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Non-viral dried powders for respiratory gene delivery prepared by cationic and chitosan loaded liposomes

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ABSTRACT

The aim of this work was to investigate lipid-based dried powders as transfection competent carriers capable of promoting the expression of therapeutic genes. The lipid-based vectors were prepared by combining different cationic lipids 1,2-dioleoyl-3-trimethylammoniumpropane chloride (DOTAP), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 3β(*N*(*N*′,*N*-dimethylaminoethane) carbamoyl) cholesterol hydrochloride (DC-Chol) or by mixing of anionic lipids (1,2-dimyristoyl-*sn*-glycero-3-phospocholine (DMPC), 1,2-dimyristoyl-*sn*-glycero-3-phospho-rac-glycerol sodium salt (DMPG) and chitosan salts. Spray drying of the formulations was performed using carbohydrates as thermoprotectant excipients and some aminoacids as aerosolisation enhancers. Both the lipidic vectors and the dried powders were characterized for morphology, size, zeta potential (*Z*-potential) and a yield of the process. Agarose gel electrophoresis was used to examine the structural integrity of dehydrated plasmid DNA (pDNA). The biological functionality of the powders was quantified using the in vitro cell transfection. Among the several lipids and lipid–polymer mixtures tested, the best-selected formulations had spherical shape, narrow size distribution (mean diameter < 220 nm, P.I. < 0.250), a positive zeta-potential (>25 mV) with a good yield of the process (>65%). The set-up spray drying parameters allowed to obtain good yield of the process (>50%) and spherically shaped particles with the volume-weighted mean diameter (d[4,3]) < 6 μm in the respirable range. The set-up conditions for the preparation of the lipid dried powders did not adversely affect the structural integrity of the encapsulated pDNA. The powders kept a good transfection efficiency as compared to the fresh colloidal formulations. Lipid-based spray dried powders allowed the development of stable and viable formulations for respiratory gene delivery. In vitro dispersibility and deposition studies are in progress to determine the aerosolisation properties of the powders.

1. Introduction

Gene therapy is considered a powerful tool for curing disease by replacing defective gene, substituting missing genes or silencing unwanted gene expression (Opalinska and Gewirtz, 2002). As viruses have naturally evolved the necessary mechanism to introduce DNA or RNA into the target cells population, viral vectors are the most-effective gene carriers. However, there are many problems to be addressed related to viral transfection and the major concerns are the safety issues, such as recombination, oncogenic potential and immunogenicity (Kootstra and Verma, 2003). It is assumed that non-viral delivery systems, if effective, may improve the safety of gene delivery for therapeutic purposes. In recent years, gene transfection with natural or synthetic cationic polymers or lipids has seemed to be an attractive alternative (Niidome and Huang, 2002; Dinçer et al., 2005; Li and Huang, 2006). Cationic liposomes are widely used for almost animal cells because they have non-specific ionic interaction with cells and low-toxicity properties (Karmali and Chaudhuri, 2007). Therefore, many polymeric cationic systems such as gelatine, polyethylenimine (PEI), poly(L-lysine), cationic dendrimers and chitosan have been studied for in vitro as well as in vivo applications (Merdan et al., 2002). In particular, because of the numerous chitosan properties suitable for pharmaceutical applications (Kato et al., 2003; Kumar et al., 2004), considerable research efforts have been directed towards the development of chitosan/DNA complexes as promising gene carriers. By combining chitosan and liposomal characteristics, suitable carriers with specific, prolonged and controlled release have already been obtained for peptides and proteins (Galović Rengel et al., 2002; Guo et al., 2003; Takeuchi et al., 2005): the most part of the published papers is referring to chitosan-coated liposomes in which the systems are formed by ionic interaction between the positively

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charged chitosan and negatively charged diacetyl phosphate on the surface of liposomes; nevertheless in order to prepare liposomes modified by chitosan addition, the reverse phase evaporation (REV) method has also been investigated (Perugini et al., 2000).

The choice of appropriate route of administration to obtain acceptable bioavailability represents a significant challenge for gene therapy. Due to its physico-chemical characteristics, DNA is typically delivered by parenteral administration (Conti et al., 2000), even if less invasive alternatives for DNA delivery have been investigated (Liaw et al., 2001). Lungs have been suggested as delivery route for peptides, proteins and DNA (Lu and Hickey, 2005; Davis and Cooper, 2007). Particles delivered to the lungs are preferentially within the size range of 0.5–7 μm to allow efficient penetration and deposition in the peripheral pulmonary regions (Hickey, 1993).

Spray drying has recently been used in developing pulmonary delivery systems for gene drugs. Dried powders are particularly promising for delivering therapeutic active macromolecules (i.e. genes, peptides, proteins) because of the many advantages they may present including an increased shelf-life, retention of biological activity, reduced drug loss upon administration, improved patient compliance and possibly more efficient delivery to pulmonary target region (Seville et al., 2007). The unique feature of spray drying lies in its ability to involve both particles formation and drying in a continuous single step, while the major concerns are the high working temperatures, the shearing stresses and the absorption phenomenon that may contribute to the thermal and mechanical degradation of the active molecules. The stability of the molecules can be considerably improved by optimising the operating parameters in spray drying (i.e. drying air temperature and liquid spraying rate). Moreover, stabilising adjuvants, such as disaccharides and polyols, included in the solution/suspension are necessary to protect the molecules integrity during the spray drying.

Regarding gene delivery, spray dried powders with various addictives have been formulated for the transfection of plasmid DNA (pDNA) in the lungs (Seville et al., 2002; Li et al., 2003, 2005a,b) and the effectiveness of protective agents has been studied to obtain stable pDNA-based powders and to avoid plasmid degradation during the spray drying process. Moreover, the DNA condensation induced by cationic agents, such as polymers or lipids, may provide to minimise damage to pDNA (Kuo and Hwang, 2004).

The aim of this work was to investigate lipid-based dried powders as transfection competent carriers capable of promoting the expression of therapeutic genes. For this reason, liposomal formulations were optimized in view of their use in the preparation of lipid/polycation/DNA (LPD) complexes. The lipid-based vectors were prepared by combining different cationic lipids (1,2-dioleoyl-3-trimethylammoniumpropane chloride (DOTAP), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 3β(*N*(*N*′,*N*-dimethylaminoethane) carbamoyl) cholesterol hydrochloride 3β(*N*(*N*′,*N*-dimethylaminoethane) carbamoyl) cholesterol hydrochloride (DC-Chol) or by mixing of anionic lipids (1,2-Dimyristoyl-sn-Glycero-3-Phospocholine (DMPC), DMPG) and chitosan salts. Subsequently, the pDNA condensation induced by cationic liposomes was set-up optimising the best ratio composition in LPD complexes and both the liposomes and the LPD vectors were characterized in terms of particles morphology, size distribution, zeta potential (*Z*-potential), physical integrity of loaded pDNA and capability to protect pDNA against the nucleases degradation.

The second part of the work was aimed to investigate the process of spray drying as potential one-step method for preparing stable and respirable non-viral gene delivery systems for pulmonary administration. Spray drying of the LPD complexes was performed using carbohydrates as thermoprotective additives and some amino acids as aerosolisation enhancers. Dried powders were physico-chemically characterized; moreover the structural integrity of dehydrated pDNA, even after incubation with DNase I, and their bioavailability were assessed by agarose gel electrophoresis and MTT assay, respectively. The biological functionality of powders was quantified in comparison to positive control (Lipofectamine™) and freshly prepared LPD vectors using the reporter gene for firefly luciferase in cultured cells (HEC 293).

2. Materials and methods

2.1. Materials

The cationic lipids considered were: DOTAP, M_w 698.6 Da; DOPE, M_w 786.1 Da; DC-Chol, M_w 537.3 Da; DOPC, M_w 744.1 Da; they were purchased from Sigma–Aldrich Chemical Company, UK.

The anionic lipids considered were: DMPC, M_w 678 Da purchased from Northern Lipids Inc., Canada; 1,2-dimyristoyl-*sn*-glycero-3-phospho-*rac*-glycerol sodium salt (DMPG, M_w 688 Da) donated from Lipoid GmbH, Germany.

The commercially available medical grade chitosan considered were: chitosan chloride salts, namely Protasan UP CL_{113} with viscosity < 20 mPa s, M_w < 150 kDa, DA 75–90% (CL113) and Protasan UP CL_{213} with viscosity 20–200 mPa s, M_w 150–400 kDa, DA 75–90% (CL213), chitosan glutamate salts, namely Protasan UP G_{113} with viscosity < 20 mPa s, M_w < 200 kDa, DA 75–90% (G113) and Protasan UP G_{213} with viscosity 20–200 mPa s, M_w 200–600 kDa, DA 75–90% (G213); they were purchased from Novamatrix, FMC BioPolymer, Norway.

g-WIZ-luciferase high expression pDNA control encoding firefly luciferase was developed by Gene Therapy Systems (San Diego, CA, USA) with modified promoters for increased protein expression. pDNA pGW418323 (unknown encoded transgene; size: 5256 pb) was obtained from Promega, UK.

Lipofectamine™2000 (Lipofectamine) was purchased from Invitrogen, UK: the formulations for the transfection of nucleic acids into eukaryotic cells were prepared as stated by manufacturer. The Dual-Glo™ Luciferase Assay System (with reporter lysis buffer) and DNAse I were purchased in kit form from Promega Co., USA.

Protamine sulphate (Salmine, Grade X from salmon), DL-arginine hydrochloride (Arg, M_w 210.7 Da), D-lysine monohydrochloride (Lys, M_w 182.65 Da), D(+) trehalose dehydrate (M_w 378.3 Da), α-lactose monohydrate (M_w 360.3 Da), Cibacron Brilliant Red 3B-A (M_w 995.23 Da, dye content 50%), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium (MTT, M_w 335.43 Da), bicinchonic acid protein assay kit (BCA assay) and chitosanase were purchased from Sigma–Aldrich Chemical Company (UK). L-Hystidine dihydrochloride (His, M_w 222.08 Da) and L-threonine (Thr, M_w 119.12 Da) were purchased from Fluka Biochemika, UK. Phosphate-buffered saline (Dulbecco A) (PBS) was purchased from Oxoid (UK). The epithelial cell line, HEC 293 (ATCC: CRL-1573) was obtained from the American Type Culture Collection (ATCC), USA.

2.2. Methods

2.2.1. Preparation of liposomes with cationic lipids

Liposomes were prepared by thin film hydration method (Mozafari, 2005). Briefly, DOTAP, DOPC, DC-Chol and DOPE mixtures characterized by different molar ratios (Table 1) were dissolved in chloroform. The total lipid concentration was fixed at 5 mM. The lipid films were re-hydrated with 10 mM PBS (pH 7.4) and subsequently sonicated for 5 min to prepare small unilamellar liposomes. Liposomal formulations displaying good morphology, the best size distribution, stable *Z*-potential and the highest preparation yields were selected for pDNA complexation.

Table 1
Characterization of the liposomal formulations obtained by different mixtures of cationic lipids

Composition (molar ratio)	Mean diameter (nm)	Polydispersity index (PI)	*Z*-potential (mV)	Yield of the process (%)
Dotap	125.41 ± 3.54	0.216 ± 0.012	52.93 ± 6.87	85.32 ± 3.22
1Dotap:1DOPC	135.33 ± 13.22	0.276 ± 0.034	77.95 ± 5.66	67.22 ± 7.65
2Dotap:1DOPC	141.55 ± 34.22	0.246 ± 0.044	75.87 ± 4.76	81.23 ± 3.44
3Dotap:1DOPC	126.67 ± 54.88	0.181 ± 0.021	72.37 ± 6.77	56.21 ± 6.65
1Dotap:1DC-Chol	128.29 ± 32.44	0.179 ± 0.067	45.85 ± 3.22	51.17 ± 9.87
2Dotap:1DC-Chol	137.12 ± 12.36	0.108 ± 0.022	41.11 ± 4.32	34.29 ± 8.77
3Dotap:1DC-Chol	114.91 ± 24.32	0.216 ± 0.022	57.81 ± 8.88	75.16 ± 7.66
1Dotap:1DOPE	134.44 ± 20.98	0.351 ± 0.012	58.11 ± 9.87	62.36 ± 4.65
2Dotap:1DOPE	141.16 ± 4.56	0.223 ± 0.011	69.33 ± 10.33	77.86 ± 2.33
3Dotap:1DOPE	120.68 ± 11.22	0.296 ± 0.032	65.71 ± 8.97	44.24 ± 3.45
DOPE	130.69 ± 4.56	0.120 ± 0.021	65.65 ± 4	45.93 ± 6.78
1DOPE:1DOPC	169.91 ± 35.78	0.355 ± 0.012	65.35 ± 2.33	45.33 ± 9.87
2DOPE:1DOPC	220.82 ± 43.21	0.489 ± 0.045	45.66 ± 3.44	51.22 ± 9.78
3DOPE:1DOPC	231.13 ± 43.34	0.644 ± 0.078	67.56 ± 3.22	54.12 ± 6.78
1DOPE:1DC-Chol	156.24 ± 22.45	0.214 ± 0.012	88.83 ± 1.34	70.82 ± 5.45
2DOPE:1DC-Chol	176.16 ± 21.34	0.237 ± 0.034	73.76 ± 2.44	34.32 ± 4.56
3DOPE:1DC-Chol	149.17 ± 21.67	0.191 ± 0.022	75.66 ± 7.56	33.21 ± 17.34
2DOPE:1Dotap	129.59 ± 6.89	0.239 ± 0.033	70.44 ± 4.55	76.52 ± 2.43
3DOPE:1Dotap	169.21 ± 9.99	0.289 ± 0.043	79.91 ± 7.88	54.12 ± 5.43

2.2.2. Preparation of chitosan loaded liposomes

Chitosan was encapsulated into liposomes by REV method (Perugini et al., 2000). DMPC and DMPG (16:1 molar ratio) were dissolved in $CHCl_3$ and CH_3OH (9:1, v/v; organic phase). 1 ml of chitosan solution (concentrations ranging between 0.0625 and 0.5%) was rapidly injected into lipid solution and the mixture was mechanically agitated by vortex for 5 min, then dried under vacuum (750 mm of Hg) until a gel was formed. Vacuum was released and the tube was subjected to vigorous mechanical agitation on vortex mixer for 5 min. When the gel collapsed to fluid, it was again fitted to rotary flash evaporator for the removal of organic solvent. A cycle of 10 min drying with rotary evaporator and 5 min vortexing was again repeated twice. Final concentrated liposomal suspension was subjected to complete removal of last traces of organic solvent and diluted to the suitable lipid concentration by water. The amount of chitosan reacted with liposomes was evaluated analyzing the supernatant after ultracentrifugation by colorimetric assay with anionic reactive dye Cibacron Brilliant Red (Muzzarelli, 1998). Liposomal formulations displaying the best technological properties were selected for pDNA complexation.

2.2.3. Formation of lipid/polycation/DNA (LPD) complexes

LPD were prepared by sequential addition (with 10 min incubation at each step) of protamine (1 mg/ml stock in sterile purified water) followed by positively charged liposomes to pDNA (pDNA; 1 mg/ml stock in buffer) to achieve a liposomes/protamine/pDNA weight (w/w/w) ratio of 3:2:1 with liposomes obtained from cationic lipids or 5:2:1 with chitosan loaded liposomes. All the complexes were freshly prepared before use.

2.2.4. Characterization of liposomes and LPD complexes

The morphology of liposomes and LPD complexes were examined by transmission electron microscopy (TEM, Philips CM12, Philips, The Netherlands). The colloidal suspensions were negatively stained with 1% uranyl acetate and placed on copper grids for viewing by TEM. Diameter, polydispersity and *Z*-potential of liposomes and LPD complexes were measured by PCS using a Zetasizer apparatus (Malvern Instruments, UK). Measurements on vesicles dispersed in 10 mM KCl were run in triplicate for a single batch and results were the average of six measurements. The yield of the preparation process was calculated as the ratio (%) between the final weight after freeze-drying (Virtis, UK) and the theoretical amount of lipids of liposomes or LPD complexes suspension.

2.2.5. Preparation of LPD-based dried powders by spray drying

LPD complexes, typically comprising 120 μg of pDNA in 15 ml of 3% (w/v) trehalose or lactose solutions, were spray dried by a Mini Büchi B-191 laboratory spray dryer to produce dried powders. The operating conditions employed were: inlet $T = 165\,^{\circ}C$; outlet $T = 85\,^{\circ}C$; spray flow = 800 l/min; aspirator setting = 80%; pump setting = 5 ml/min. To produce amino acid–sugar–LPD powders, lysine (Lys), threonine (Thr), histidine (His) or arginine (Arg) (concentration ranging between 0.1 and 0.6%, w/v) were added to 3% (w/v) sugar solution. Dried powders displaying the best size distribution and the highest spray drying yields were selected for further investigation.

2.2.6. Characterization of LPD-based dried powders

The selected dried powders were sputter-layered with gold and then examined for their morphology by scanning electron microscopy (SEM, Philips XL20, Philips, The Netherlands). Laser diffraction (Mastersizer 2000; Malvern Instruments, UK) was used to analyze particle size distribution of the selected dried powders formulations. Dried samples were dispersed in chloroform and sonicated for 15 s before measurements.

2.2.7. Agarose gel electrophoresis

Gel electrophoresis was used to assess the restrictive access to ethidium bromide intercalation offered by the degree of pDNA complexation. Agarose gels were prepared with 1% (w/v) agarose solution in 0.5× Tris–Acetate buffer (TAE, 0.04 M Tris-Acetate, 0.002 M EDTA) with 0.01% ethidium bromide. The electrophoresis was carried out for 45 min at 80 V. The volume of sample loaded in the well was 20 μl of vesicles suspension containing 20 ng of DNA. The resultant gels were visualised under UV transilluminator at wavelength of 365 nm.

2.2.8. Determination of DNA complexation efficiency

Samples were centrifuged at 50,000 rpm for 2 h (Ultracentrifuge LS-75 with rotor type 60Ti, Beckman, USA). Then, supernatants

were collected and concentrated using Vivaspin concentrators (5 ml, $M_w$co 100 kDa, Vivascience AG, Germany). The concentrated supernatants were analysed both on agarose gel electrophoresis and by spectrophotometry (UV–Visible Spectrophotometer Cary 3E, Varian, USA) at 260 nm for the presence of free DNA (Bozkir and Saka, 2004).

Moreover, to highlight the actual complexation of pDNA in LPD complexes, the samples were subjected to the *digestion protocol*. Briefly, LPD were incubated with a buffer system (10 mM Tris–HCl at pH 8, 1 mM EDTA, 0.5% sodium dodecyl sulphate (SDS)) for 1 h at 37 °C to release pDNA from the condensing moieties, vortexed and centrifuged before gel loading. LPD complexes prepared with chitosan loaded liposomes were subjected to the concomitant chitosanase digestion for 4 h at 37 °C. After this treatment, samples were loaded onto the 1% agarose gel.

2.2.9. Protective effect of LPD complexes and dried powders for pDNA in the presence of nucleases

The effect of protection of pDNA from nucleases degradation was examined using DNase I as model enzyme. Naked pDNA (1 μg in Tris–EDTA buffer), LPD complexes or dried powders (1–8 μl equivalent to 1 μg of pDNA) were incubated with DNase I (1 u) at 37 °C under shaking for 30 min. The reaction was stopped by adding DNase stop solution and subsequent incubation at 65 °C for 10 min to inactivate the DNase. The pDNA was then extracted by the *digestion protocol* from the complexes and analyzed by agarose gel electrophoresis.

2.2.10. Ex vivo biocompatibility evaluation of LPD complexes and dried powders toxicity

The ex-vivo studies on the metabolism of cultured HEC293 were assessed with a 3-(4,5-dimethyl-2-thiazolyl)-2,5 diphenyl-2*H*-tetrazolium (MTT) assay (Mosmann, 1983), using 96-well cell culture cluster with 10,000 HEC293 cells plated in contact to different amounts of formulation corresponding to the total lipid amounts from 0.117 to 50 μg/ml. Cell viability was calculated as percentage of untreated cells (controls).

2.2.11. Ex vivo cell transfection

One day before transfection, the cells (HEC293) were seeded in 48-well plate with a concentration of 50,000 cells/well. When the confluence of cell culture reached 80%, media were withdrawn and discarded. PBS was added in each well for washing, and then discarded. Cell culture medium (200 μl) was added in each well. Different types of formulations (50 μl) containing 1 μg of pDNA control were added in each well ($n = 3$). Transfection using lipofectamine as positive controls was carried out as described in the manufacturer's instructions. The plates were then incubated at 37 °C and 5% CO_2 for 3–5 h. Then, the medium was removed and wells were washed with PBS. Fresh medium (500 μl) was added to each well.

Two days after cells transfection, media were removed from the wells. 500 μl of PBS were added to each well for washing and then removed. The Promega Lysis buffer (5×) was added to each well. The plate was left to incubate for 30 min at room temperature. 20 μl of lysate were taken from each well and added to 50 μl of Dual-Glo™ Luciferase Assay System into a 96-well black plate. The plate containing the cell lysates was assayed for fluorescent intensity using a Wallac Victor2 microplate reader (1420 Multi-label counter, Wallac Oy, Finland) giving relative light units (RLU). The lysate (20 μl) was taken for the determination of protein amount with BCA assay. The RLU were normalized to the protein amount in the cell extracts as RLU/mg protein.

2.3. Statistical analysis

Data were expressed as mean ± standard deviation (S.D.). Comparison of mean values was performed using one-way analysis of variance (ANOVA). A statistically significant difference was considered when $P < 0.05$.

3. Results and discussion

3.1. Preparation and characterization of liposomes

Liposomes prepared starting from cationic lipids were easily obtained by thin film hydration method, keeping constant the amount of lipids but varying the lipid molar ratio considered in the batch (Table 1). Liposomes had a similar size (114.91 ± 24.32 to 231.13 ± 43.34 nm) with a suitably low polydispersity index (PI) and positive surface charge ranging from 41.11 ± 4.32 to 88.83 ± 1.34 mV. The yields of the preparation process presented a quite broad values distribution (about 33.21–85.32%): liposomal formulations with yields higher than 70% were selected (Table 1). In order to obtain vesicles added with chitosan, the REV method was considered: chitosan was added to vesicles during their preparation and it was supposed to be both entrapped inside the liposomes and layered on their surface (chitosan loaded liposomes). Different types and concentrations of polymer were tested (Table 2).

Anionic liposomes (without chitosan) with made with DMPC e DMPG presented a mean diameter of 121.2 ± 34.3 nm (PI = 0.293) and a negative *Z*-potential of −38.1 ± 2.1 mV.

In order to include chitosan to the into vesicles by REV method, the polymer concentration should be not very low, since the viscosity of the polymers solution has to be high enough to stabilize the water/oil/water emulsion before the organic solvent evaporation (Perugini et al., 2000). The main characteristics of liposomes prepared by this method are presented in Table 2.

The mean sizes of chitosan loaded liposomes ranged from 216.41 ± 22.72 to 1317.89 ± 624.56 nm displaying a unimodal population only in the cases of batches Loaded5 and Loaded11. The other batches showed quite high PIs as to highlight a multimodal size distribution. Moreover, it is possible to note that only for the above-cited batches, the mean sizes of liposomes were suitably comparable to the mean diameters of the preparations obtained with cationic lipids. Moreover, it is possible to note that G113 and CL113 seemed the best candidates more suitable for the preparation of the chitosan loaded liposomes: one possible explanation is the lower M_w of these polymers and subsequently the more suitable viscosity these polymers solutions at the selected concentration (0.25%), that could suitably affect the stability of the chitosan-lipid emulsion during the liposomes preparation.

Decreasing the concentration of chitosan solution employed, there was a reduction of the liposomes sizes till to a certain polymer concentration value (0.25%). Below this value, it seemed that the double emulsion produced in this preparation method became deeply physically instable till to precipitation of the polymer during the process when the polymer concentration was of 0.0625%, and the vesicles size kept to increase together with high PI values.

The *Z*-potential values of the different colloidal systems varied, as expected, according to different composition: they ranged from 27.61 ± 2.72 to 53.23 ± 3.74 mV. The increase in surface charge inherent to naked DMPC–DMPG liposomes could be attributed to the formation of complexes with positively charged chitosan chains and supported the conclusion that chitosan was both entrapped inside and adsorbed onto liposomes.

Table 3 resumes the characteristics of the selected liposomal formulations. The yields of the preparation process were quite suitable

Table 2
Composition and main characteristics of the cationic liposomes obtained by chitosan loading

Batch	Type of chitosan	Concentration of polymer (%)	Mean diameter (nm)	Polydispersity index (PI)	*Z*-potential (mV)
Loaded1	G213	0.5	1088.33 ± 32.71	1	45.51 ± 14.56
Loaded2	G213	0.25	687.32 ± 234.56	0.466 ± 0.098	39.63 ± 3.55
Loaded3	G213	0.125	823.23 ± 456.67	0.876 ± 0.123	36.44 ± 2.45
Loaded4	G113	0.5	979.56 ± 664.66	1	51.66 ± 10.88
Loaded5	G113	0.25	216.91 ± 23.45	0.150 ± 0.021	27.61 ± 2.72
Loaded6	G113	0.125	983.52 ± 543.22	0.761 ± 0.156	35.17 ± 9.08
Loaded7	CL213	0.5	755.54 ± 134.56	0.656 ± 0.167	40.51 ± 9.65
Loaded8	CL213	0.25	507.16 ± 234.67	0.476 ± 0.098	33.41 ± 9.34
Loaded9	CL213	0.125	846.78 ± 567.78	0.998 ± 0.119	46.56 ± 8.56
Loaded10	CL113	0.5	1056.78 ± 234.67	1	53.23 ± 3.74
Loaded11	CL113	0.25	216.41 ± 22.72	0.191 ± 0.022	36.81 ± 9.56
Loaded12	CL113	0.125	1317.89 ± 624.56	1	41.23 ± 13.22

Different types of chitosan were considered, namely chitosan glutamate at high (G213) and low (G113) M_w and chitosan chloride at high (CL213) and low (CL113) M_w in different concentrations.

ranging between 67.81 and 85.32% and the percentages of chitosan reacted in liposomes preparation for L6 and L7 were 16.61 and 17.33%, respectively.

Fig. 1 shows TEM images of some selected liposomal formulations: liposomes revealed homogeneous and perfectly spherical and regular shapes.

The stability of the liposomal suspensions in terms of mean size, PI and morphological shape was evaluated in static storage conditions at 4 °C. Liposomes prepared by thin film hydration method (L1–L5) presented a really good stability for the above cited parameters up to 4 weeks in suspension without precipitation or aggregation. Chitosan loaded liposomes (L6–L7) had a lower stability in the cited storage conditions and they already tended to coalescence after 1-week storage: this could be explained by the labile nature of the chitosan-lipid emulsion employed for the preparation of the chitosan loaded liposomes systems (non-reported data).

3.2. Preparation and characterization of LPD complexes

The selected liposome formulations (L1–L7) were tested to determine the optimum amount of complexed pDNA they could accommodate. The liposomes (40 μg) were complexed with increasing amounts of pDNA and the size and the *Z*-potential of the resulting LPD complexes were measured. The results showed that upon complexation of increasing amounts of pDNA, the size of LPD complexes starts to increase. The vesicles remained stable up to 20 and 8 μg of pDNA per 40 μg of liposomes for formulations prepared with cationic lipids and with chitosan-lipid mixtures, respectively. At higher amounts of pDNA, the size of LPD complexes sharply rose till to aggregates formation.

As examples, Fig. 2(a and b) presents the results of the optimization of pDNA amount to be loaded by L1 and L6; the loading capacity of the batches L2–L5 and L7 are suitably similar to L1 and L6, respectively (data not shown).

In addition, measurements of surface charge of LPD complexes revealed a reduction in *Z*-potential with increasing pDNA amount loaded, as the result of the pDNA negative charge. At 20 and 8 μg of pDNA added for batches L1–L5 and for batches L6 and L7, respectively, the *Z*-potential was still strongly positive (about 25–30 mV) and suitable for colloidal stability.

In order to investigate the actual complexation efficiency, a fixed amount of pDNA (20 μg) was incubated with different amounts of liposomes considered in the pDNA:liposomes (w/w) ratio ranging from 1:1 to 1:4 for L1–L5 and from 1:4 to 1:7 for L6 and L7, keeping in account the ratio indications obtained by the previous tests. The batches were sized and after centrifugation, the free pDNA in the supernatant determined by UV spectroscopy at 260 nm. The optimum liposomes:protamine:pDNA mass (w/w/w) ratios of 3:2:1 for liposomes obtained from cationic lipids and of 5:2:1 for chitosan loaded liposomes were assessed: these ratios led to suitable size and *Z*-potential values and to pDNA complexation efficiencies of about 100%.

Moreover, the samples were centrifuged to pellet LPD complexes and the supernatants were collected and analyzed by agarose gel electrophoresis for the presence of free pDNA released from the complexes. Supernatants of LPD complexes resulted pDNA-free, confirming the pDNA complexation efficiency that approached 100% and suggesting the strength of the adsorption interactions between liposomes and pDNA (non-reported data).

Table 4 resumes the characteristics of the LPD complexes prepared in terms of mean size, PI and *Z*-potential and Fig. 3 reports TEM images of the non-viral carriers: it is possible to highlight that the LPD complexes still showed the spherical shape even if they presented higher PI values after pDNA adsorption with respect to the liposomes.

The optimal ratio of complex formation between pDNA and liposomes was confirmed also by the direct electrophoresis of

Table 3
Composition and main characteristics of the selected liposomes obtained by cationic lipids (L1–L5) and by chitosan loading (L6 and L7, corresponding respectively to batch Loaded11 and Loaded5 presented in Table 2)

Batch	Composition (molar ratio)	Mean diameter (nm)	Polydispersity index	*Z*-potential (mV)	Yield of the process (%)
L1	Dotap	125.41 ± 3.54	0.216 ± 0.012	52.93 ± 6.87	85.32 ± 3.22
L2	2Dotap:1DOPC	141.55 ± 34.22	0.246 ± 0.044	75.87 ± 4.76	81.23 ± 3.44
L3	3Dotap:1DC-Chol	114.91 ± 24.32	0.216 ± 0.022	57.81 ± 8.88	75.16 ± 7.66
L4	2Dotap:1DOPE	141.16 ± 4.56	0.223 ± 0.011	69.33 ± 10.33	77.86 ± 2.33
L5	1DOPE:1DC-Chol	156.24 ± 22.45	0.214 ± 0.012	88.83 ± 1.34	70.82 ± 5.45
L6	1DMPC:1DMPG + CL	216.41 ± 22.72	0.191 ± 0.022	36.81 ± 9.56	70.51 ± 3.56
L7	1DMPC:1DMPG + G	216.91 ± 23.45	0.150 ± 0.021	27.61 ± 2.72	67.81 ± 7.76

The presence of chitosan in the formulation is here indicated as +G and +CL after the lipidic composition when chitosan glutamate at low M_w (G113) and chitosan chloride at low M_w (CL113) are respectively included in liposomes.

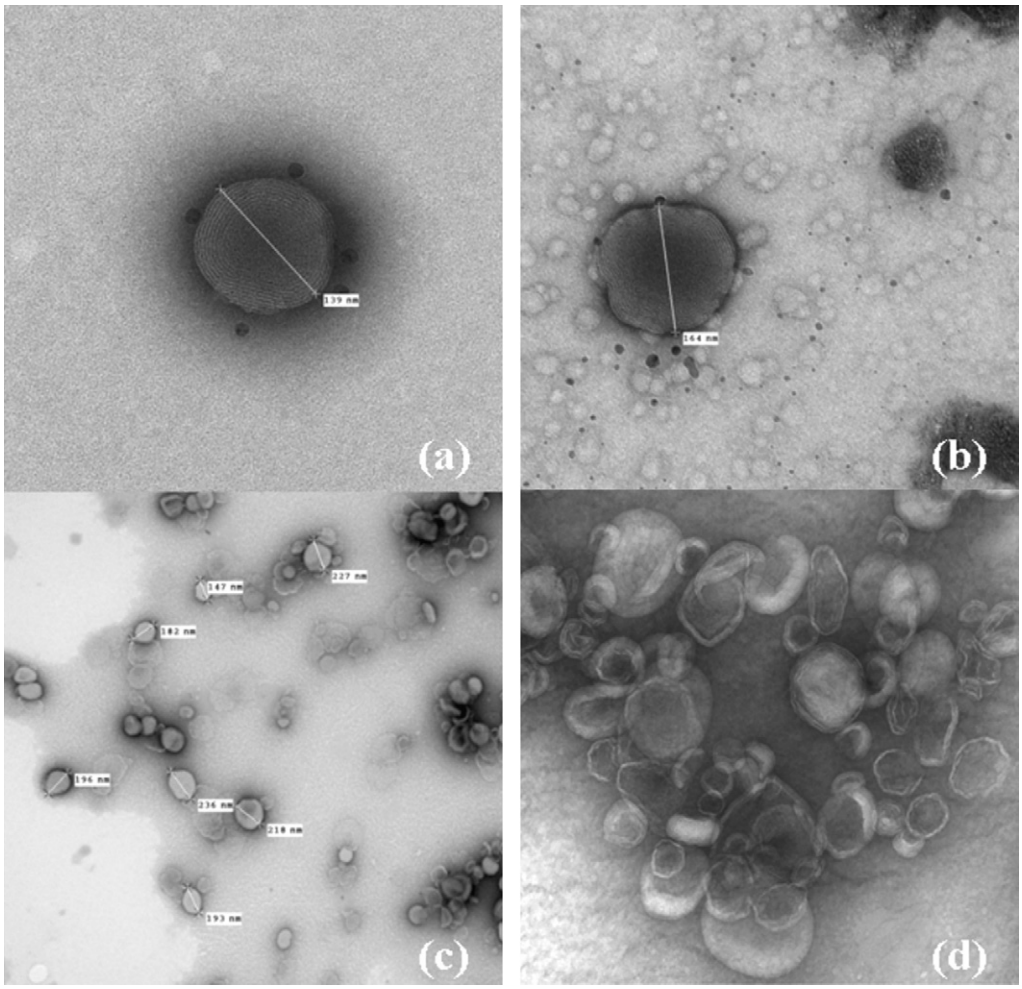

Fig. 1. TEM photomicrographs of selected liposomal formulations: (a) L2 (magnification 180,000×); (b) L3 (magnification 135,000×); (c) L6 (magnification 24,500×); (d) L7 (magnification 93,000×). TEM images show the distinctive single vesicles, each still possessing similar nanometric dimension and a size range that confirm the size measurements by PCS ($n = 6$).

complexes on agarose gel (Fig. 4): when pDNA was mixed with liposomes, the electrostatic interaction drove the formation of complexes. Migration of pDNA on agarose gel was retarded because of the charge neutralization and/or increase in molecular size the complexes (Fig. 4, lanes 3–9).

By the ethidium bromide exclusion gel, the lack of detectable fluorescent signal for the selected formulations indicated that the lipids or the mixture lipid-chitosan in the LPD complexes enclosed the pDNA; only after incubation in the *digestion buffer* to liberate pDNA, the fluorescent signals appeared equivalent to the signal obtained from the naked pDNA (non-reported data).

Another important property of LPD should be their resistance to the degradation by nucleases. Upon the in vivo administration, DNA is exposed to nucleases both prior and subsequent to cell uptake and it is crucial for the carrier to provide adequate protection to the loaded pDNA.

To this purpose, samples of free pDNA and LPD complexes were incubated with DNase I, a nuclease known to degrade any accessible DNA. Following the incubation, the pDNA was purified from the vesicles and analyzed on 1% agarose gel.

As the gel shows (Fig. 5), when free pDNA was exposed to the nuclease, it was entirely degraded, resulting in the absence of a band in the gel (Fig. 5, lane 3). When liposomes were exposed to the enzyme, pDNA was protected from the degradation by its complexation with the vesicles. Indeed, there was no reduction in band intensity after DNase I treatment for any of the LPD complexes prepared.

Table 4
Composition and main characteristics of the selected LPD complexes obtained by cationic liposomes (LPD1–LPD5) and by chitosan loaded liposomes (LPD6 and LPD7)

Batch	Liposomes batch	Mean diameter (nm)	Polydispersity index	*Z*-potential (mV)	Yield of pDNA complexation (%)
LPD1	L1	205.21 ± 23.44	0.216 ± 0.012	30.92 ± 2.34	100.23 ± 1.23
LPD2	L2	231.53 ± 12.45	0.286 ± 0.022	54.84 ± 4.33	99.91 ± 2.32
LPD3	L3	254.94 ± 32.13	0.316 ± 0.023	32.82 ± 4.33	97.81 ± 2.11
LPD4	L4	241.15 ± 12.35	0.293 ± 0.034	40.32 ± 5.43	98.04 ± 1.22
LPD5	L5	256.22 ± 23.45	0.254 ± 0.034	50.82 ± 1.22	98.95 ± 1.34
LPD6	L6	337.83 ± 34.55	0.296 ± 0.054	24.44 ± 4.33	96.52 ± 1.23
LPD7	L7	360.93 ± 56.61	0.265 ± 0.033	20.65 ± 4.67	94.71 ± 3.22

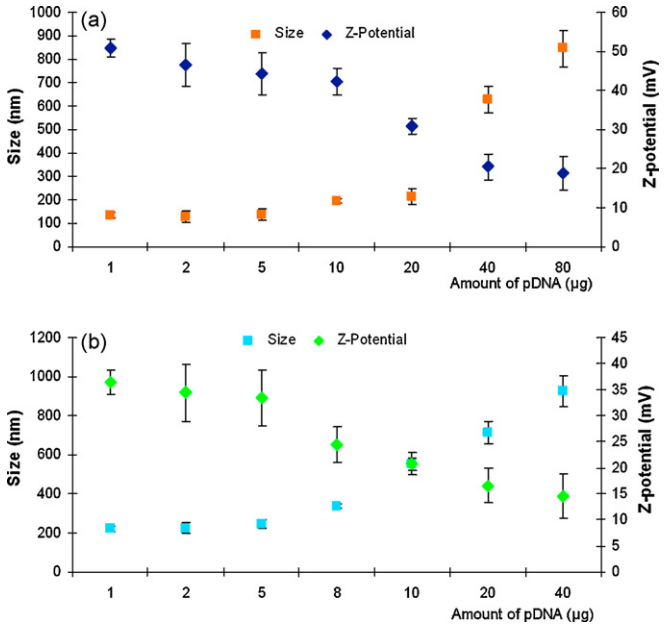

Fig. 2. Optimization of the pDNA amount to be complexed by cationic liposomes (i.e. L1, panel a) and by chitosan loaded liposomes (i.e. L6, panel b): mean size (nm) (left *y*-axis) and *Z*-potential values (mV) (right *y*-axis) are plotted vs. the amount of pDNA (μg) considered.

Table 5
Selected additives for the spray-drying process of the LPD complexes

Batch	Sugar (concentration 3%, w/v)	Aminoacid (concentration %, w/v)
SD1	Trehalose	Arginine (0.3%)
SD2	Trehalose	Lysine (0.3%)
SD3	Trehalose	Hystidine (0.6%)
SD4	Lactose	Arginine (0.6%)
SD5	Lactose	Lysine (0.6%)
SD6	Lactose	Histidine (0.6%)

3.3. *Preparation and characterization of LPD-based dried powders*

Thanks to a preliminary study, the more suitable mixtures of additives for the spray drying process were selected (Table 5):

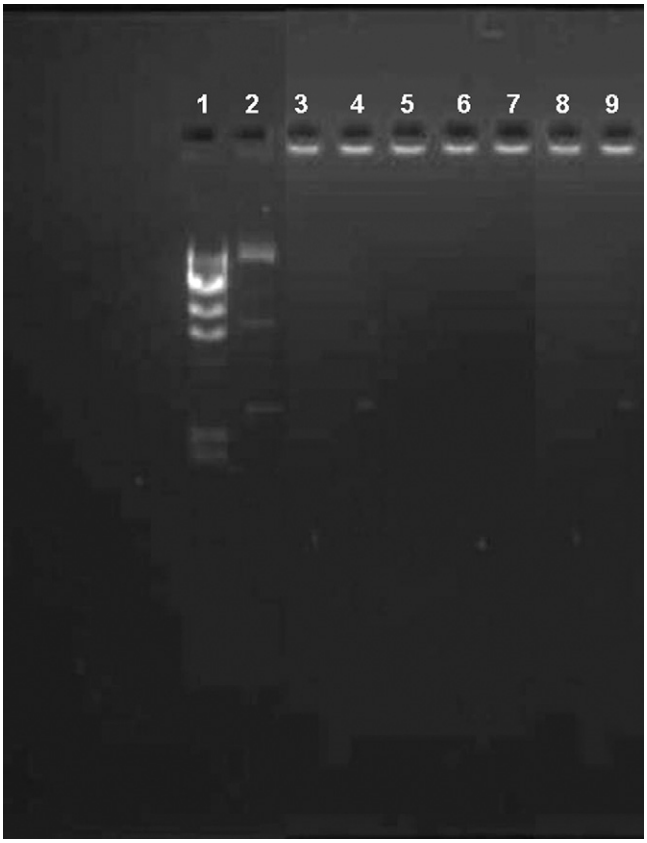

Fig. 4. Electrophoretic analysis of LPD complexes prepared with L1–L7. Lane 1: standard pDNA (λ/HindIII) as marker; lane 2: pDNA pGW418323 as control; lane 3–9: LPD1–LPD7 complexes.

regarding sugars, lactose-based powders generally presented lower yields of the preparation process with respect to trehalose-based powders (data not shown). In any case, thanks to the addition of amino acids in the lactose solution before the spray drying, an improved yield (%) and more spherical shape of the lactose-based powders were obtained, leading to similar shape, comparable size distribution and yield (%) for lactose-based and trehalose-based powders.

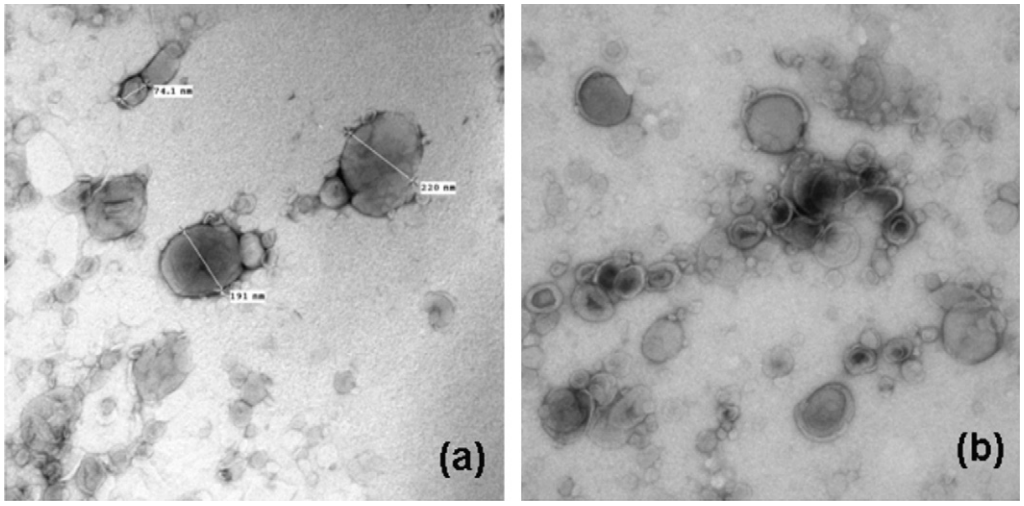

Fig. 3. TEM photomicrograph of LPD prepared with L1 (a) and L6 (b) (magnification 65,000×): the image shows of few distinctive single vesicles, each possessing a typical spherical shape and even if non-homogeneous nanometric dimension.

Table 6
Particles size distribution and yield of the preparation process of LPD-based spray-dried powders

Batch	Liposomes composition	Spray drying condition	D10 (μm)	D50 (μm)	D90 (μm)	*d*[4,3] (μm)	Span	Yield (%)
L1-SD1	L1	SD1	1.64 ± 0.72	3.52 ± 0.98	6.97 ± 1.20	3.94 ± 0.71	1.36 ± 0.56	49.56 ± 2.11
L1-SD3	L1	SD3	1.73 ± 0.31	3.42 ± 0.88	6.36 ± 2.11	3.79 ± 0.56	1.35 ± 0.43	50.01 ± 4.33
L1-SD4	L1	SD4	1.96 ± 0.45	4.42 ± 1.21	7.56 ± 3.11	4.97 ± 0.97	1.39 ± 0.23	51.21 ± 3.24
L2-SD1	L2	SD1	1.67 ± 0.43	3.72 ± 1.01	8.82 ± 3.21	5.00 ± 0.78	1.93 ± 0.98	60.21 ± 3.67
L2-SD2	L2	SD2	1.86 ± 0.23	4.20 ± 2.11	8.42 ± 2.45	4.75 ± 1.20	1.56 ± 0.45	55.23 ± 5.67
L3-SD1	L3	SD1	1.64 ± 0.32	3.21 ± 1.21	6.24 ± 2.56	3.59 ± 0.88	1.31 ± 0.76	53.21 ± 2.45
L3-SD4	L3	SD4	1.52 ± 0.11	3.09 ± 1.01	6.01 ± 1.45	3.31 ± 0.56	1.45 ± 0.68	49.51 ± 3.21
L4-SD1	L4	SD1	1.86 ± 0.56	4.20 ± 1.45	8.42 ± 3.21	4.75 ± 1.78	1.45 ± 0.56	51.23 ± 1.98
L5-SD1	L5	SD1	1.31 ± 0.72	3.10 ± 0.98	6.55 ± 1.20	3.44 ± 0.71	1.26 ± 0.52	48.56 ± 2.11
L5-SD4	L5	SD4	1.53 ± 0.31	3.02 ± 0.88	5.97 ± 2.11	3.56 ± 0.56	1.24 ± 0.43	49.01 ± 4.33
L6-SD1	L6	SD1	2.01 ± 0.42	4.80 ± 0.98	7.79 ± 1.20	3.98 ± 0.21	1.46 ± 0.59	48.26 ± 2.14
L6-SD3	L6	SD3	2.53 ± 0.56	4.02 ± 0.32	6.97 ± 2.01	4.56 ± 0.56	1.31 ± 0.34	44.01 ± 6.54
L7-SD1	L7	SD1	1.91 ± 0.62	3.95 ± 0.38	6.45 ± 1.20	3.74 ± 0.71	1.45 ± 0.78	45.56 ± 6.51

D [*x*]: particle diameter at *x*% of the volume distribution. Span: width of the volume distribution, relative to the median diameter (calculated from (*D*[90] – *D*[10])/*D*[50]). *d*[4,3]: volume-weighted mean diameter.

The set-up parameters of spray drying and the selected mixtures of additives used allowed to obtain good yield of the process (>50%) and spherically shaped particles with the majority of the spheres under 5 μm diameter (Table 6).

When LPD vectors were added to the amino acid–sugar solution to spray drying, SEM images show that powder particles were perfectly spherical with a smooth surface. The particles appeared really close one to each other with limited inter-particle space (Fig. 6).

Spray dried powders demonstrated a unimodal particle size distribution with the majority of particles forming a population with the diameter smaller than 10 μm, as shown by SEM images (Fig. 6).

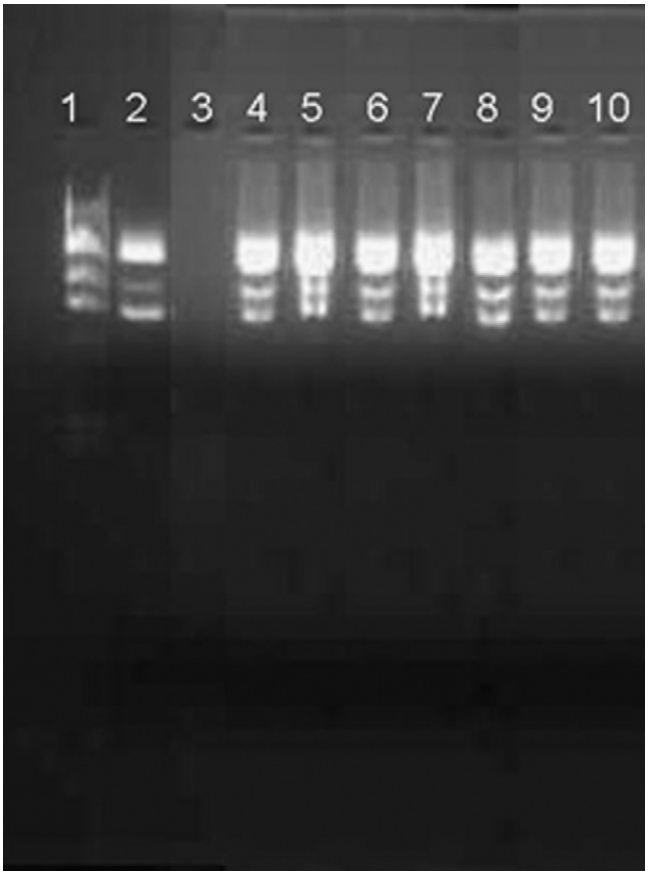

Fig. 5. Structural integrity of pDNA in LPD complexes after incubation with DNase I. Lane 1: standard pDNA (λ/HindIII) as marker; lane 2: pDNA pGW418323 as control; lane 3: pDNA pGW418323 plus DNase I; lanes 4–10: LPD1–LPD7 plus DNase I for 30 min (after SDS or SDS/chitosanase digestion).

The laser diffraction particle sizing data for the best formulations are tabulated in Table 6: all the batches presented a mean diameter over volume distribution (*d*[4,3]) < 6 μm; this feature could let to avoid the inertial impaction in the oropharingeal cavity and highlighted these powders as potentially efficient for drug delivery to air-ways.

The structural integrity of the LPD complexes and the subsequent stability of pDNA after the harsh spray drying conditions were investigated by agarose gel electrophoresis.

The lack of detectable fluorescent signal of the samples before their incubation in the digestion buffer indicated that free pDNA was not present in the spray dried powders, rather the pDNA was still well complexed in LPD vectors. After the incubation in the digestion buffer, pDNA became accessible to ethidium bromide intercalation and fluorescent bands were revealed (Fig. 7).

Generally, when LPD complexes were prepared with chitosan loaded liposomes, it was possible to highlight an increased degradation of complexed pDNA. This phenomenon could be due to the lower stability of chitosan loaded liposomes, as found in the preliminary stability studies. One possible explanation is that chitosan in aqueous solution tends to swell partially disrupting the LPD complex and exposing pDNA, which is subsequently destroyed during the spray drying process (Fig. 7, lanes 14–16).

As reported in the literature (Li et al., 2005a,b), the addition of amino acids in the spray drying suspensions does not adversely affect the stability of LPD: fluorescent signals approached approximately the control for the most part of formulations. A slight reduction in signals intensity was obtained from spray dried powders containing Thr and His as amino acids (Fig. 7, lane 4). The isoelectric points of Thr and His are respectively 5.64 and 7.47 and the preparation of aqueous solutions of these amino acids at pH 7.4 resulted in a slight overall negative charge for Thr and in a neutral charge for His. These ions could interact with the cationic LPD complexes and led to the partial release of pDNA.

On the other side, Arg and Lys present a basic side group that resulted in the generation of positively charged species at pH 7.4. The cationic molecules should be repelled from the LPD complexes, avoiding any adverse effects to the stability of the LPD complexes.

To complete this part of the work, the stability of LPD-based dried powders against DNase I was investigated (non-reported data). While the free pDNA degraded completely within 30 min, spray dried LPD complexes well-protected pDNA against the disrupting effects of DNase I. However, there were differences among the samples in regard to the DNase stability: as expected, dried LPD complexes containing chitosan showed a higher susceptibility to the nuclease activity (non-reported data).

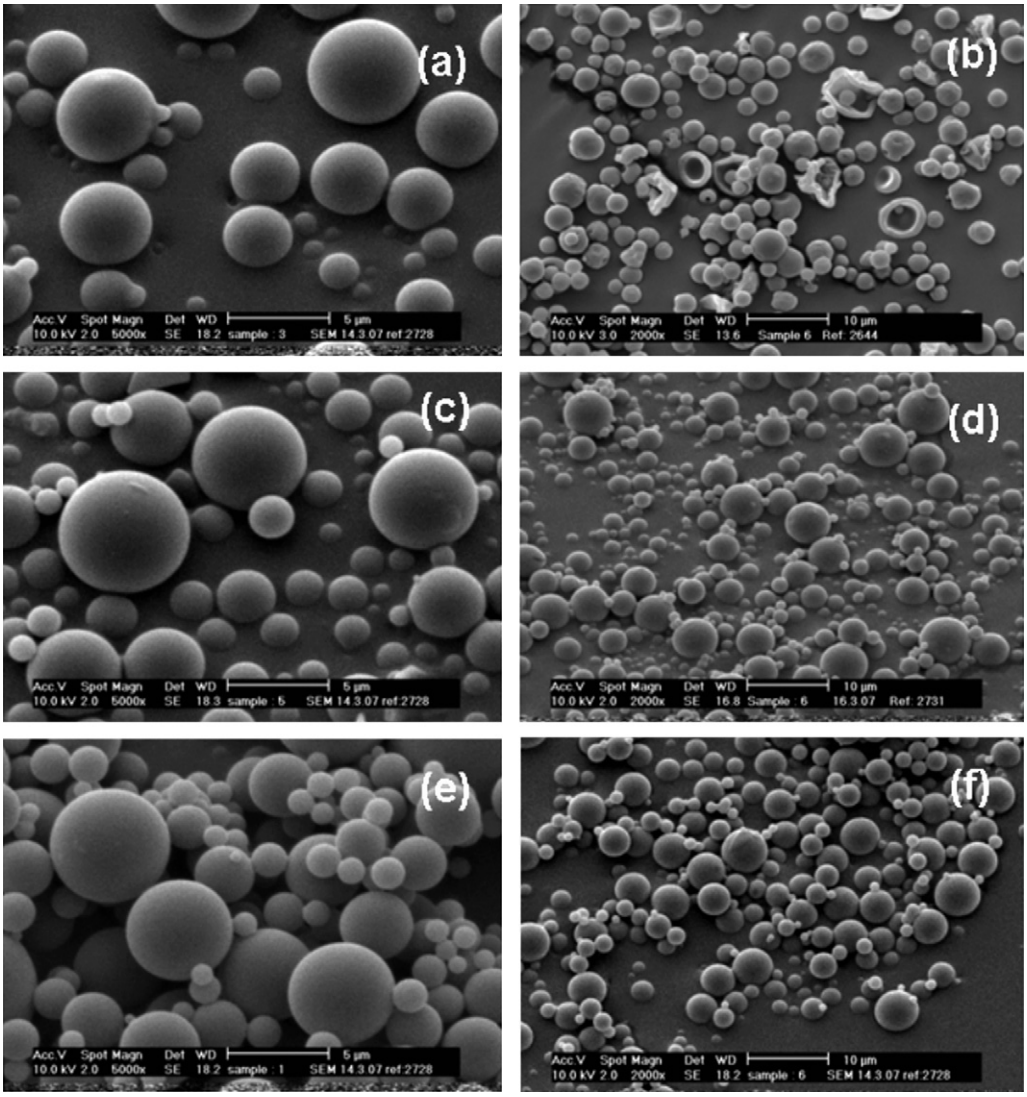

Fig. 6. Scanning electron microscopy of spray dried powders. LPD1-SD1 (a), LPD3-SD1 (b) and LPD3-SD4 (c), LPD5-SD4 (d), LPD6-SD1 (e) and LPD7-SD1 (f). Scale bars: 5 μm at the left side and 10 μm at the right side of the figure.

3.4. Ex vivo biocompatibility evaluation of LPD complexes and dried powders toxicity

One of the prerequisites for the success of gene therapy is to have a safe and efficient delivery system; therefore, it is important to assess to what extent cytotoxicity is affecting the transfection efficiency. Concerning lipoplex-induced toxicity, these non-viral vectors cause changes to cells, including cell shrinking, reduced number of mitoses, and vacuolisation of cytoplasm (Lu et al., 2006). This toxicity may, in part, result from the large size or high positive *Z*-potential of the lipoplexes required for their up-take (Dass, 2002).

For these reasons, as a preliminary step of the transfection studies, different amounts of LPD complexes and the corresponding amounts of dried powders were incubated with cultured HEC 293 cells to verify their toxicity towards these cells. The total lipid amount in contact with cells was considered ranging from 0.117 to 50 μg/ml.

The highest concentrations tested, corresponding to 35, 40, 45 and 50 μg lipid/ml, deeply affected the cells metabolism: the availability values collected represented about 62, 55, 34 and 28% of the controls values. For the sample amounts corresponding to lipid concentration lower than 30 μg/ml, treated cells got bioavailability values superimposable to controls, revealing that this concentration could be considered the safety limit for the ex vivo studies.

3.5. Ex vivo evaluation of transfection efficiency

Lipoplex- and LPD complex-mediated gene delivery systems are currently one of the most favourable means to achieve transgene expression in cells in culture. Transfection via lipoplex, also referred as lipofection, is a multifunctional process and many important aspects of this process are crucial to get successful results. Some of these factors include the medium composition, the type of cells considered, the characteristics of lipoplexes/LPD vectors and the plasmid nucleotide composition and its properties.

Considering the results of physico-chemical studies performed previously, liposomes reported in Table 3 and their derived dried powders were chosen to be tested comparatively for the transfection efficiency in HEC 293 cell line. Complexes were prepared with gWIZ-pDNA encoding for firefly luciferase (Sullivan et al., 2003).

Expression level of gWIZ encoding luciferase reporter gene in HEC 293 cells transfected by LPD complexes and LPD spray dried powders obtained by different mixtures of addictives are reported in Fig. 8(a and b), respectively.

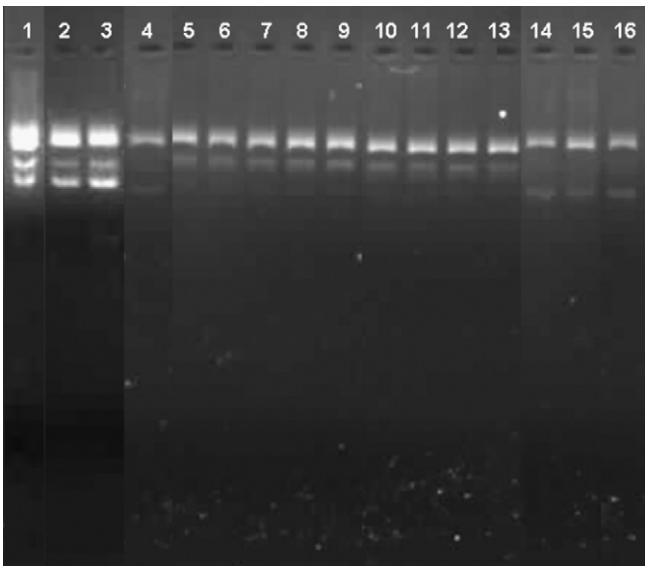

Fig. 7. Electrophoretic analysis of spray dried LPD complexes prepared with LPD1–LPD7. Lane 1: standard pDNA (λ/HindIII) as marker; lane 2: pDNA pGW418323 as control; lanes 3–16: L1-SD1, L1-SD3, L1-SD4, L2-SD1, L2-SD2, L3-SD1, L3-SD3, L4-SD1, L5-SD1, L5-SD4, L6-SD1, L6-SD3, L7-SD1, L7-SD4.

Referring to Fig. 8(a), all the LPD complexes showed a satisfactory level of transfection efficiency with an order of complexes tested as LPD1, LPD3, LPD4, LPD5 (>10^8 RLU/mg protein) > LPD2 (about 10^8 RLU/mg protein) > LPD6, LPD7 (about 10^7 RLU/mg protein). Complexes prepared with cationic lipids showed higher level of transfection efficiency which were comparable to the positive control (lipofectamine). LPD1–LPD5 presented a size distribution ranging from 205.2 to 250.2 nm and *Z*-potential values ranging from 30 to 50 mV: the combination of these two characteristics could be relevant to the increase of the transfection efficiency. It was already shown that the size and the *Z*-potential values of cationic lipidic complexes are very important in their transfection capacity (Ozgel and Akbuğa, 2006).

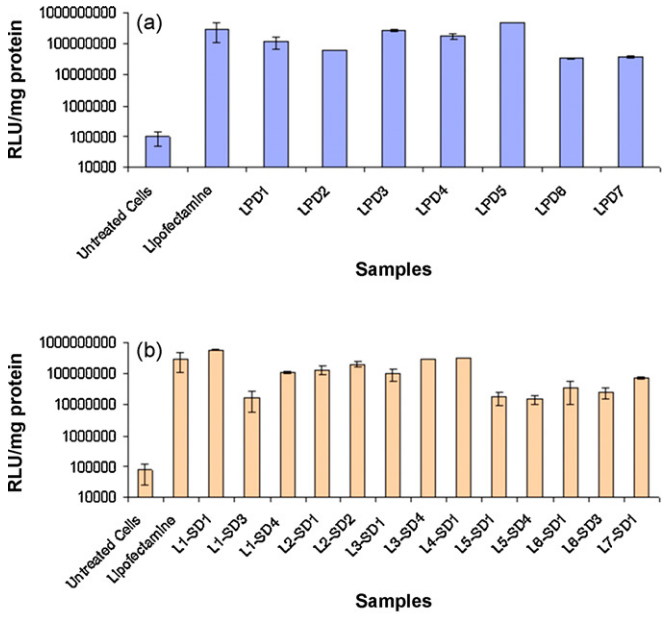

Fig. 8. Expression level of gWIZ encoding luciferase reporter gene in HEC 293 cells transfected by LPD1–LPD7 (a) and by LPD spray dried powders (b). The number of cells is 50,000 per well. Lipofectamine (with 1 μg of pDNA) was used as positive control. Negative control was the equal amount of cells without transfection reagent (untreated cells).

On the other hand, LPD6 and LPD7 showed a transfection efficiency 10-fold lower than LPD1–LPD5 and they were characterized by bigger sizes (>335 nm) and lower *Z*-potential values (about 20–25 mV): especially these lower *Z*-potentials could be the cause of the reduced transfection efficiency. Moreover, regarding the stability of the complexes, the batches prepared with chitosan loaded liposomes presented lower stability in suspension.

The transfection efficiencies of resuspended dried powders prepared by batches listed in Table 6 and containing the luciferase report gene are shown in Fig. 8(b). The spray drying process and the inclusion of sugars and amino acids in the formulations did not negatively affect the transfection efficiency: the results showed that the expression levels of luciferase of samples prepared with LPD1–LPD5 were superimposable to the Lipofectamine values, even if the presence of His as spray drying addictive slightly reduced the effective transfection as well as the use of DOPE in liposomes preparation would have seemed unsuitable for spray dried LPD complexes.

For powders containing chitosan, the expression levels of luciferase were lower than for the other powders, but the spray drying process did not affect their transfection efficiency as these data were similar to the ones collected for freshly prepared LPD6 and LPD7.

4. Conclusions

The preparation of lipid-based dried powders as transfection competent carriers was carried out by combining different cationic lipids (DOTAP, DOPE, DOPC and DC-Chol) or by mixing of anionic lipids (DMPC, DMPG) and chitosan salts. Selected liposomes presented spherical shape, narrow size distribution (PI ranging from 0.150 to 0.233) and positive zeta potential (27.6–88.8 mV) with a suitable yield of the preparation process (67.8–85.3%). The same main characteristics were maintained by LPD complexes prepared by an optimized liposomes/protamine/pDNA mass ratio (w/w/w) of 3:2:1 with liposomes obtained from cationic lipids or 5:2:1 with chitosan loaded liposomes: these conditions led to pDNA complexation efficiencies approaching 100%. LPD complexes prepared effectively condensed pDNA, protecting it from enzymatic degradation.

The set-up conditions for the preparation of the lipid dried powders in terms of spray drying parameters and stabilizing adjuvants had the ability to protect the structural integrity of the encapsulated pDNA. Powders were characterized by good yield of the preparation process (>45%), spherically shapes with the majority of the spheres under 5 μm diameter and therefore, in the respirable range (d(4,3(< 6 μm) and ability to preserve pDNA against DNase I degradation.

The selected LPD complexes and the dried powders presented a really good biocompatibility when incubated with HEC 293 cells (up to 30 μg of liposomes/ml) and kept a good transfection efficiency as compared to the positive control (lipofectamine). In any case, when chitosan was included in the dried powders, results showed that the expression levels of luciferase were slightly reduced, but this could be due to the higher instability of the chitosan loaded liposomes and not to the spray drying process.

This work provided a practical approach for the development of stable and viable formulations for respiratory gene delivery obtained by spray drying. Further studies are due to establish the aerosolisation properties of the powders (in vitro dispersibility and deposition studies) before considering these formulations as transfection vectors in animal studies.

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References

Bozkir, A., Saka, O.M., 2004. Chitosan nanoparticles for plasmid DNA delivery: effect of chitosan molecular structure on formulation and release characteristics. Drug Del. 11, 107–112.

Conti, S., Polonelli, L., Frazzi, R., Artusi, M., Bettini, R., Cocconi, D., Colombo, P., 2000. Controlled delivery of biotechnological products. Curr. Pharm. Biotechnol. 1, 313–323.

Dass, C.R., 2002. Cytotoxicity issues pertinent to lipoplex-mediated gene therapy in vivo. J. Pharm. Pharmacol. 54, 593–601.

Davis, P.D., Cooper, M.J., 2007. Vectors for airway gene delivery. AAPS PharmSciTech. 9 (Article 2).

Dinçer, S., Türk, M., Pişkin, E., 2005. Intelligent polymers as non-viral vectors. Gene Ther. 12, S139–S145.

Galović Rengel, R., Barisić, K., Pavelić, Z., Zanić Grubisić, T., Cepelak, I., Filipović-Grcić, J., 2002. High efficiency entrapment of superoxide dismutase into mucoadhesive chitosan-coated liposomes. Eur. J. Pharmaceut. Sci. 15, 441–448.

Guo, J., Ping, Q., Jiang, G., Huang, L., Tong, Y., 2003. Chitosan-coated liposomes: characterisation and interaction with leuprolide. Int. J. Pharmaceut. 260, 167–173.

Hickey, A.J., 1993. Characteristics influencing the effective administration of drugs as inhalation aerosols. Chest 103, 657–658.

Karmali, P.P., Chaudhuri, A., 2007. Cationic liposomes as non-viral carriers of gene medicines: resolved issues, open questions and future promises. Med. Res. Rev. 27, 696–722.

Kato, Y., Onischi, H., Machida, Y., 2003. Application of chitin and chitosan derivatives in the pharmaceutical field. Curr. Pharm. Biotechnol. 4, 303–309.

Kootstra, N.A., Verma, I.M., 2003. Gene therapy with viral vectors. Annu. Rev. Pharmacol. Toxicol. 43, 413–439.

Kumar, M.N., Muzzarelli, R.A.A., Muzzarelli, C., Sashiwa, H., Domb, A.J., 2004. Chitosan chemistry and pharmaceutical perspectives. Chem. Rev. 104, 6017–6084.

Kuo, J.S., Hwang, R., 2004. Preparation of DNA dry powder for non-viral gene delivery by spray-freeze drying: effect of protective agents (polyethyleneimine and sugars) on the stability of DNA. J. Pharm. Pharmacol. 56, 27–33.

Li, S.D., Huang, L., 2006. Gene therapy progress and prospects: non-viral gene therapy by systemic delivery. Gene Ther. 13, 1313–1319.

Li, H.Y., Neill, H., Innocent, R., Seville, P.C., Williamson, I.J., Birchall, J.C., 2003. Enhanced dispersibility and deposition of spray dried powders for pulmonary gene delivery. J. Drug Target. 11, 425–432.

Li, H.Y., Seville, P.C., Williamson, I.J., Birchall, J.C., 2005a. The use of absorption enhancers to enhance the dispersibility of spray dried powders for pulmonary gene delivery. J. Gene Med. 7, 1035–1043.

Li, H.Y., Seville, P.C., Williamson, I.J., Birchall, J.C., 2005b. The use of amino acids to enhance the aerosolisation of spray dried powders for pulmonary gene delivery. J. Med. Chem. 7, 343–353.

Liaw, J., Chang, S.F., Hsiao, F.C., 2001. In vivo gene delivery into ocular tissues by eye drops of PEO–PPO–PEO polymeric micelles. Gene Ther. 8, 999–1004.

Lu, D., Hickey, A.J., 2005. Liposomal dry powders as aerosol for pulmonary delivery of proteins. AAPS PharmSciTech. 6 (Article 80).

Lu, H., Zhang, S., Wang, B., Cui, S., Yan, J., 2006. Toxicity of cationic lipids and cationic polymers in gene delivery. J. Control. Release 114, 100–109.

Merdan, T., Kopecek, J., Kissel, T., et al., 2002. Prospects for cationic polymers in gene and oligonucleotide therapy against cancer. Adv. Drug Deliv. Rev. 54, 715–758.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.

Mozafari, M.R., 2005. Liposomes: an overview of manufacturing techniques. Cell. Mol. Biol. Lett. 10, 711–719.

Muzzarelli, R.A.A., 1998. The colorimetric determination of chitosan. Anal. Biochem. 260, 255–257.

Niidome, T., Huang, L., 2002. Gene therapy progress and prospects: non-viral vectors. Gene Ther. 9, 1647–1652.

Opalinska, J.B., Gewirtz, A.M., 2002. Nucleic-acid therapeutics: basic principles and recent applications. Nat. Rev. Drug Discov. 1, 503–514.

Ozgel, G., Akbuğa, J., 2006. In vitro characterisation and transfection of IL-2 gene complexes. Int. J. Pharm. 315, 44–51.

Perugini, P., Genta, I., Pavanetto, F., Conti, B., Scalia, S., Baruffini, A., 2000. Study on glycolic acid delivery by liposomes and microspheres. Int. J. Pharmaceut. 196, 51–61.

Seville, P.C., Kellaway, I.W., Birchall, J.C., 2002. Preparation of dry powder dispersion for non-viral gene delivery by freeze-drying and spray drying. J. Gene Med. 4, 428–437.

Seville, P.C., Li, H.Y., Learoyd, T.P., 2007. Spray dried powders for pulmonary drug delivery. Crit. Rev. Ther. Drug Carrier Syst. 24, 307–360.

Sullivan, S., Gong, Y., Hughes, J., 2003. Cationic liposomes in gene delivery. In: Torchilin, V.P., Weissing, V. (Eds.), Liposomes. A Practical Approach, 2nd Ed. Oxford University Press, New York, pp. 289–301.

Takeuchi, H., Matsui, Y., Sugihara, H., Yamamoto, H., Kawashima, Y., 2005. Effectiveness of submicron-sized, chitosan-coated liposomes in oral administration of peptide drugs. Int. J. Pharmaceut. 303, 160–170.