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RESEARCH ARTICLE

Cationic and anionic lipoplexes inhibit gene transfection by electroporation *in vivo*

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Abstract

Background Nonviral gene therapy still suffers from low efficiency. Methods that would lead to higher gene expression level of longer duration would be a major advance in this field. Lipidic vectors and physical methods have been investigated separately, and both induced gene expression improvement.

Methods We sought to combine both chemical and physical methods. Cationic or anionic lipids can potentially destabilize the cell membrane and could consequently enhance gene delivery by a physical method such as electrotransfer. A plasmid model encoding luciferase was used, either free or associated with differently-charged lipoplexes before electrotransfer.

Results Electrotransfer alone strongly enhanced gene expression after intramuscular and intradermal injection of naked DNA. On the other hand, cationic and anionic lipoplex formulations decreased gene expression after electrotransfer, whereas poorly-charged thiourea-based complexes, brought no benefit. Pre-injection of the lipids, followed by administration of naked DNA, did not modified gene expression induced by electroporation in the skin.

Conclusions The results obtained in the present study suggest that packing of DNA plasmid in lipoplexes strongly decreases the efficiency of gene electrotransfer, independently of the lipoplex charge. Non-aggregating complexes, such as poorly-charged thiourea-based complexes, should be preferred to increase DNA release. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords electrotransfer; gene therapy; lipoplexes; lyophilization; nonviral vectors; uptake of DNA complexes

Introduction

Gene therapy represents a suitable alternative to protein therapy in terms of toxicity, clearance rates and manufacturing costs. The efficient and safe delivery of plasmids to target cells upon direct *in vivo* administration remains one of the most important challenges for the development of nonviral gene therapy. This had led to efforts in the development of chemical nonviral vectors, as well as alternative physical techniques to deliver 'naked' plasmid DNA. However, the level of transgenic protein expression is still insufficient for several therapeutic applications and this field requires the optimization of the different technologies to increase gene expression level and hence protein production level. For this purpose, we chose to combine a physical plasmid delivery method (i.e. electrotransfer, which is found to be potent for gene transfer) with chemical DNA binding vectors.

As far as electrotransfer (or electroporation) is concerned, the application of several electric pulses induces membrane destabilization and DNA electrophoresis [1]. Electrotransfer represents one of the most efficient methods for gene transfection *in vivo*, in particular in the muscle [2]. As an example, the comparison of the restoration of muscle function using insulin growth factor (IGF)-1 protein injected systemically versus electrotransfered gene encoding IGF-1 to the mouse tibialis anterior muscle indicated that electrotransfered gene induced higher IGF-1 levels and better signs of muscle restoration [3]. Moreover, a longer-term hematocrit increase was achieved when using an erythropoietin encoding gene electrotranfered into the tibialis mouse muscle of β-thalassemic mice [4].

The advantages of chemical vectors include possible repeated administration, the absence of a theoretical size limit for the gene, simpler development and improved safety/toxicity profiles. Formulated DNA has also been developed to protect DNA from degradation, help its way through the cell membrane and increase the level of gene expression [5]. It also allows targeting by grafting of specific ligands. For example, $\alpha v\beta 3$ integrins have been targeted by multiligands systems improving particle accumulation [6] or DNA transfection efficacy [7] in tumors overexpressing this receptor. The incorporation of degradable lipids allows an increased liberation of the DNA from its lipid/DNA structure. DNA particles present a high flexibility, such as the use of differently-charged lipids. DNA/lipid complexes, also called lipoplexes, have been well described in the case of cationic lipids [8]. Few anionic lipoplexes have also been reported because it appears challenging to associate DNA to anionic lipids [9]. We previously reported the two-step preparation of anionic lipoplexes [10] and, in the present study, report a procedure to prepare them in one step. Concerning neutral lipids able to interact with DNA, few have been described. Among them, two are able to transfect cells, namely glycosylated assemblies [11] and the thiourea-based lipids that we developed [12].

Two tissues were chosen for this study: the muscle and the skin. The muscle represents an excellent tissue for gene transfer because it allows a stable gene expression and the production of functional proteins either locally or by secretion into the bloodstream [13]. The skin was also chosen because DNA delivery has previously been achieved in the skin in conjunction with physical methods [14–17].

The association of formulated DNA to electrotransfer could be of some interest. Destabilization of the membranes could facilitate the internalization of the lipid/DNA complexes. Alternatively, membrane destabilization could be enhanced by lipid/DNA complexes synergistically with electroporation, thus increasing the amount of internalized DNA. Cationic lipids have been shown to inhibit transfection by electroporation *in vitro* [18], as well as in the skin *in vivo* [19], although anionic phospholipids have been shown to enhance transdermal transport of water soluble molecules by electroporation [20]. We chose to evaluate cationic, weakly-charged and anionic lipidic systems in combination with DNA electrotransfer *in vivo*, in mouse muscle and skin.

Materials and methods

Materials

The solvents (analytical grade) were purchased from Carlo Erba-SDS (Peypin, France). The lipid cholesterol (5-cholesten-3β-ol) was purchased from Sigma (St Louis MO, USA). L-α-dioleoyl phosphatidylethanolamine (DOPE) was purchased from Aventi Polar Lipids (Alabaster AL, USA). The cationic lipid 2-{3-[bis-(3-amino-propyl)-amino]-propylamino}-*N*-ditetradecylcarbamoylmethyl-acetamide or RPR 209 120 (DMAPAP), the tetracarboxylated cholesterol compound [(2-{cholesteryloxycarbonyl-[2-(bis-carboxymethyl-carbamoyloxy)-ethyl]-amino}-ethoxycarbonyl)-carboxymethyl-amino]-acetic acid (CCTC) and the lipopolythiourea *N*-[1,3-Bis(2,3-dihydroxy-propylthiocarbamoylamino)propan-2-yl]-2-(didecylcarbamoylmethoxy)acetamide (DDSTU) were synthesized as described previously [4,8,10].

Plasmid DNA

Plasmid encoding Luciferase pGL3CMVluc was purified using 'Endofree plasmid Giga kits' (Qiagen, Hilden, Germany). Endotoxin levels were determined using the LAL assay (Lonza, Verviers, Belgium) and were below the detection limits (<0.05 EU/ml). The quality of the plasmid was assessed by calculating the ratio of light absorption (260 nm/280 nm) and by visualization on ethidium bromide-stained 1% agarose gel. Light absorption at 260 nm was used to determine DNA concentration.

Formulation and physico-chemical characterization of the cationic, neutral and anionic lipoplexes

Preparation of the cationic liposomes DMAPAP/DOPE 1/1 [+]

Lipid DMAPAP (5.05 mg, 6×10^{-3} mmol, 1 equivalent) and lipid DOPE (4.47 mg, 6×10^{-3} mmol, 1 equivalent) were each dissolved in ethanol (250 µl). The ethanolic lipid solutions were combined and mixed thoroughly by vortexing. This mixture was dropped on stirred water (5 ml) and the suspension was left to stir vigorously overnight. The excess solvent was removed under vacuum and 720 µl of a liposome suspension at 8.3 mM was obtained.

Preparation of the DDSTU liposomes [0]

Lipid DDSTU (5.9 mg, 7.85×10^{-3} mmol), was dissolved in ethanol (600 µl). The lipidic solution was then dropped

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on stirred water (6 ml). The suspension was stirred overnight. The excess solvent was removed under vacuum and 350 µl of a liposome suspension at 22.5 mM was obtained.

Preparation of the anionic liposomes DMAPAP/CCTC/cholesterol 1.5/1/1 [−]
Lipid DMAPAP (3.1 mg, 3.68×10^{-3} mmol, 1.5 equivalent), lipid CCTC (2.05 mg, 2.45×10^{-3} mmol, 1 equivalent) and lipid cholesterol (0.95 mg, 2.45×10^{-3} mmol, 1 equivalent) were each dissolved in ethanol (150 µl, 150 µl and 200 µl, respectively). The ethanolic lipid solutions were combined and vortexed. This mixture was dropped on stirred water (5 ml) and the suspension was left to stir vigorously overnight. The excess solvent was removed under vacuum and 670 µl of a liposome suspension at 8.3 mM was obtained.

Preparation of lipoplexes
One milliliter of each lipoplexes at 5 µg of DNA/30 µl of liposome in sucrose 7.5% were made: 500 µl of plasmid DNA pGL3CMVluc (0.34 g/l in sucrose 15%) was dropped to 500 µl of each liposome preparations at room temperature with 10 s of vortexing, and left for 1 h at temperature.

Total lipids/DNA ratios (w/w) were 19, 22 and 38, respectively, for the complexes DMAPAP/DOPE/DNA [+], DDSTU/DNA [0] and DMAPAP/CCTC/cholesterol/DNA [−].

Lipoplex lyophilization
Lipoplexes (1 ml of each preparation at 5 µg of DNA/30 µl) were frozen in liquid nitrogen. Then each vial was put into a flask which was connected to the lyophilizer (Christ Alpha 2–4 LD; Christ, Osterode, Germany). Lyophilized lipoplexes samples were rehydrated with water (1 ml) before use.

Size and zeta potential measurements
Particle diameter and zeta potential were determined by dynamic light scattering (DLS) on a Zeta Sizer NanoSeries Malvern (Malvern Instruments, Orsay, France). Samples were diluted in water for size measurements and in HEPES buffer (20 mM, pH 7.4) for zeta potential determination.

Transmission electron microscopy (TEM)
A drop of nonlyophilized complexes was loaded on a Formvar/carbon copper grid (200 mesh; Agar Scientific, Stansted, UK) and allowed to dry for 2 min. After excess removal with a filter paper, uranyl acetate (2% in H_2O) was then dropped on the complexes and allowed to dry for 2 min. Excess was removed by a filter paper, then the grid was observed on a JEOL, JEM 100S electronic microscope (Jeol, Tokyo, Japan).

Gel retardation experiments
Five microliters of bromophenol blue were added to 20 µl of each lipoplex and the mixtures were loaded in wells on a 0.8% agarose gel in TBE buffer (1 M Tris, 0.9 M boric acid, 0.01 M ethylenediaminetetraacetic acid, pH 8) at 80 V/cm. DNA was revealed with ethidium bromide and visualized under ultraviolet light.

Sterility and endotoxin content

To avoid any risk of contamination that would change the *in vivo* responses, we elaborated a 'clean-in lab' protocol. The whole protocol was performed in a sterile environment with autoclaved instruments. Endotoxin content was then evaluated. Data obtained from Cambrex (Charles City, IA, USA), indicated a level of endotoxin lower than 0.05 EU/ml of DNA, which is considered as acceptable by the Qiagen purification kit.

In vitro transfections

B16 murine melanoma cells were grown into Dulbecco's midified Eagle's medium supplemented with 10% fetal bovine serum, 1% antibiotics (penicillin and streptomycin) and 1% glutamine. One day before transfection, cells were treated by trypsin and seeded into 24-well plates (45 000 cells/well) and incubated 24 h at 37 °C. Fifty microliters of lipoplexes (corresponding to 0.5 µg of DNA) were loaded on each well and the plates were incubated at 37 °C for 24 h. Then the cells were washed twice with phosphate-buffered saline (PBS) and treated with 200 µl of a lysis buffer (Promega, Madison WI, USA). After 15 min, the cells were centrifuged for 5 min at 8000 ***g***.

Ten microliters of supernatant and 10 µl of iodoacetamide were added in a 96-well plate which was incubated at 37 °C for 1 h. Protein quantification was performed with the BCA protein Assay Kit (Pierce, Rockford, IL, USA) using bovine serum albumin as a reference. Absorbance was read at 562 nm on a EL340 spectrometer (Bio-Tek Instruments, Winooski, VT, USA). Luciferase activity was quantified using a commercial kit Luciferase assay system (Promega). On 10 µl of the supernatant of lysed cells, 50 µl of the luciferin substrate was injected via an injector and the luminescence was read on a Wallac Victor2 1420 Multilabel Counter (Perkin Elmer, Villebon-sur-Yvette, France).

Plasmid DNA membrane interaction and cellular uptake

Prior to the experiment Chinese hamster ovary (CHO) cells were plated on 2-chamber Lab-Tek II culture dish (Nunc, Rochester, NY, USA) for visualization under the microscope at a density of 50 000 cells per chamber and cells were allowed to adhere and grow for 16 h. On the day of the experiment, 3 µg of free or complexed

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rhodamine-labeled plasmid DNA was diluted in 500 µl of pulsation buffer (10 mM K_2HPO_4/KH_2PO_4 buffer, 1 mM $MgCl_2$, 250 mM sucrose, pH 7.4) and loaded in one chamber of the Lab-Tek. Electrotransfer was realized as: ten pulses of 5-ms duration at a frequency of 1 Hz and at a field intensity of 0.5 kV/cm, applied to the adherent cells. After 5 min, the cells were rinsed with PBS and, after the addition of growth medium, the cells were incubated for 24 h at 37 °C. The uptake of plasmid DNA after 24 h was then observed with an inverted fluorescent microscope (Leica DMIRB; Leica Microsystems, Wetzlar, Germany) and images were acquired with a camera (Quantem 512 SC; Photometrics, Tucson, AZ, USA). Alternatively, CHO cells in suspension were also electropulsated with naked plasmid DNA and the different complexes. The experiment was processed: 500 000 cells were resuspended in pulsation buffer containing 3 µg of rhodamine-labeled plasmid DNA complexed or not with different compounds; suspension cells were placed between the electrodes and ten pulses of 5-ms duration at 0.5 kV/cm and 1 Hz were applied. The interaction of the plasmid DNA with the cell membrane was observed by microscopy as described above.

Combination of intramuscular injection of formulated DNA and electrotransfer evaluation of luciferase expression in the mouse tibial cranial muscle

In vivo studies were performed using 6–8-week-old female BalbC/J mice. Prior to all procedures (treatment and evaluation of the transgene expression), the animals were anesthetized by intraperitoneal injection of ketamine and xylazine (Bayer Pharma, Puteaux, France) (100 and 10 mg/kg, respectively). Studies were conducted in accordance with the recommendations of the European Convention for the Protection of Vertebrates Animals used for Experimentation and the Ethic Committee on Animal Care and Experimentation of Université de Paris Descartes.

Each tibial muscle of anesthetized mouse was injected longitudinally with 30 µl of a solution with DNA coding luciferase (5 µg) ± the different products tested. Approximately 20 s after injection, eight square-wave electric pulses of 190 V/cm (20 ms each) at 2 Hz were applied through two stainless steel plate electrodes, placed 3.6–4.0 mm apart at each side of the leg. Electrical contact with the shaved leg skin was ensured by means of conductive gel. Electric pulses were generated by an ECM 830 electropulsator (BTX, San Diego, CA, USA). Evaluation of luciferase expression was performed 4 days later by optical imaging. For this purpose Luciferin (Promega) diluted in PBS was injected locally into the tibial cranial muscle at a dose of 100 µg/40 µl, which is in large excess relative to expected luciferase amount [21]. Optical imaging was performed with a cooled intensified CCD camera (Biospace; Photo Imager, Paris, France) placed in a black box. This camera allows real time measurements (one acquisition each 0.04 s). Operating temperature was set at −16 °C. We confirmed that, after substrate injection in muscle, luminescence rapidly decreased and chose to take the measurement at 2 minutes after substrate injection. Luminescence levels were integrated in region of interest (ROI) drawn by hand around luminescence zones corresponding to the tibial cranial muscle estimated from superposed optical image of the mice (software β Vision+; Biospace Mesure, Paris, France). ROI were very similar from one experiment to the other. The results are expressed as c.p.m./muscle.

Combination of intradermal injection of formulated DNA formulations and electrotransfer: evaluation of luciferase expression in mouse skin

Female NMRI mice, 6 weeks old (Université Catholique de Louvain, Brussels, Belgium). were anesthetized with 40 µl of a mixture of ketamine 50 mg/ml (Ketalar, Pfizer, Brussels, Belgium) and xylazine 5.6 mg/ml (Sigma). The skin of the abdomen was depilated 1 day prior to the experiments with a depilatory cream (Veet for sensitive skin; Veet, Brussels, Belgium). Experimental protocols in mice were approved by the Ethical Committee for Animal Care and Use of the faculty of Medicine of the Université catholique de Louvain.

Lyophilized DNA samples were reconstituted with sterile water. For intradermal electrotransfer, the plasmid (5 µg), formulated or not, was injected into the dermis using a Hamilton syringe with a 30-gauge needle. We injected 15 µl intradermally in two different sites, with a distance of approximately 5 mm. Then, a cutaneous fold was performed and the sites of injection were placed between plate electrodes, 2 mm spaced (IGEA, Carpi, Italy) as described previously [14,17]. A short high voltage (HV) pulse (700 V/cm 100 µs), immediately followed by a low voltage (LV) pulse (200 V/cm 400 ms) was applied approximately 1 min after plasmid injection. There was no time interval between HV pulse and LV pulse. The pulses were delivered by a Cliniporator system (Cliniporator; IGEA). Conductive gel was used to ensure electrical contact with the skin (ultrasound transmission gel; EKO-GEL, Egna, Italy).

Two days after the electrotransfer, the mice were sacrificed and the electrotransfered areas of the skin were removed. The samples were cut into pieces and homogenized in 1 ml of cell culture lysis reagent solution (CCLR; Promega) containing a protease inhibitor cocktail (Roche, Mannheim, Germany) using a Duall® tissue grinder (Cofraz, Essene, Belgium). After centrifugation at 12 000 ***g*** for 10 min at 4 °C, we assessed the luciferase activity of 10 µl of the supernatant (diluted in CCLR if needed) after the addition of 50 µl of Luciferase Assay Substrate (Promega), using a TD-20/20 luminometer (Promega). The results obtained were expressed in relative light units (RLU).

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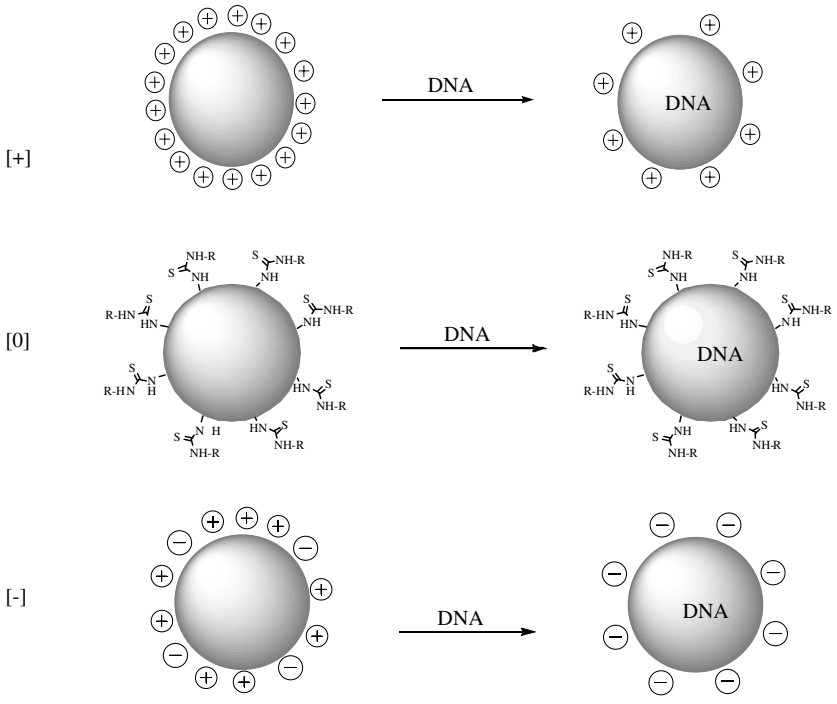

Scheme 1. Schematized cationic [+], poorly-charged [0] and anionic [−] formulations of plasmid DNA lipoplexes. R is a diol group in the thiourea lipid

Results

Physico-chemical characterization of the cationic, neutral and anionic lipoplexes

Formulation of the lipoplexes

The cationic lipoplexes [+] are based on a lipopolyamine DMAPAP that we have extensively studied [22]. Less-charged particles [0] are based on lipopolythiourea lipids DDSTU described earlier that are able to form hydrogen bonds with DNA [12]. The anionic lipoplexes [−] are based on the cationic lipid DMAPAP into which cholesterol and an anionic cholesterol (CCTC) have been added [10]. The ratio between the lipids has been studied aiming to obtain slightly cationic particles. Addition of the plasmid to these particles led to the formation of anionic lipoplexes (Scheme 1).

Particles were formed by the ethanolic injection method described previously [22]. With this method, unilamellar structures around a hundred nanometers are spontaneously formed. We evaluated the size of the particles at the same time as increasing the amount of DNA in the complexes and modifying the lipid/DNA ratio. The colloidal stability of the particles was maintained when the formulations [+], [0] and [−] were composed of 5 μg of DNA in 30 μl with total lipids/DNA ratios (w/w) of 19, 22 and 38, respectively. Thus, to inject stable nanometer scale lipid/DNA complexes, 5 μg of DNA in 30 μl of each complex were used for *in vivo* studies. After lipoplex formation, formulations were freeze dried and stored at room temperature until use. To maintain their structure during the lyophilization process, lipoplexes were prepared in 7.5% sucrose.

Size and zeta potential of the particles

The size of the particle, as well as DNA association, was checked prior and after lyophilization.

The size of the lipoplexes was in the range of 120–210 nm. Polydispersity index was in the range 0.15–0.25 for all the data given in Table 1. As expected, the zeta potential of the lipoplexes was positive for the cationic lipoplexes and negative for anionic lipoplexes. Lipopolythiourea exhibit a zeta potential that was slightly positive, in the range 0–20 mV, as usually obtained for these particles [12]. The size and zeta potential of the lipoplexes were maintained after lyophilization (Table 1).

TEM

As shown in Figure 1, the complexes were well dispersed. The size of the particles observed by TEM was slightly lower than that found by light scattering. This was expected because TEM displays the real particle diameter, which is smaller than the hydrodynamic diameter measured by DLS. The standard error deviation for the cationic complexes found by DLS measures was demonstrated by the presence of smaller particles observed by TEM. The homogeneity of the neutral and anionic suspensions was confirmed by TEM.

Gel retardation

As shown in the agarose gel electrophoresis, free plasmid DNA migrated in the gel when DNA associated with the

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Table 1. Hydrodynamic diameter (nm) and zeta potential (mV) in HEPES buffer (20 mM, pH 7.4) of cationic lipoplexes [+], neutral lipoplexes [0] and anionic lipoplexes [−] before and after lyophilization

	Size (nm)			Zeta potential (mV)	
	Lipids/DNA ratio (w/w)	Before lyophilization	After lyophilization	Before lyophilization	After lyophilization
[+]	19	130 ± 45	114 ± 40	28 ± 10	39 ± 5
[0]	22	210 ± 10	220 ± 10	20 ± 1	19 ± 1
[−]	38	116 ± 35	107 ± 30	−19 ± 1	−21 ± 2

Data are given as the mean of three runs.

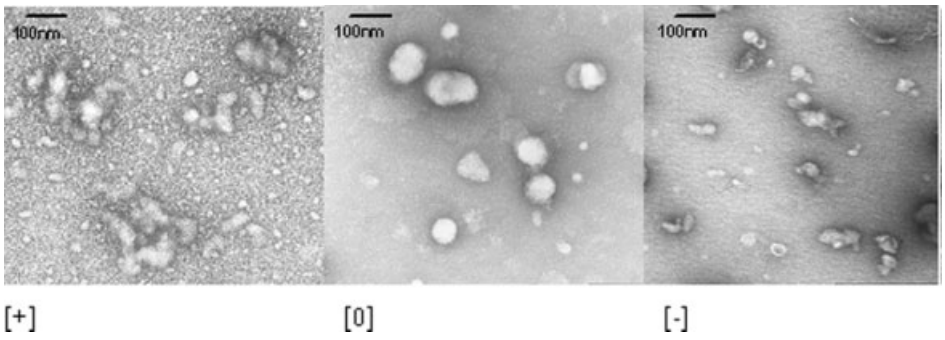

Figure 1. TEM of the cationic [+], neutral [0] and anionic [−] lipoplexes

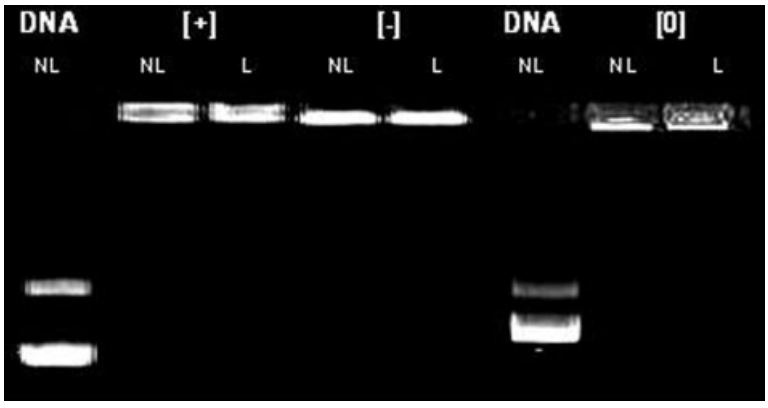

Figure 2. Migration of lipid/DNA complexes [+], [0] and [−] on a 0.8% agarose gel in TBE (×1). Complexes were revealed with ethidium bromide staining. Nonlyophilized (NL) and lyophilized (L) complexes were loaded on the gel

complexes was totally retained in the upper wells, indicating that DNA was condensed by the lipids. This was verified for the three type of lipoplexes ([+], [0] and [−]) before and after lyophilization (Figure 2).

In vitro transfection studies: effect of the lyophilization process

In vitro experiments were performed on a B16 cell line to check the transfection efficiency of the three different formulations before and after lyophilization. As shown in Figure 3, the three formulations were able to transfect cells and the impact of the lyophilization process was not significant. A slight decrease was observed for all formulations at the ratio shown, which never exceeded a factor of 1.5 between nonlyophilized and lyophilized formulations. The lower RLU related to luciferase level obtained for the anionic formulation is a result of anionic DNA lipoplexes being less prone to interact with cell membranes.

Combination of electrotransfer and formulated DNA *in vitro*: interaction and uptake influence of the lipids

Rhodamine-labeled DNA was introduced into the lipoplexes to follow their interaction and intracellular fate on adherent and non-adherent cells, when submitted or not to electric field. Merged contrast phase and fluorescent images are shown in Figure 4.

Interaction at $t = 0$ is only shown for suspension cells (Figure 4, right). Interaction of rhodamine-labeled plasmid DNA with the membrane, as revealed by the formation of fluorescent spots on the periphery of the cells, could be observed only for naked plasmid DNA.

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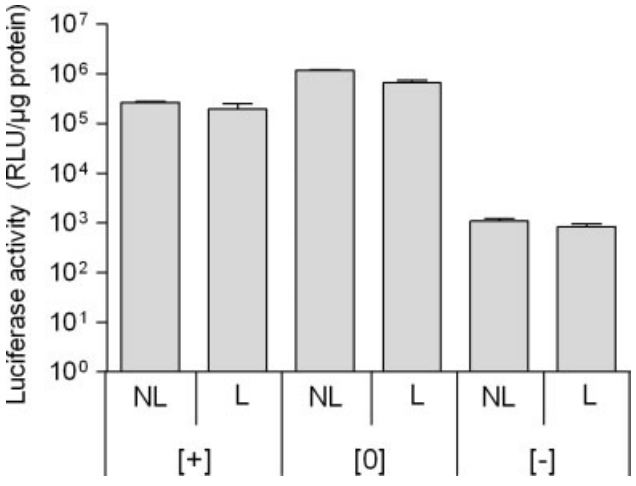

Figure 3. Luciferase expression mediated by the cationic, neutral and anionic formulations without lyophilization (NL) or after the complexes had been lyophilized and rehydrated (L) ($n = 3$)

Cationic and anionic lipoplexes formed aggregates with the plasmid DNA in the culture medium. For the anionic lipoplexes, very large clusters were found after the electric pulse in the vicinity of the cells, and cationic clusters were found everywhere even on the cover slide. For the thiourea-based lipoplexes, few spots attributed to DNA were found at the membrane level.

Uptake ($t = 24$ h) of naked plasmid DNA and complexes was studied on control cells (Figure 4, left) and submitted to electric field adherent cells (Figure 4, middle). We observed that, for naked plasmid, DNA uptake only took place with electrotransfer. By contrast, with thiourea-based complexes and cationic lipolexes, uptake took place even without electrotransfer. Only the uptake of thiourea lipoplexes appeared to be favored by the electrotransfer application. With cationic lipids/DNA complexes, the presence of aggregates could be observed that likely prevent the release of plasmid DNA inside the cells. Aggregates could also be observed at the periphery of cells with anionic lipids/DNA complexes. The overall uptake for these complexes was poor.

Combination of electrotransfer and formulated DNA for gene expression into the muscle

We had previously shown that the combination of cationic formulation and electrotransfer into the tibial cranial muscle resulted in the inhibition of muscle transfection [23]. That is why we chose, in the present study, to solely evaluate the combination of electrotransfer and neutral formulation, electrotransfer and anionic formulations.

As shown in Figure 5, the anionic formulation significantly inhibited the transfection compared to naked DNA. On the other hand, the thiourea-based formulation did not significantly modify the transfection level.

Combination of electrotransfer and formulated DNA for gene expression into the skin

We also studied the intradermal injection of luciferase encoding plasmids, either formulated or not. The luciferase expression in the skin was evaluated *ex vivo*. First, we showed that, without electrotransfer, reporter gene expression was slightly higher after the injection of naked DNA compared to cationic or anionic [−] lipoplexes. Electrotransfer of these formulations did not improve luciferase expression, which remained lower than the expression after injection of naked DNA (Figure 6A). The differences obtained were not significant; however, the trend of lower luciferase expression when plasmid was encapsulated into particles indicated that there was no advantage to include the DNA plasmid in lipoplexes before electrotransfer. Noteworthy, this trend was independent of the lipoplex charges.

The combination of electrotransfer with formulation is of interest because of the possible destabilization of cell membrane by the lipoplexes, which could facilitate plasmid DNA cell entry. To test this hypothesis, we first injected empty cationic or anionic formulations, and then naked DNA. Electrotransfer was applied after these two injections. As previously shown, electrotransfer applied post-DNA injection dramatically increased luciferase expression compared to plasmid injection without electrical pulses [16]. The pre-injection of empty cationic or anionic lipidic particles did not modify the level of gene expression (Figure 6B).

Discussion

Nonviral vectors are quite efficient with respect to transfecting cells *in vitro*. However, vectors reported to induce high transfection *in vivo* are rare. Because the level of protein expression was still quite low, we had speculated that the use of physical methods in conjunction with the advantageous properties of lipid delivery would help to improve the protein production level. To test this hypothesis, we combined the effect of DNA-based lipoplexes with electroporation to enhance DNA permeation through the plasma membrane. Three different formulations were developed for this project. DNA lipoplexes of cationic, poorly-charged and anionic zeta potential were formed to provide either increased cell membrane destabilization or facilitated DNA diffusion, which could improve DNA transfection mediated by electrotransfer.

Cationic and uncharged formulations have been reported previously [8,12]. Anionic complexes have been formed from a mixture of anionic and cationic lipids, in which the ratio was appropriately chosen to allow the formulation of DNA in one step, without aggregation. The ratio was chosen to maintain a cationic charge to the particles prior to DNA addition, and to obtain a global

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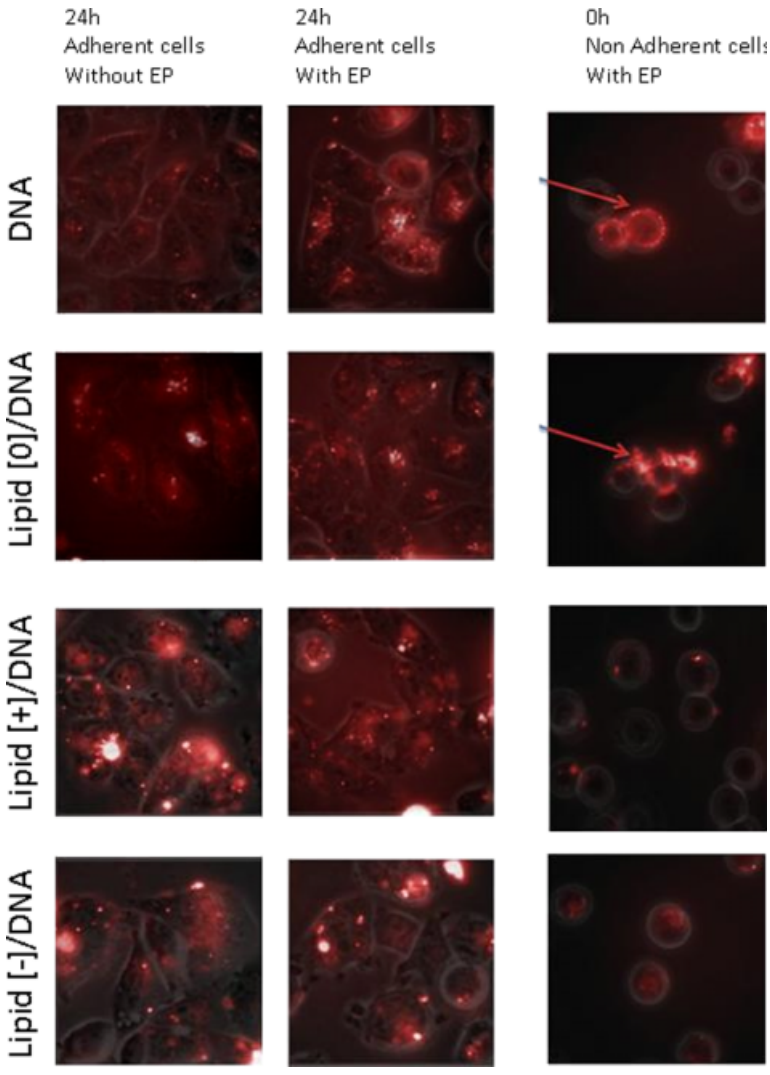

Figure 4. Interaction and uptake of plasmid DNA and complexes in CHO cells under electropulsation. Merged phase contrast and fluorescent images obtained by setting the transparency of the phase contrast images to 80%. Suspension cells (right columns) and adherent cells (two left columns) were electropulsated in the presence of plasmid DNA complexed or not with neutral [O], cationic [+] and anionic lipids [−]. On suspension cells, DNA membrane interaction was observed directly after electropulsation. On adherent cells, the uptake was observed 24 h after electropulsation

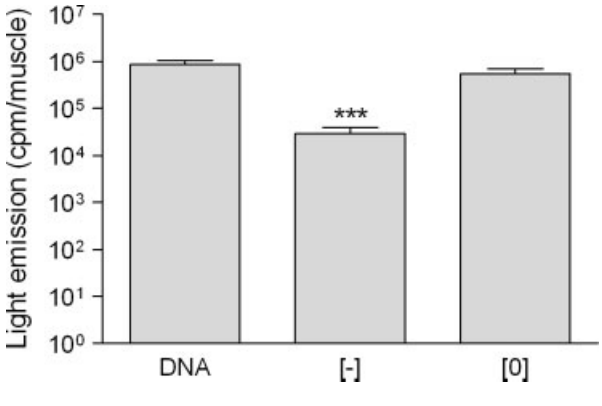

Figure 5. Tibial cranial muscle mouse electrotransfer of DNA or formulated DNA ($n = 5$)

anionic charge post-DNA addition. This allowed stable and homogeneous particles to be obtained. A 'clean-in lab' protocol has been worked out for the preparation of endotoxin-free lipoplexes. Moreover, the lyophilization of the lipoplexes has been optimized to provide reproducible and endotoxin-free batches of lipoplexes.

The results obtained in the present study indicate that we did not obtain any positive effects of the combination of both physical and chemical DNA delivery methods compared to free DNA and electrotransfer. This correlated with the findings of the earlier study by Coulberson *et al*. [18] with respect to the combination of lipids, peptides or viruses with electroporation *in vitro*. The DNA electroporation mechanism is poorly known. However, it is clear that DNA has to reach the cell membrane to be integrated into the cell. Anionic phospholipids have been shown to enhance the transdermal delivery of water soluble molecules such as dextrans through the epidermis of porcine skin [20]. However, the large size of the present DNA lipoplex particles compared to free DNA might reduce DNA mobility under the electrophoretic

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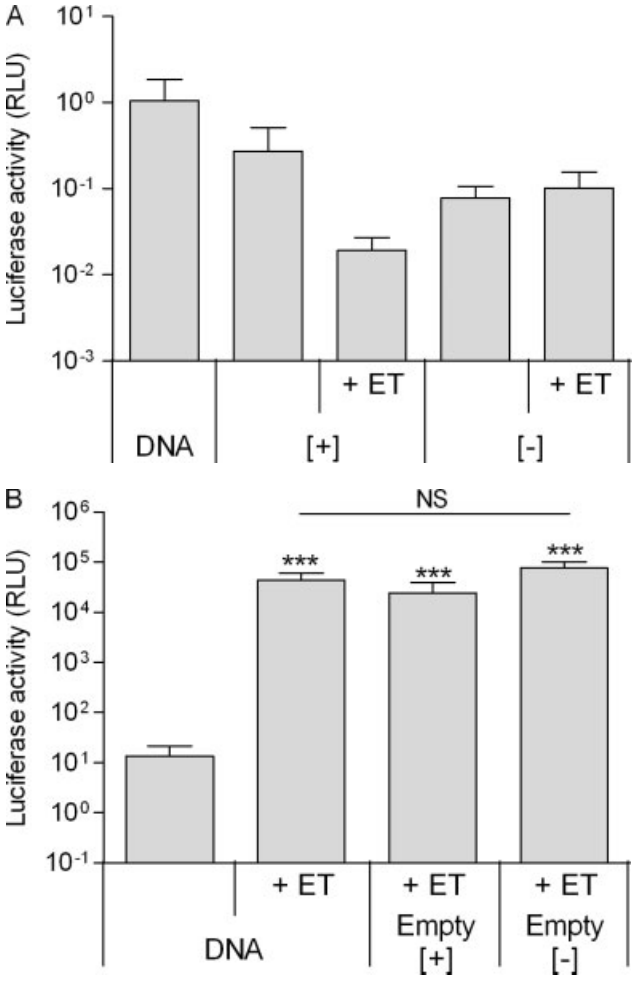

Figure 6. (A) Luciferase expression in the skin after injection of naked or formulated DNA with (+ET) or without electrotransfer ($n = 5$). (B) Luciferase expression in the skin after electrotransfer of naked DNA with or without pre-injection of empty cationic or anionic formulation ($n = 5$)

field, even though the particles display a non-neutral charge.

The fact that DNA transfection is not inhibited in the skin when injected in the presence of the lipids but not complexed tends to indicate that increased diffusion was not achieved by these lipids, in contrast to the increased diffusion reported for liposomes in the case of topical application [20]. The nature of the lipid might be an issue. Indeed, polyoxyethylene and glyceryl dilaurate, comprising the non-ionic liposomes reported in the literature [20], also appear to act as skin penetration enhancers, whereas the synthetic anionic cholesterol and cationic lipid that we used did not appear to act in the same way. A different liposome composition could be envisioned to provide both permeabilization and increased tissue diffusion.

Chemical vectors might interfere at different levels: reduce electrophoretic mobility, decrease DNA uptake by the cell during electroporation or, if incorporated into the cells, limit DNA release from the complexes and DNA nucleus uptake.

Reduced mobility was demonstrated by the retention of DNA in the wells during gel electrophoresis and from the zeta potential (electrophoretic mobility) of the particles exhibiting values of +40, +20 and −20, whereas DNA would be approximately −50 mV under these conditions.

Increased DNA uptake in the presence of electroporation could only be demonstrated for naked DNA. The uptake studies conducted *in vitro* could demonstrate an effect of the formulation on DNA membrane interaction, but not on DNA uptake, because electrotransferred DNA was efficiently internalized by itself. Only the combination of neutral complexes and electroporation appeared to increase the uptake of DNA *in vitro*, which could explain why this formulation did not inhibit DNA transfection in the muscle as much as the cationic or anionic complexes did.

Moreover, aggregates were mostly found for cationic and anionic lipoplexes, which will more than likely limit intracellular DNA release. Thiourea-based complexes were shown to release DNA more efficiently than cationic-based complexes, which should also affect the results [24]. Finally, cationic and anionic complexes were shown to form aggregates in the culture medium that obviously interfere with DNA mobility and release, leading to the inhibition of DNA transfection mediated by electrotransfer. Despite the fact that thiourea complexes led to DNA uptake in the presence of electroporation and to DNA release, no synergy could be obtained from the association of thiourea complexes and electrotransfer.

In conclusion, we have succeeded in preparing endotoxin-free, lyophilized preparations of anionic, uncharged and cationic lipoplexes for animal studies. The three formulations formed nanometer range particles in which DNA was condensed. The association of these particulate forms of DNA to physical methods such as electrotransfer reduced (or did not affect) DNA transfection compared to electrotransferred naked DNA. From these results, we conclude that, when associated with a physical delivery method, DNA should be free and mobile for the protein to be expressed.

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