of the lipids DOTAP/Chol (1:0.9 molar ratio) by rotary evaporation and then to hydrate the film with the polyplexes (PEI/DNA) at a N/P ratio of 4. *Strategies 2 and 3* were performed by drying a chloroform solution of the lipids (DOTAP/Chol) and PEI by rotary evaporation. In strategy 2 the film was hydrated with water, followed by the addition of the plasmid. In strategy 3, the mixture of lipids and PEI were directly hydrated with the plasmid. *Strategies 4 and 5* were to formulate the polyplexes PEI/DNA at a N/P ratio of 4 and, after a 15-min incubation, to add different amounts of preformed cationic liposomes, to obtain complexes at different lipid/DNA molar ratios (2/1, 5/1 and 17/1). Both strategies are similar, differing only in the order of addition of plasmid to obtain the polyplexes: PEI added to DNA (strategy 4) or DNA added to PEI (strategy 5). All the complexes were extruded through polycarbonate membranes with 100 nm pore diameter using a Liposofast device (Avestin, Toronto, ON, Canada) to obtain a uniform size distribution.

2.4. DNA/PEI condensation assays

The binding of PEI to DNA was examined using a quenching method based on ethidium bromide, whose

fluorescence is dramatically enhanced upon binding to DNA and quenched when displaced by higher affinity compounds or by condensation of the DNA structure [21]. The assays were carried out in 96-well plates in 10 mM Hepes, 10% (w/v) glucose buffer (pH 7.4). DNA (0.6 μg) was mixed with 1.2 μg of ethidium bromide, then increasing amounts of PEI were added to the wells and incubated for 10 min in the darkness. Fluorescence was read in an LS 50 spectrofluorimeter (Perkin-Elmer, Mountain View, CA, USA), at excitation and emission wavelengths of 520 and 600 nm, respectively. The relative fluorescence values were determined as follows: $F_r = (F_{obs} - F_e) \times 100/(F_0 - F_e)$, where F_r is the relative fluorescence, F_{obs} is the measured fluorescence, F_e is the fluorescence of ethidium bromide in the absence of DNA under the given buffer conditions, and F_0 is the initial fluorescence in the absence of the polycation.

2.5. Particle size and ζ-potential measurements

The particle size of complexes was measured by dynamic light scattering, and the overall charge by ζ-potential measurements, using a particle analyzer (Zeta Nano Series, Malvern Instruments, Spain). Samples of the prepared complexes were diluted in distilled water and were measured at least three times immediately after preparation of complexes.

2.6. DNAse I protection assays

Lipopolyplexes were prepared at lipid/DNA molar ratios of 5/1 and 17/1 at a final pDNA concentration of 25 μg/ml in 100 μl of total volume and incubated for 30 min at room temperature. DNAse I (1 U/μg of DNA) was added to each sample and the mixtures were incubated at 37 °C for 30 min. Ten microliters of an EDTA solution (0.25 M) was added to inactivate the DNAse. Lipopolyplexes were disassembled by adding 60 μl of SDS (20%) and 40 μl of 4 N NaCl to the samples, which were then incubated for 10 min at room temperature. After centrifugation at 12,000*g* for 1 min at 4 °C, complexes were disassembled by the addition of heparin (0.9% w/v) and analyzed by agarose gel electrophoresis. The integrity of the plasmid in each formulation was compared with untreated DNA as a control.

2.7. In vitro transfection activity

For transfection, cells were seeded in medium in 48-well culture plates (Iwaki Microplate, Japan), and incubated for 24 h at 37 °C in 5% CO_2. After this, the medium was removed and 0.2 ml of the complexes (containing 1 μg of plasmid) and 0.3 ml of fetal bovine serum were added to each well. After a 4-h incubation in 60% FBS, the complex was replaced and the cells were further incubated for 48 h in medium containing 10% FBS. Cells were washed with phosphate-buffered saline (PBS) and lysed using 100 μl of Reporter Lysis Buffer (Promega, Madison, WI, USA) at room temperature for 10 min, followed by two freeze-thaw cycles. The cell lysate was centrifuged for 2 min at 12,000*g* to pellet 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, 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 were expressed as nanograms of luciferase (based on a standard curve for luciferase activity) per milligram of protein. In vitro IL-12 levels were determined by an ELISA kit for murine IL-12p70 (BD OptEIA ELISA sets, Pharmingen, San Diego, CA, USA) following the manufacturer's instructions.

2.8. Cell viability

Cell viability was quantified by a modified Alamar blue assay. Briefly, 2 ml of 10% (v/v) Alamar blue dye in DME-HG (Dulbecco's modified Eagle's medium-high glucose) supplemented with 10% (v/v) FBS medium was added to each well 48 h post-transfection. After 2.5 h of incubation at 37 °C, 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 $\times 100/(A_{570} - A_{600})$ of control cells.

3. Results

3.1. Characterization of lipopolyplexes

To characterize and evaluate the influence of the protocol of preparation, the molecular weight and type of PEI, and the lipid/DNA molar ratio, different lipopolyplexes were prepared with five different strategies by using PEI 800, 25 and 22 kDa, and DOTAP and cholesterol as lipids, at three different lipid/DNA molar ratios 2/1, 5/1 and 17/1, as described in Section 2.

First, in order to prepare complexes in which DNA was completely condensed by PEI, the corresponding condensation studies were performed, by measuring the decrease in the ethidium bromide fluorescence upon its expulsion from DNA. Different N/P ratios of PEI/DNA and different amounts of lipid were analyzed. As shown in Fig. 1, the fluorescence decreased with increasing N/P ratio. At N/P ratios of 4–10 the fluorescence reached a plateau, with the DNA being condensed to less than 10% of the uncomplexed DNA, suggesting that DNA is completely condensed at these ratios. The same behaviour was observed with all 3 polymers of PEI 800, 25 and 22 kDa. Increasing amounts of lipid added to polyplexes at an N/P ratio of 4 did not show any additional effect on the condensation of the plasmid.

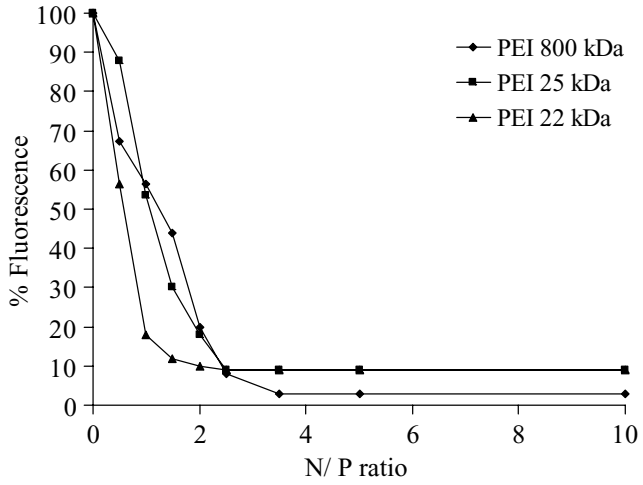

Fig. 1. PEI/DNA condensation assay. DNA condensation measured as a decrease in fluorescence of EtBr added to polyplexes.

Complexes were characterized in terms of particle size and ζ-potential. At a lipid/DNA ratio of 2/1, the largest lipopolyplexes were those prepared using Strategy 2 (281 nm), while at the higher ratios, Strategy 3 produced the largest lipopolyplexes (356 and 295 nm for 5/1 and 17/1 ratios, respectively) (Table 1). The smallest particle size (124 nm) was obtained using Strategy 5 at the 2/1 lipid/DNA ratio. Most of the other complexes had an average particle size of about 200 nm. Regarding the ζ-potential measurements, it is interesting to note that at the 2/1 lipid/DNA ratio, the smallest lipopolyplexes (prepared by Strategy 5) also had the highest net positive charge (ζ-potential = 68 mV), while the largest ones (Strategy 2) had the lowest ζ-potential (34 mV) (Table 2). Nevertheless, all the preparation strategies resulted in net positively charged complexes. For each strategy, increasing the lipid/DNA ratio appeared to increase the ζ-potential (Table 2).

In order to study the influence of the molecular weight and type of PEI on the particle size and ζ-potential of lipopolyplexes, complexes were prepared by protocol 4 at a lipid/DNA molar ratio of 17/1. Table 3 shows that complexes prepared with PEI 800 of high molecular weight were bigger in particle size compared to lipopolyplexes containing PEI 25 or PEI 22. No differences in the particle size were observed between lipopolyplexes prepared with branched (25 kDa) or linear (22 kDa) PEI of similar molecular weight. Regarding the ζ-potential measurements, however, complexes prepared with the branched PEIs had a slightly higher ζ-potential compared to the linear PEI.

3.2. Protection of DNA inside lipopolyplexes against DNAse

To assess the role of our complexes in protecting the DNA from attack by degrading enzymes in vivo, an in vitro DNAse I protection assay was performed by agarose gel electrophoresis of the samples. Fig. 2 shows that naked DNA was degraded quickly (lane 2), while the plasmid inside the lipopolyplexes prepared at lipid/DNA molar ratio 5/1 and 17/1 was protected (lanes 3 and 4), as indicated by the presence of supercoiled DNA.

3.3. In vitro transfection activity

Transfection activity mediated by lipopolyplexes was first examined as a function of the protocol of formulation and the lipid/DNA molar ratio of the complexes, in the presence of 60% FBS. As shown in Fig. 3 lipopolyplexes formulated with protocols 3 and 4 were more effective in transfecting HepG2 cells than complexes generated with the other strategies, 1, 2 and 5, independently of the lipid/DNA molar ratio used to prepare them. The highest expression was achieved with complexes prepared by strategy 4 at a molar ratio lipid/DNA of 17/1, which showed a 16-, 28-, 1.3- and 25-fold increased transfection compared to lipopolyplexes formulated by the strategies 1, 2, 3 and 5, respectively, prepared at the same molar ratio. On the other hand, transfection activity increased by increasing the lipid/DNA molar ratio in the lipopolyplexes, independently of the protocol used to prepare the complexes. A 1.7- and 33-fold increased transfection efficiency was observed with complexes prepared at 17/1 molar ratio compared to lipopolyplexes at 5/1 and 2/1 molar ratio respectively, by using protocol 4 in the formulation of complexes. It is interesting to note that lipopolyplexes prepared with protocols 3 and 4 showed much higher transfection activity than conventional polyplexes or lipoplexes, particularly at lipid/DNA ratios of 5/1 and 17/1. No measurable luciferase expression was observed with the naked DNA.

We also investigated the effect of the molecular weight and type of polymer (linear or branched) on polyplex- and lipopolyplex-mediated gene transfer into HepG2 cells in the presence of 60% FBS. For these experiments, lipopolyplexes were formulated using strategy 4 at a lipid/DNA molar ratio of 17/1. As shown in Fig. 4, no significant differences were found in the levels of gene expression by using PEI 800 or 25 kDa. Complexes formed with linear PEI (22 kDa) were slightly more effective than lipopolyplexes containing branched PEI (800 or 25 kDa) in trans-

Table 1
Particle size of lipopolyplexes, prepared with PEI 800 kDa, as a function of the protocol of formulation at indicated lipid/DNA molar ratios

Molar ratio (lipid/DNA)	Particle size (nm)				
	Strategy 1	Strategy 2	Strategy 3	Strategy 4	Strategy 5
2/1	188 ± 8	281 ± 10	229 ± 14	157 ± 3	124 ± 2
5/1	187 ± 6	232 ± 8	356 ± 11	181 ± 4	183 ± 1
17/1	205 ± 4	211 ± 20	295 ± 2	214 ± 8	201 ± 8

Table 2
ζ-Potential of lipopolyplexes, prepared with PEI 800 kDa, as a function of the protocol of formulation at indicated lipid/DNA molar ratios

Molar ratio (lipid/DNA)	ζ-Potential (mV)				
	Strategy 1	Strategy 2	Strategy 3	Strategy 4	Strategy 5
2/1	55 ± 3	34 ± 4	35 ± 1	57 ± 2	68 ± 1
5/1	57 ± 1	48 ± 1	54 ± 3	68 ± 1	71 ± 3
17/1	69 ± 2	49 ± 2	59 ± 2	69 ± 1	72 ± 1

Table 3
Influence of molecular weight and type of PEI (branched of linear) on the particle size and ζ-potential of lipopolyplexes

	Particle size (nm)	ζ-Potential (mV)
PEI 800 branched	214 ± 8	69 ± 1
PEI 25 branched	134 ± 4	60 ± 4
PEI 22 linear	147 ± 2	51 ± 2

Complexes were prepared by protocol 4 at a lipid/DNA molar ratio of 17/1. Results are expressed as means ± SD of three independent experiments.

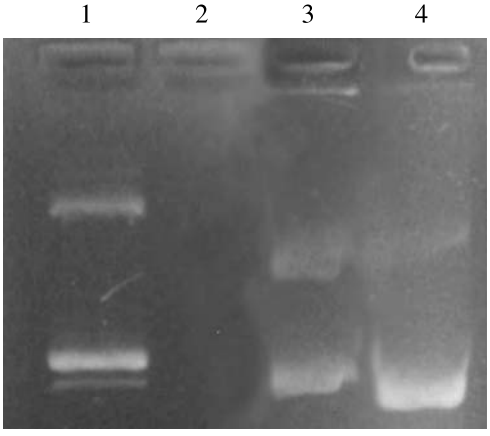

Fig. 2. DNAse I protection assay. Stability to degradation by DNAse I of naked DNA and DNA formulated in lipopolyplexes, containing PEI 800, with different amounts of cationic lipid. Untreated DNA (lane 1), DNA treated with DNAse I: naked plasmid (lane 2), DNA inside lipopolyplexes prepared at 5/1 (lane 3) and 17/1 (lane 4) lipid/DNA molar ratios.

fecting the cells. The lipopolyplexes were much more effective in gene transfer than conventional polyplexes. For example, lipopolyplexes containing PEI 22 kDa resulted in 26 times higher luciferase expression compared to the polyplex prepared with the same polymer.

3.4. Viability studies

Cell viability following transfection was assessed to evaluate whether lipopolyplexes formulated with different protocols and amounts of lipid were toxic to the cells. Toxicity assays showed a viability between 80% and 90% in all transfected wells, independently of the protocol and the lipid/DNA molar ratio used in the formulation of complexes (Fig. 5). Similarly, different molecular weight or type of PEI (branched or linear) did not have an effect on the toxicity of the complexes in transfected cells, being the viability higher than 80% in all cases (Fig. 6). The relative cytotoxicity of the complexes was also assessed by the total amount of extractable cellular proteins in the cell lysate per well, and confirmed the results with the Alamar blue assay (data not shown).

3.5. Interleukin-12 gene delivery

To evaluate the capacity of our complexes to transfect liver cancer cells to express a therapeutic gene, lipopolyplexes were prepared in the presence of pCMVIL-12. The levels of expressed IL-12 demonstrated high transfection

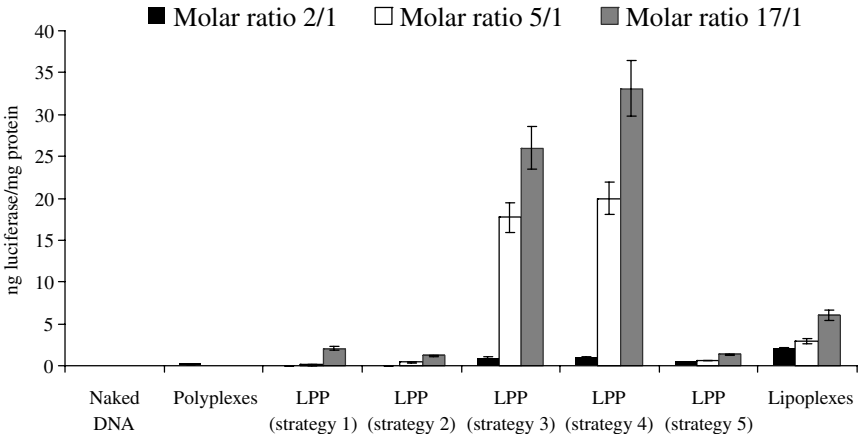

Fig. 3. In vitro transfection activity in HepG2 cells by polyplexes, lipoplexes and lipopolyplexes (LPP) in the presence of 60% FBS. Complexes were formulated with branched PEI of 800 kDa containing 1 μg of pCMVLuc. The data represent means ± SD of three wells and are representative of three independent experiments.

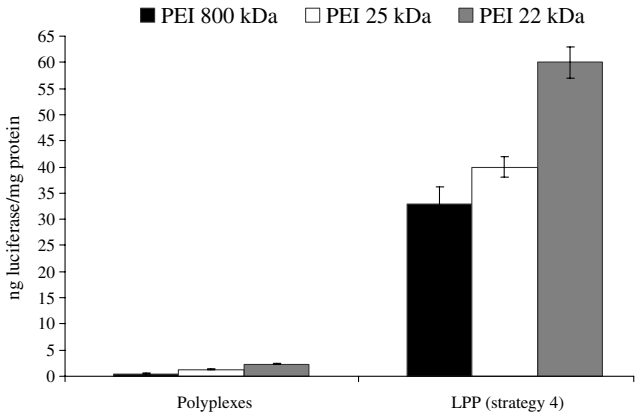

Fig. 4. In vitro transfection activity by polyplexes and lipopolyplexes (LPP) in HepG2 cells in the presence of 60% FBS. Complexes were prepared with different types of PEI, at a lipid/DNA molar ratio of 17/1, containing 1 μg of pCMVLuc. The data represent means ± SD of three wells and are representative of three independent experiments.

efficacy of the complexes (Fig. 7). Lipopolyplexes prepared with linear PEI 22 kDa mediated higher IL-12 expression than lipopolyplexes containing PEI 800 or 25 kDa, in accordance with the results obtained with the plasmid encoding luciferase. Transfection activity of lipoplexes and polyplexes in the presence of the therapeutic gene was lower than that of the lipopopolyplexes.

4. Discussion

The most important considerations in the development of an optimal non-viral delivery system include (i) adequate encapsulation of therapeutic agents, (ii) physical and chemical stability of the formulation and (iii) optimal delivery to cells without significant cytotoxicity. We have therefore explored the combined use of lipoplexes and polyplexes for efficient gene delivery to liver cancer cells, in the presence of high concentrations of serum to mimic in vivo conditions. We have prepared and evaluated different lipopolyplexes comprising DOTAP/Chol liposomes, PEI and DNA, using five different protocols. The cationic lipid DOTAP was selected because of its low toxicity [22] and better hepatocyte transfection properties as compared with other cationic lipids [23].

First, as shown in Fig. 1, the ability of PEI and DOTAP/Chol liposomes to condense DNA was studied by measuring the decrease in the ethidium bromide fluorescence upon its expulsion from DNA. PEI 800, 25 and 22 kDa were very effective in condensing DNA, leading to a complete condensation of the plasmid at N/P ratios above 4. Addition of DOTAP/Chol liposomes did not further condense DNA. It is interesting to note that the condensation of polyanionic DNA into small particles is an important prerequisite for gene delivery employing polycations. The size of condensed DNA is critical for in vivo delivery, because the particle size influences not only the biodistribution but also the efficiency of cellular uptake through endocytosis [24]. Therefore, particle size determination is a useful tool for characterizing colloidal drug delivery systems. In this respect, our complexes showed, by light-scattering measurements, a unimodal size distribution with average diameters in the range of 124 to 356 nm, depending on the method of preparation and the lipid/DNA ratio. Our results indicate some dependence on the protocol of formulation and the lipid/DNA molar ratio (Table 1). Lipopolyplexes formed with branched PEI of 800 kDa had larger diameters than those formed with branched PEI of 25 kDa, while no influence of the type of PEI (linear of 22 kDa or branched of 25 kDa) was detected on the particle size (Table 3).

ζ-Potential measurements give information about the surface properties of the carrier and therefore help to determine how the constituent molecules are organized. As expected, increasing the lipid/DNA ratios resulted in higher ζ-potentials due to an excess of positive surface charge. The lipopolyplexes generated in this study had net positive ζ-potentials, indicating that they can interact readily with negatively charged cell surfaces, which is likely to facilitate internalization. Cell culture experiments indicated that transfection activity of DNA increases by increasing the lipid/DNA molar ratio in the lipopolyplexes (Fig. 4).

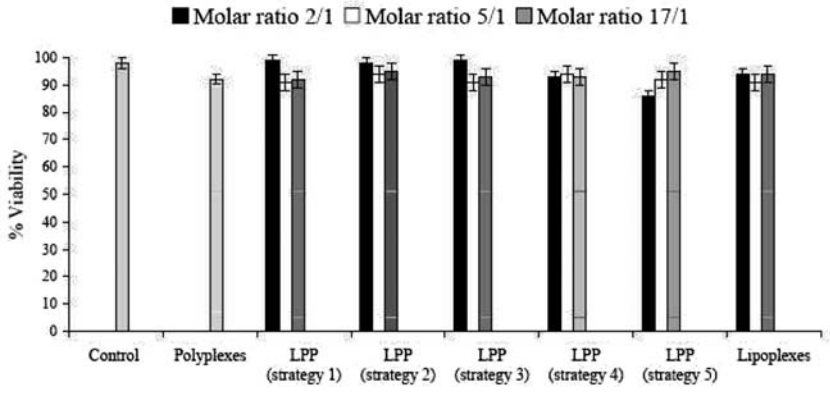

Fig. 5. Cytotoxicity assay. Effect of lipoplexes, polyplexes and lipopolyplexes (LPP) prepared by different protocols, on the viability of HepG2 cells. The data represent means ± SD of three wells and are representative of three independent experiments.

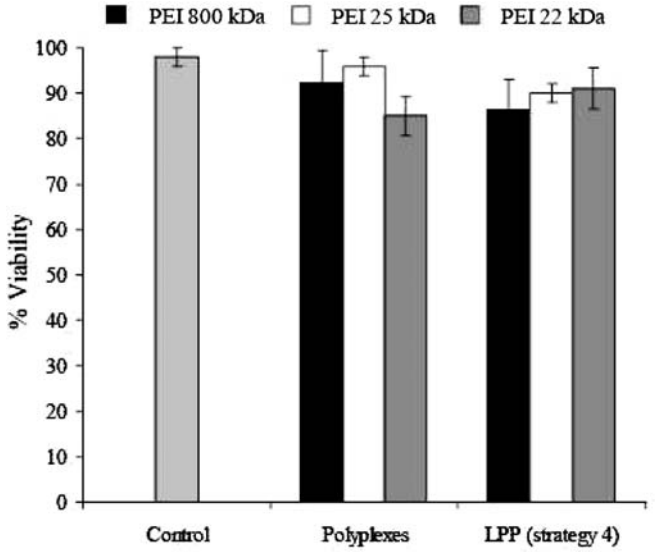

Fig. 6. Cell viability. Effect of polyplexes and lipopolyplexes (LPP), prepared with PEI of 800, 25 and 22 kDa, at a lipid/DNA molar ratio of 17/1, on the viability of HepG2 cells. The data represent means ± SD of three wells and are representative of three independent experiments.

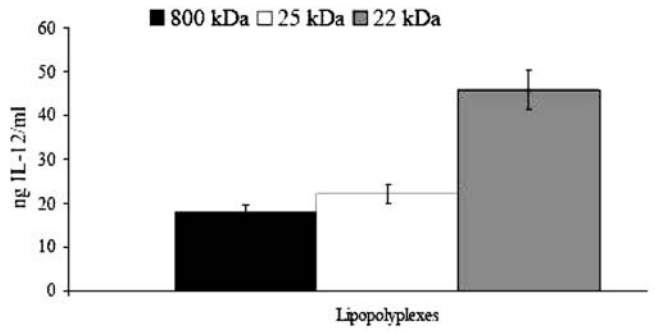

Fig. 7. IL-12 gene expression in vitro. Transfection of HepG2 cells by lipopolyplexes (LPP), in the presence of 60% FBS. Complexes were formulated by protocol 4 at a lipid/DNA molar ratio of 17/1, containing 1μg of pCMVIL-12. The data represent means ± SD of three wells and are representative of three independent experiments.

The rate limiting step in PEI-mediated cell transfection is the transfer of PEI–DNA complexes from the lysosomal compartment to the nucleus [8]. In this respect, our experiments with DNAse I indicated that naked DNA is quickly degraded, while complexation of DNA in the lipopolyplexes protects the plasmid from degradation (Fig. 2). An ideal transfection agent should protect DNA from degradation by nucleases in serum and the cytoplasm during the delivery process, as well as maintain the stability of DNA during the formulation.

Lipopolyplexes formed with protocols 3 and 4 were more effective in transfecting HepG2 cells than lipopolyplexes generated with the other strategies. The complexes formed by these strategies most likely have a different overall structure than those prepared by the other methods, having the DNA more available on the surface of the complex. At the same time, lipopolyplexes composed of DOTAP/Chol and PEI were more efficient than those of just only PEI (polyplexes) or DOTAP/Chol (lipoplexes). This higher transfection activity can be explained by the association of PEI with cationic liposomes, which lead to an altered intracellular behaviour of complexes. In addition, the conformation of the DNA complex, the proton-sponge effect of PEI and the membrane fusion mechanisms are important factors that contribute to the transfection process [25,26]. PEI improves the intracellular kinetics of DNA, while DOTAP/Chol is responsible for the cellular uptake of the complexes. However, it is important to note that this synergy is not universal, as demonstrated by Lampela et al. [27,28], who showed that the level of synergism varied in different cell lines and with the presence of serum. Other authors have studied the combination of polycations and cationic lipids; however chloroquine treatment was essential for obtaining high efficiency of gene transfer [29], or the experiments were done in the absence or in a very low amount of serum [18,19]. Although transfer of naked DNA appears to be a simple method to introduce therapeutic DNA into cancer cells in tumours, it can be used only with certain tissues and requires large amounts of DNA. In our experiments, no luciferase gene expression was detected with naked DNA.

The influence of the molecular weight and type of PEI (branched or linear) in transfection activity was also studied in approaches to optimize further this system for in vivo applications. Lipopolyplexes formulated with linear PEI were the most effective in transfecting liver cancer cells, as shown in Fig. 4. The easy intracellular dissociation of the complex with DNA in the cytoplasm, which is correlated with the high gene expression, could be the reason for this effectiveness. The efficiency of linear and branched PEI has been compared by different authors, obtaining different results for the influence of branching [11,30–34].

It is already known that, the anionic compounds present in serum often complex with positively charged transfection reagents, resulting in decreased transfection efficiency [35,36]. In fact, most in vitro transfection studies in the literature have been carried out in the absence of serum, or in the presence of at most 20% serum. To better simulate biological conditions, all the transfection experiments in this study were performed in the presence of 60% FBS. The described differences in transfection activity between the different lipopolyplex formulations cannot be explained by the different toxicities of the formulations, since cell viability was similar in all cases, independently of the protocol, molar ratio lipid/DNA, and molecular weight or type of PEI used in the formulation of complexes (Figs. 5 and 6). The low amount of PEI needed for the formulations in this study may have contributed to the high viability of the cells (80–90%).

Finally, transfection studies by lipopolyplexes containing the IL-12 gene, a cytokine with potent antitumour activity [37,38], demonstrated that this vector is able to deliver therapeutic genes, and that the levels of gene expression obtained with complexes formulated with linear PEI

22 kDa were higher than that with the branched polymer of 25 or 800 kDa (Fig. 7).

In summary, we have developed an efficient gene carrier by combining PEI and DOTAP/Chol liposomes. The significant advantages conferred by these complexes include (i) small particle size to improve transfection efficiency in vivo, (ii) reproducibility of transfection efficacy, (iii) decreased cytotoxicity, (iv) efficient transfection of liver cancer cells in the presence of 60% of serum, and (v) stability of the complexes. Since both PEI and liposomes can be easily modified with various ligands, these lipopolyplexes might be useful in the design of targeted carriers specific for cell-surface receptors, and the delivery of therapeutic genes. Studies on the suitability of this non-viral vector for gene transfer in vivo are in progress.

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