Linear DNA Low Efficiency Transfection by Liposome Can Be Improved by the Use of Cationic Lipid as Charge Neutralizer

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A plasmid expressing the β-galactosidase enzyme was used to transfect Vero cells in order to evaluate the efficiency of a liposome-mediated transfection by circular and linear DNA. The results obtained showed a low rate of transfection by linear DNA:liposome complexes. To explore whether the structure of the complexes was interfering with the transfection, atomic force microscopy (AFM) was used. It has confirmed the difference between the linear and circular condensates: whereas the circular DNA:liposome complexes presented compact spherical or cylindrical structures of about 100−800 nm, the linear DNA showed pearl necklace-like structures, with pearls varying from 250 to 400 nm. On the basis of the theory proposed by Kuhn et al. (1999), low concentrations of cationic amphihile were used to neutralize or reverse the DNA charge in order to improve the transfection efficiency of the *linear* DNA. Using this method, we were able to obtain the expression of the transgene without an associated toxicity observed with the linear DNA liposome delivery.

Introduction

Transfection of DNA into eukaryotic cells has been used for different purposes. Gene therapy (*1*) and DNA immunization (*2*) are some of the clinical applications of this technology. Delivery of DNA to the target cells could be mediated by different vehicles: viral vectors, liposomes, cationic lipids, and other reagents are used to overcome the membrane barrier. The DNA transfection methods must take into account the negative charge present on the cell membrane, the stability of the DNA in the cytoplasm, the DNA transport to the cell nucleus, and the expression of the target gene (*3*).

Liposomes are one of the strategies proposed to facilitate the DNA delivery (*4*). Several studies have demonstrated that endocytosis is the major pathway through which the DNA enters the cell when associated with liposomes (*5, 6*). These results are contrary to the mechanism originally proposed by Felgner (*7*), fusion of the lipoplex with the cellular membrane followed by the release of the DNA into the cytoplasm. The use of liposomes in genetic immunization has recently been reviewed in ref *8*. The main advantages of this approach are the induction of humoral and cellular immune response greater than by the injection of DNA alone, the DNA protection from nucleases, and the priming of professional antigen presenting cells (APCs).

Besides the vehicle used for transfection, the DNA topology can influence the gene delivery and protein expression. Comparisons between circular and linear DNA have been performed demonstrating differences of the transfection efficiency (*9, 10*), as well as of the sustained protein expression using viral (*11*) and nonviral (*12*) gene delivery methods. Although circular supercoiled DNA has demonstrated a higher efficiency of transfection when analyzed by protein expressing cell number, linear DNA has been described as more stable in vivo (*12*) and in vitro (*10*) experiments. Moreover, minimal linear DNA transfection has been proposed as a method of choice to vaccinate large animals, avoiding plasmid antibiotic resistance genes (*13*).

Two main aspects of transfection were evaluated in this work: the analysis of liposome:DNA complexes, comparing linearized and circular forms, and the use of an alternative method of transfection based on minimal concentration of cationic amphiphile.

Our results indicate that under the same experimental conditions, the linear DNA-liposome mediated transfection exhibits a much lower efficiency than the complex in which DNA appears in a circular form. To evaluate the differences observed, atomic force microscopy (AFM) was used to analyze the DNA complexes. Next, following the work of Kuhn et al. (*14*), we employed a new strategy based on charge neutralization or reversal by a *minimal* concentration of cationic amphiphile. This approach demonstrated itself to be quite efficient for transfection of the linear DNA with a very low toxicity.

Materials and Methods

Cells. Vero cells (ATCC/CCL-81) used for plasmid transfection were cultured and maintained in Dulbecco's modified Eagle's medium (D-MEM, SIGMA). Cultivation was performed with 10% supplementation of fetal bovine serum (FBS) and 2% was used to maintenance of cells in D-MEM.

Plasmid. The pCH110 (Pharmacia) plasmid was used for transfection. It is a eukaryotic vector that expresses the β-galactosidase enzyme under the control of SV40 promoter. To linearize the plasmid, *Bam*HI digestion was performed. The DNA quantitation was obtained spectrophotometrically and confirmed by the comparison with a high DNA mass ladder (GIBCO BRL). This measurement was done using the Scion Image software (Scion Corporation). Briefly, a standard curve was generated with the ladder, and the bands observed were

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Table 1. DNA and Lipofectamine Ratios Used for Analysis

DNA topology	DNA (μg):Lipofectamine (μL) ratio	transfection[a]
circular	2:15	yes
linear	2:15	no
	2:30	no
	2:45	no
	2:75	yes

[a] Detection of blue cells.

measured in pixels. Finally, the pixel value obtained with the linearized plasmid was then compared to the values of the standard curve and determined by linear interpolation.

Transfection. Circular and linearized (see above) pCH110 were used to transfect Vero cells in 25 cm^2 flasks. Vero cells (5×10^5/25 cm^2) were trypsinized 15 h before transfection to obtain around 80% of confluence. Transfections were carried out with Lipofectamine (Invitrogen), as recommended by the manufacturer; we shall refer to this as the manufacturer-recommended concentration (MRC). Briefly, 2 μg of circular or linearized plasmid was incubated with 15 μL of Lipofectamine for 45 min at room temperature in a total volume of 200 μL of DMEM without serum. The complex, in a total volume of 2 mL, was then incubated with the cells at 37 °C during 5 h, when 10% FCS DMEM was added. Twenty-four hours after transfection the medium was replaced by freshly prepared 10% FCS DMEM. To evaluate transfection efficiency, the cells were submitted to detection of β-galactosidase activity by staining with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), as described previously (*15*). The percentage of blue cells expressing the enzyme was estimated by inverted optical microscopy visualization.

To overcome linearized plasmid transfection inefficiency by Lipofectamine, we used an alternative method to transfect the linearized pCH110. The theory of this method was described elsewhere (*14*). Basically, a cationic lipid, dimethyldioctadecylammonium bromide (DDAB) (Sigma), at low concentrations is used to neutralize the DNA charge. Both circular and linearized plasmid DNA were submitted separately to transfection in a proportion of 2 μg of DNA to 35 μg of DDAB. DNA and DDAB were incubated at room temperature in 1 mL of a 154 mM NaCl solution for 10 min. The next steps were the same as those described for liposome transfection.

Atomic Force Microscopy. The complex DNA:liposome was analyzed by atomic force microscopy (AFM) in a NanoScope III (Digital Instruments). Complex formation was performed in a final volume of 1 mL of Milli Q water. The proportion of DNA:Lipofectamine was the same as that used to transfect Vero cells, as well as the incubation period before analysis. The mixture was deposited in a mica support and air-dried. The D scanner with a range of 1−100 μm was used for all samples. The scanning rate was 0.5−1.0 Hz.

Gel Electrophoresis. The formation of DNA:Lipofectamine complex was also analyzed by gel electrophoresis (*16*). Linearized and circular plasmids were incubated with Lipofectamine in the proportion indicated by the manufacturer (MRC) and the conditions established for the transfection experiment. Briefly, 350 ng of DNA was incubated with 2.6 μL of Lipofectamine in D-MEM for 45 min at room temperature in a final volume of 30 μL. Different linear DNA:Lipofectamine ratios (Table 1) were also submitted to incubation to evaluate complex formation: two to five times more Lipofectamine than the MRC was used. Finally, the samples were submitted to electrophoresis on a 1% agarose gel and staining by ethidium bromide and were visualized by UV transillumination.

Results

Liposome Transfection Efficiency of Circular and Linearized pCH110. Linearized DNA has been described as more stable than a circular plasmid when used to transfect eukaryotic cells, leading to a more sustained expression of the target gene. To evaluate the transfection efficiency of the liposome-mediated methods, the two topologies were transfected using Lipofectamine. Under the same conditions, no β-galactosidase enzyme activity was observed in Vero cells transfected with the linear DNA:liposome complexes. On the other hand, using a light microscope, the presence of transfected blue cells was estimated to be around 40% for circular DNA. A vacuolization of the cells was also observed, but not with the linear DNA complex (data not shown). The toxicity associated with the circular DNA transfection provides another motivation for improving the efficiency of the methods based on the linear DNA topology.

Atomic Force Microscopy Analysis of the DNA:Liposome Complex. To evaluate whether the inefficiency of the linear DNA transfection is due to an impaired complex formation, the DNA:liposome complexes were analyzed by the AFM (Figure 1). The images obtained have demonstrated different patterns of association between the lipofectamine and the circular and the linear DNA. The circular complexes were found to be composed of compact structures with variable sizes: spheres of about 100−500 nm in diameter and cylinders of 300−800 nm long (Figure 1, A and B). On the other hand, linear DNA showed pearl necklace-like structures with pearls varying from 250 to 400 nm (Figure 1, C and D).

Eletrophoretic Mobility of Linear and Circular DNA: Liposome Complexes at Different Ratios. When associated with liposomes, the circular DNA was very efficient at transfecting the eukaryotic cells. Under the same conditions, the transfection efficiency of the linear complexes dropped all the way down to zero. To better understand this dramatic difference, an eletrophoretic mobility analysis of the two types of complexes was performed. It was found that during the electrophoresis, the circular DNA:liposome complexes were retained inside the gel. There was also no, or very little, incorporation of the ethidium bromide, signifying that the condensed DNA is fully protected (dressed) by the associated liposomes (Figure 2, lane 3).

In the case of the linear DNA, no transfection was observed when using the same amount of Lipofectamine. The AFM showed the condensate structures to be very different from those of the circular plasmid. Furthermore, during the electrophoresis there was no or very little retention of the linear DNA inside the agarose gel (Figure 2, lane 4). The incorporation of the ethidium bromide was signaled by a strong fluorescence, comparable in its intensity to that of pure linear DNA *without any* Lipofectamine (see Figure 2, compare lanes 2 and 4). This suggests that there is only a very partial blockage of the DNA by the associated Lipofectamine. This, again, is consistent with our earlier AFM observations that showed that linear DNA: Lipofectamine complexes retained their extended conformation. We next used two, three, and five times the manufactured recommended concentration (MRC) of Lipofectamine to try to transfect the linearized plasmid. The DNA retention started only when the concentration of the Lipofectamine was at least three times the MRC (Figure 2, lane 6). Furthermore, even at five times the MRC there was still some fluorescence, signifying only a partial blockage of the linear DNA by the associated Lipofectamine. The fraction of the DNA that was retained in the gel (at five times the MRC; Figure 2, lane 7) was then used

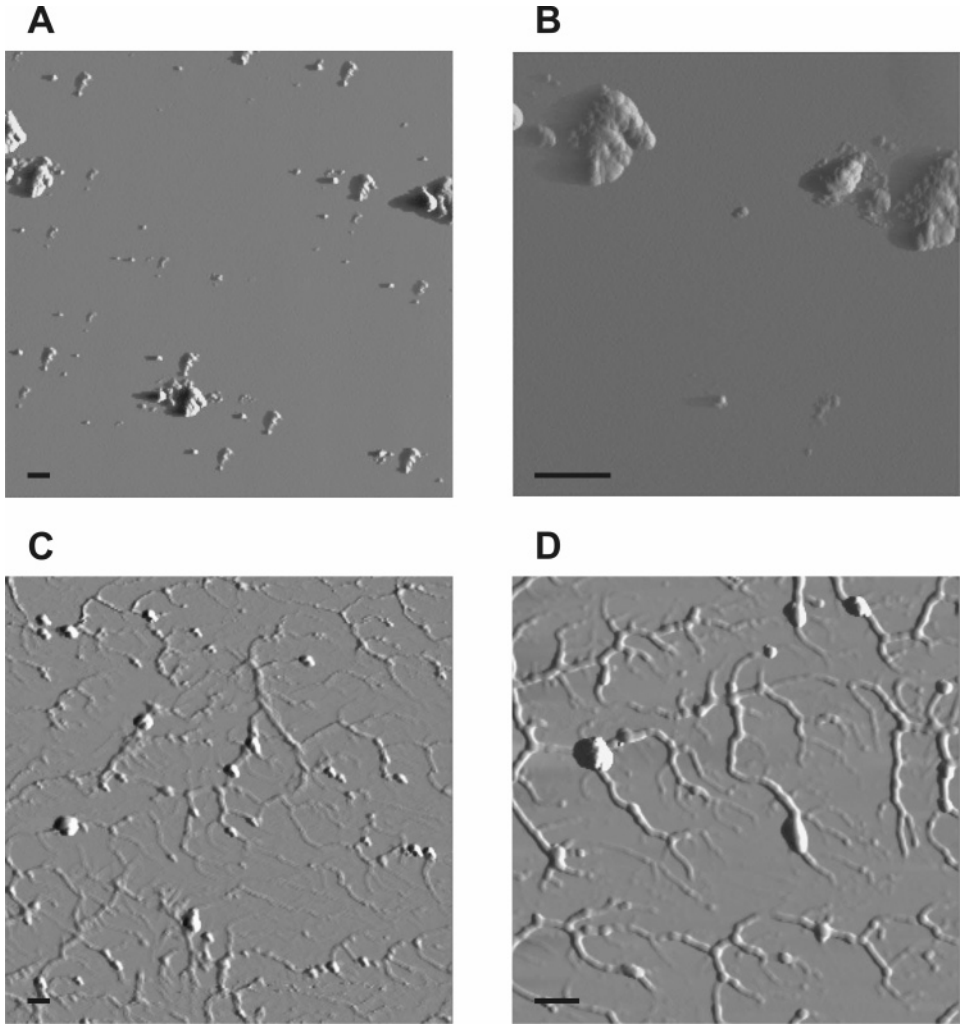

Figure 1. Analysis of DNA:Lipofectamine complexes by atomic force microscopy. Circular DNA:Lipofectamine complex images (A and B) showed irregular compact structures of 100−800 nm, and the linear DNA:Lipofectamine complex images (C and D) presented pearl necklace-like structures with pearls varying from 250 to 400 nm. The bars in the left lower corners represent 500 nm.

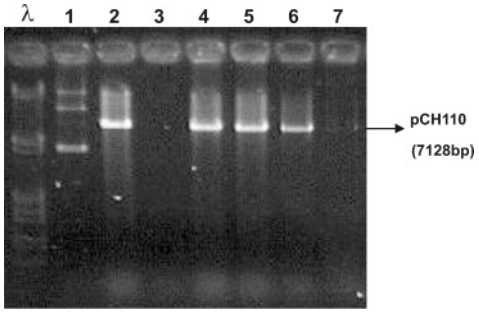

Figure 2. Eletrophoretic mobility of the linear DNA:Lipofectamine complex. Agar gel electrophoresis of the linear DNA in association with an increasing concentration of Lipofectamine (as compared to the manufacturer recommended proportions (MRC, 2 μg DNA:15 μL Lipofectamine)): 1× (lane 4), 2× (lane 5), 3× (lane 6), and 5× (lane 7). λ = DNA marker (*Eco*RI and *Hind*III digested); lane 1 = circular DNA; lane 2 = linear DNA; lane 3 = circular DNA:Lipofectamine complex (1×, 350 ng:2.6 μL).

to transfect the Vero cells. In this case, it was possible to visualize some transfected blue cells (less than 5%), however, with a very high toxicity to the cell monolayer (Figure 3A).

DDAB-Mediated Transfection. On the basis of the theory proposed by Kuhn and colleagues (*14*), Vero cells were also transfected with the linear DNA and the amphiphile molecule DDAB. Ten to fifteen percent of the cells were expressing β-galactosidase activity, as detected by incubation with the substrate. Moreover, the pattern of expression was different when compared to the one observed with the Lipofectamine transfected cells (using circular plasmid), for which the blue intensity and the distribution in the cytoplasm were higher (Figure 3B). The DDAB transfected cells presented a less intense color with a more granular distribution (Figure 3C).

Discussion

The low efficiency of the DNA uptake and its stability in the cytoplasm of the transfected eukaryotic cells are some of the major causes of low protein expression, despite the amount of injected DNA (*17*). Endocytosis has been proposed as the mechanism through which the DNA:liposome complexes enter the cells. The lipid composition and the vesicle size are both important for the transfection efficiency (*18*). A combination of neutral and cationic lipids has been used to avoid the DNA degradation (*19*) as, for example, in the case of Lipofectamine, the commercial liposome used in this work.

The complex size is also an important feature for an efficient cell transfection. The estimated size of the liposome:DNA condensates is around 400 nm (*20*). Our AFM analysis, however, showed very different patterns of the DNA complexation when comparing circular and linear topologies: large compact aggregates were observed with circular DNA, and necklace-like structures with the linear plasmids. The different geometry of the condensates might also account for the dramatic disparity

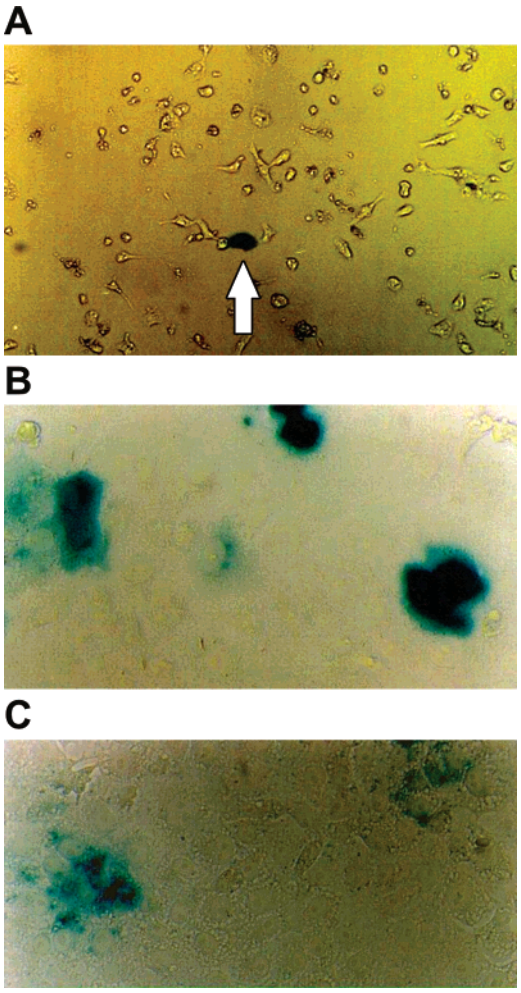

Figure 3. Vero cells transfected by Lipofectamine (A and B) and DDAB (C). Excess of Lipofectamine (5 × MRC) was shown to be toxic to the cells as visualized by the cell morphology and the integrity of the monolayer (A; 100×). This, however, was the concentration necessary to obtain blue cells (white arrow) when using linear DNA. Circular DNA:Lipofectamine complex (1 × MRC) transfection showed a more intense staining (B; 320×), when comparing to DDAB transfection presenting a granular staining pattern (C; 320×). In both cases (B and C), cell monolayer integrity was maintained.

in the transfection efficiency between the two topologies. While the transfection of the more compact circular plasmids is likely to go through endocytosis, the pathway of entry of extended linearized DNA aggregates might be quite different.

To improve the linear DNA transfection efficiency, we have tried to increase the concentration of the Lipofectamine by as much as 5-fold the MRC value. Except for a higher toxicity, no improvement in the transfection efficiency was observed.

Considering the minimal linear DNA as a better choice for the purpose of the DNA vaccination (*12*, *13*) and a very high toxicity of the liposome formulations for its transfection, the use of other vehicles must be explored. One such possibility has been studied in this work: use of the *minimal* concentration of the cationic lipid. It should be noted that the cationic lipid chosen by us, DDAB, also takes part of the liposome formulation (*16*), however, at concentrations 2 orders of magnitude higher than the ones used by us, 55 μM as compared to 13.4 mM for the *same* quantity of the DNA. Therefore, one advantage of using amphiphile molecules at low concentrations is the reduced toxicity and increased efficiency in the case of *linear* DNA transfection. Again, we would like to stress that to transfect linear DNA using Lipofectamine was practically impossible.

When comparing liposome and DDAB transfected cells with circular DNA, a different pattern of β-galactosidase expression was observed with light microscopy visualization. The blue staining was more intense with the liposome than with the DDAB-mediated transfection. This and the inefficacy of Lipofectamine in transfecting linear DNA suggests different pathways of DNA cellular uptake between the two methods. In addition, the analysis of ethidium bromide incorporation and the electrophoresis of the linear DNA:DDAB complexes showed no differences compared to DNA alone (data not shown), suggesting that the linear DNA:DDAB complexes retain their noncompact structure. More studies are necessary to elucidate the mechanism by which the linear DNA:DDAB complexes enter the cells.

To conclude, use of amphiphile molecules at low concentrations can be a viable alternative for transfecting linear DNA without a high toxicity associated with the liposome-mediated delivery.

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