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An Insight into the Use of Cationic Peptides for Plasmid DNA Delivery in Cells

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ABSTRACT

In this work, we contribute an insight into the ability of cationic peptides for the delivery of plasmid DNA in cells. Although most peptides used for cellular transfection are cationic, not all of them possess this potential. Using plasmid DNA bearing reporter genes and cells of the breast cancer MDA-MB 435 line, we show at first that only peptides in an α-helical structure can give high levels whereas peptides with a β-strand structure cannot. Amphipathic peptides rich in lysine, namely $L_{10}K_5$ or $L_{13}K_6$, adopting both an α-helical structure are able to be used for this task. Subsequently, we show that protamine, equally rich in basic arginine, but not having an α-helical structure, cannot alone efficiently deliver DNA. However, it improved the transfection level by cationic liposomes, undoubtedly by a condensing effect. This enhancement in transfection by protamine was not observed using the peptide $L_{13}K_6$ and this peptide did not behave as protamine to enhance the transfection level of cationic liposomes.

Keywords: cancer cells, cell penetrating peptides (CPP), DNA delivery, protamine

Abbreviations: CMV, cytomegalo virus; **CPP**, cell penetrating peptides; **DMEM**, Dulbeco's modified Eagle medium; **DMHAPC-Chol**, 3β[*N*-(*N*′,*N*′,*N*′-dimethylhydroxyethylaminopropane)-carbamoyl] cholesterol iodide; **DMSO**, dimethylsulfoxide; **DOPE**, dioleoyl phosphatidyl ethanolamine; **DTT**, dithiothreitol; **FCS**, fetal calf serum; **EGFP**, enhanced green fluorescent protein; **FTIR**, Fourier transform infrared spectroscopy; **LD**, complex cationic liposomes – DNA; **LPD**, complex of liposomes with DNA precondensed with protamine; **Lip(+)**, cationic lipid; **MTT**, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; **NLS**, nuclear localization sequence; **O.D.**, optical density; **ODN**, oligodeoxyribonucleotide; **PBS**, phosphate buffer saline; **pCMV-β**, plasmid containing β-galactosidase gene, promotor cytomegalovirus; **pD**, complex peptide LK with DNA; **PD**, complex protamine-DNA; **pPD**, complex of peptide LK with DNA precondensed with protamine; **RLU**, relative light unit; **TE**, Tris-EDTA buffer

INTRODUCTION

During the past two decades, several works have been devoted to the research of non-viral carriers for efficient DNA delivery. The advantages over viral vectors include weak toxicity, reduced immunogenicity and thus safety in their use (Schatzlein 2001). The area includes almost cationic liposomes and cationic polymers (Felgner *et al*. 1994; Boussif *et al*. 1995). In association with these carriers, peptides were initially used in a minor role for targeting (Shadidi and Sioud 2003), while the employment of cationic peptides to carry DNA was dealt with in parallel (Wyman *et al*. 1997; Planck *et al*. 1999) but has only recently been intensively investigated. In this paper, using two typical approaches, we will contribute an insight into the use of peptides for DNA delivery.

The first concerns the true transport and delivery of DNA by peptides. Since protein transduction domains (PTD) *tat* isolated from (HIV)-1 TAT (fragment 48-60) and penetratin from *Drosophila* Antennapedia homeodomain (fragment 43-58) were shown to be able to transport proteins and deliver them in an active form, there was an increase in the number of papers devoted to peptides for delivery in living cells (Gratton *et al*. 2003; Ziegler *et al*. 2005; Zorko and Langel 2005). These were developed from membrane-permeable carrier peptides, known as cell penetrating peptides (CPP) (Castano *et al*. 1999; Richards *et al*. 2005; Sauer *et al*. 2005; Kerkis *et al*. 2006). They were generally positively charged peptides, shorter than 30 amino acids, able to penetrate cell membranes and translocate different cargoes such as peptides, liposomes, oligonucleotides etc… into cells (Futaki *et al*. 2002; Coeytaux *et al*. 2003; Console *et al*. 2003; Albertshofer *et al*. 2005; Borghouts *et al*. 2005; Herbic *et al*. 2005; Melikov *et al*. 2005; Zorko and Langel 2005; Kerkis *et al*. 2006). The potential of CPP to deliver nucleic acids into cells has been reported (Planck *et al*. 1999) but still rarely. Most non-viral DNA carriers or peptides for DNA delivery were designed on the basis of their cationic character to load negatively charged nucleic acids by electrostatic binding (El-Aneed 2004), and cellular delivery of DNA was assayed using oligo-arginine peptides, histidine-rich or lysine-rich peptides (McKenzie *et al*. 2000; Rittner *et al*. 2002; Kim *et al*. 2003; Kichler *et al*. 2006).

The second concerns also the use of peptides for DNA delivery – but not directly – and necessitated a carrier such as a cationic liposome. This includes peptides which serve as a ligand, associated to the carrier for cellular targeting (Slimani *et al*. 2006) and signal peptides, associated to DNA, which, once internalized in the cell by the carrier, could conduct DNA towards the nucleus or towards the mitochondria (Zanta *et al*. 1999; Geromel *et al*. 2001).

In a previous work, some of us observed that a leucine- and lysine-rich peptide of sequence KLLKLLLKLLLKLLK ($L_{10}K_5\alpha$ or LK15α) bearing 15 amino acids, with 5 cationic charges on lysines, was able to deliver oligodeoxynucleotides (ODN) into cells (Boukhalfa-Heniche *et al*. 2004; Hernandez *et al*. 2006). In this work, we will show that this peptide can be used for delivery of plasmid DNA of 7100 base pairs. In a comparative study with a LK-peptide having the same number of amino acids (i.e. 15), but with a

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sequence favouring a β-strand conformation, we will demonstrate that the α-helical conformation of the peptide was the essential condition for the delivery of plasmid DNA in cancerous MDA-MB 435 cells.

The main directive idea often associated with the transport of DNA by cationic carriers was the condensation of DNA (Tecle *et al*. 2003). Using protamine, rich in arginine and well known for its effect on DNA compaction (Herskovits and Brahms 1976), we will show that such a condensation was not sufficient for plasmid DNA transport. A comparative study of CD spectra of protamine and LK19α will provide proof in favour of the structural effect of the peptide on transfection. However, this condensation allowed to improve the cellular delivery of DNA by cationic liposomes (Li and Huang 1997; Harvie *et al*. 2003), and we will discuss how it was possible to use protamine for DNA delivery.

MATERIALS AND METHODS

Reagents

OPTIMEM, glutamax-containing Dulbeco modified Eagle medium (DMEM), fetal calf serum (FCS), penicillin/streptomycin, trypsine-EDTA and phosphate-buffered saline (PBS) were obtained from In Vitrogen (Paisley, UK). Reagents were analytical grade and purchased from Sigma-Aldrich (Saint-Louis, MI), Carlo Erba (Milano, Italia) and Promega (Madison, WI). All other chemicals were of the purest grade available. Dioleoyl phosphatidyl ethanolamine (DOPE) was purchased from Avanti Polar Lipids. Cationic lipid 3β[*N*-(*N*′,*N*′,*N*′-dimethylhydroxyethylaminopropane)-carbamoyl] cholesterol iodide (DMHAPC-Chol) was synthesized and liposomes were prepared with DOPE as described previously (Percot *et al*. 2004).

Peptides LK15α, LK15β, LK19α and protamine

Three peptides were studied and denoted as
LK15α or $L_{10}K_5α$ KLLKLLLKLLLKLLK α-helical structure
LK19α or $L_{13}K_6α$ KLLKLLLKLLLKLLLKLLK α-helical structure
LK15β or $L_7K_8β$ KLKLKLKLKLKLKLK β-strand structure

They were synthesized at the Institut Pasteur (Paris) following a solid phase Fmoc procedure as previously described in detail (Boukhalfa-Heniche *et al*. 2004). Because of their low water solubility, all the peptides were dissolved in methyl alcohol (MeOH), generally at the concentration of 1 nmol/µl of cationic charge.

Peptides were characterized by mass spectrometry. Moreover, the secondary structures of LK15α and LK15β were investigated by CD and Raman spectroscopies (Boukhalfa-Heniche *et al*. 2004; Hernandez *et al*. 2006).

Protamine sulfate was purchased from Sigma (Ref. P4505-1G). That is an arginine-rich mixture of three peptides, YI, YII and Z, belonging to the family of clupeine, formed of 31-32 amino acids of sequence

YI	$AR_3RS_3RPIR_4PR_3TTR_4AGR_4$	20 R residues
YII	$PR_3TR_2ASRPVR_4PR_2VSR_4AR_4$	20 R residues
Z	$AR_4SR_2ASRPVR_4PR_2VSR_4AR_4$	21 R residues

There are 20-21 arginine residues in each molecule. The product is soluble in water.

Plasmid DNA, reporter genes pCMV-βgal and complexes with peptides or liposomes

pCMV-βgal plasmid used was a plasmid of 7.164 kb containing the β-galactosidase reporter gene sequence under the control of the cytomegalovirus promoter (pCMV) (Clontech, Palo Alto, CA). Plasmids were amplified in JM109 stain of *Escherichia coli*, and purified using a Qiagen Plasmid Maxi Kit (Qiagen GmbH, Hilden, Germany). The concentration of plasmid DNA was measured by UV absorption at 260 nm and plasmid DNA purity was controlled using the A_{260}/A_{280} ratio and agarose gel electrophoresis. Plasmids were conditioned in Tris-EDTA buffer (pH 8).

For the study of peptides, protamine or cationic liposomes as carriers, plasmid (0.1 µg/µl in water) was complexed at desired ratios by mixing with peptide (in MeOH), protamine (in water) or cationic liposomes (aqueous solution).

For the study of the effect of protamine on the transfection by cationic liposomes or peptides, aqueous plasmid was mixed with aqueous protamine before the complexation with cationic liposomes or peptides.

Circular dichroism spectroscopy

Samples were diluted at a concentration of 0.15 mg/ml, equivalent to 36 µM for protamine and 100 µM for LK19α.

Circular dichroism (CD) spectra from peptide samples were analyzed in the 180-250 nm range (bandwidth 1 nm) on a JASCO J-810 spectrophotometer equipped with a Peltier accessory. Samples were placed in suprasil quartz cells (with path lengths 0.01 mm or 1 mm). Each spectrum corresponds to the average of 5 scans with a speed of 100 nm/min (5 min of accumulation). CD spectra of the buffer were used as the baseline in all experiments. The measured ellipticity for each sample, $[\theta]_{observed}$, was normalized to obtain the so-called mean residue ellipticity, $[\theta]$, by using the expression : $[\theta]=[\theta]_{observed}$ /10ncl, where n, c, and l are the number of residues in the peptide, the molar concentration and the path length of sample, respectively. Normalized ellipticity was expressed in deg cm^2 $dmol^{-1}$.

Cells and growth conditions

MDA-MB 435 cells were grown on plastic ware in monolayers at 37°C in a humid atmosphere containing 5% CO_2 in air. The glutamax-containing culture medium DMEM (In Vitrogen) was supplemented with fetal calf serum (FCS, 10%) and penicillin/streptomycin (50 U/ml).

Transfection protocol and transfection level measurements

In order to determine the transfection levels, cells were seeded in a 6-well Falcon plate (4.10^5 cells per well) on the day preceding transfection. Just before transfection, culture medium was replaced with 1 ml OPTIMEM without serum, and 20 µl peptide-plasmid complexes at desired charge ratio X (containing 3 nmoles of plasmid) were added. After 6 hours of incubation, OPTIMEM was removed, replaced with culture medium containing serum. After 48 h, cells were twice washed with PBS and the transfection level was measured using the chemiluminescence of β-galactosidase in the presence of APMGD (3-(4-methoxyspiro(1,2-dioxetane-3,2′-tricyclo(3.3.1.1)decan)-4-yl)phenyl-β-D-galactopyranoside) substrate with a Tropix Galactolight Plus kit (Applied Biosystems, Bredford, MA). Following the procedure of the supplier, the transfected cells were lysed with 200 µl of a lysis solution containing 0.5 mM of dithiothreitol (DTT) freshly added. After, 20 µl of the cell extract was incubated with 200 µl of the chemiluminescent substrate reagent (diluted to 1%) before 300 µl of the light emission accelerator reagent was added. Immediately after the mix, luminometric measurement was made using a BCL luminometer (Gouteyron Technologies, Vals le Puy, France) operating at integration mode for 10 seconds. Protein was titrated by using the Bio-Rad DC Protein assay kit (Hercules, CA), in order to normalize results expressed in relative light unit per mg of protein (RLU/mg) as described previously (Percot *et al.* 2004). The base level of untransfected cells was measured and subtracted.

RESULTS

Circular dichroism results: structures of LK19α and protamine

CD spectra of protamine and LK19α are displayed in **Fig. 1**. These results clearly show that protamine is rather unstructured in water, as confirmed by a negative signal peaking at *ca*. 198 nm. However a long high wavenumber tail follows the negative peak, leading us to think about the existence of some minor populations of ordered structures (presumably

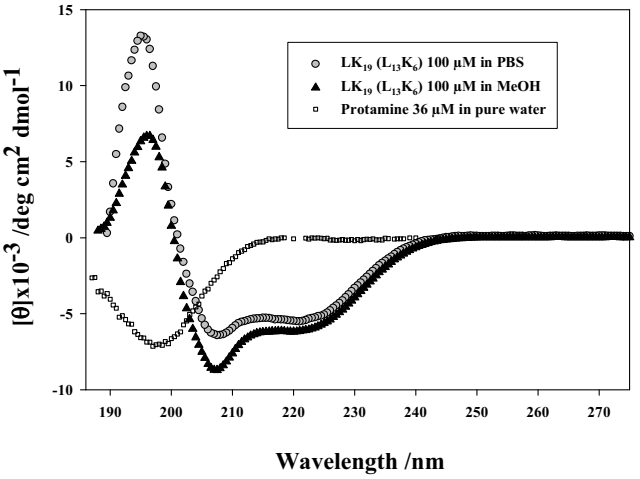

Fig. 1 CD spectra of LK19α, 100 µM in MeOH and in PBS and protamine, 36 µM in water. See text for experimental procedures.

β-type conformers) in aqueous solution. In contrast, the 19-mer LK-peptide adopts an α-helical conformation, as manifested by two negative bands at *ca*. 208 and 222 nm. The α-helical structure of this peptide is shown to be independent of its environment in solution (pure water, methanol, PBS, phosphate buffer, etc.), as it is also the case for the 15-mer LK15α (see for more details, Boukhalfa-Heniche *et al*. 2004; Hernandez *et al*. 2006). We have also previously shown that LK15β has a natural tendency to adopt a secondary structure belonging to the β-family (Hernandez *et al*. 2006), on the basis of the results obtained by optical spectroscopy.

Transfection level: Effect of helical secondary structure and chain length of the LK peptides

Transfection of MDA-MB 435 cells using peptides LK15α, LK15β and LK19α was carried out and β-galactosidase activity levels were measured.

Fig. 2 represents the transfection level of peptides LK15α, LK15β, LK19α and protamine in MDA-MB 435 cells. The results of β-galactosidase activity 48 hours after transfection are indicated. The level of naked DNA was negligible, showing that without carrier, the plasmid was not internalized in these cells. Results for peptide/DNA complexes with molar charge ratios X comprised in the range from 1 to 5 showed that the β-galactosidase activity was optimal with approximately X = 3, illustrated for LK19α.

A comparative transfection of LK15α in an α-helical form and another, LK15β, in a β-form (sequence KLKLKL KLKLKLKLK) has been undertaken (**Fig. 2A**) in order to study the effect of the peptide conformation on the ability of cellular internalization. The structures of these peptides were studied by circular dichroism spectroscopy (Boukhalfa-Heniche *et al*. 2004). In the same experimental conditions for transfection of MDA-MB 435 cells as described above and with the same molar charge peptide/DNA ratio X = 3, the results in **Fig. 2A** undoubtedly indicated that only the sequence inducing the *alpha* form gave a high level of transfection, while the peptides LK15β with a β-strand structure did not result in a noticeable level.

On the other hand, an effect of the peptide length on the cellular permeation of peptide alone was reported (Castano *et al*. 1999). Such an effect on the delivery of plasmid DNA was observed by studying another peptide with 19 amino acids, LK19α, (sequence KLLKLLLKLLLKLLLKLLK with 6 cationic charges) synthesized and assayed in comparison with LK15α. The results in **Fig. 2B** showed that the peptide length constitutes another factor affecting the transfection level. Measurements of the β-galactosidase activity induced by the longer chain LK19α also with an α structure, indicated a clear enhancement effect. The level obtained with LK19α was 2.5-fold better than LK15α.

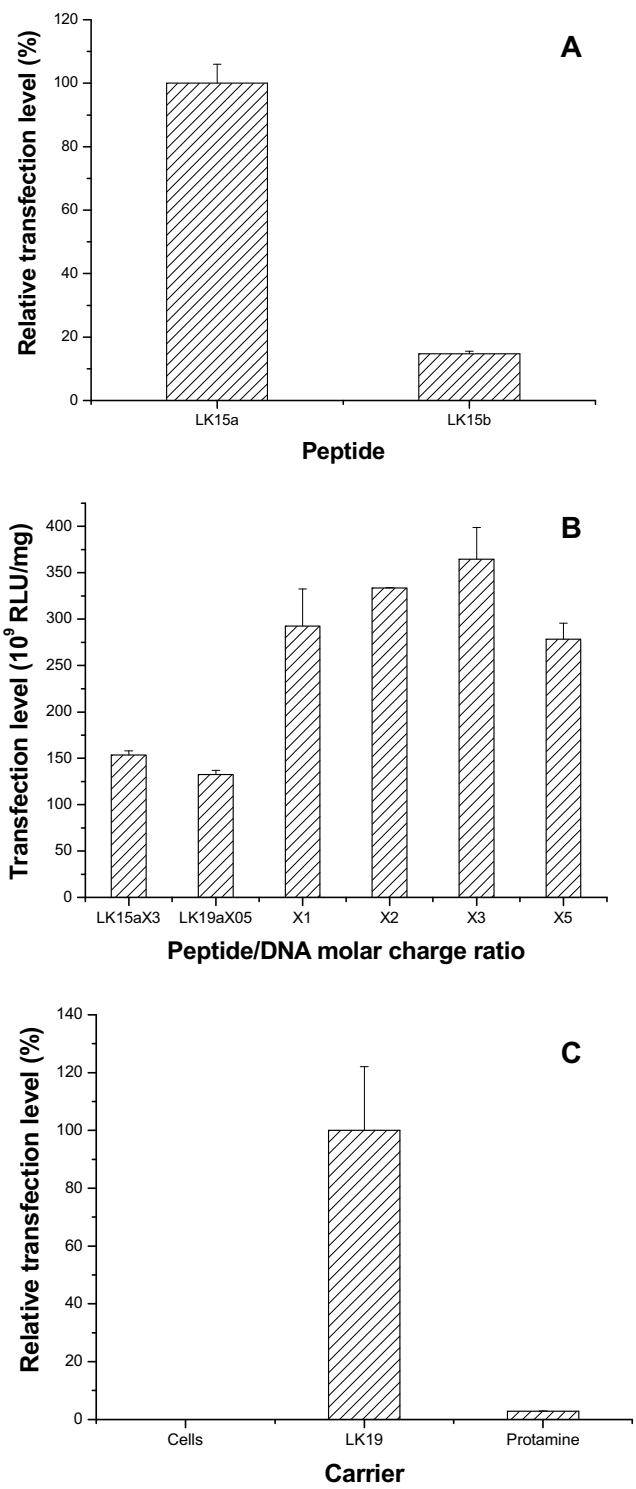

Fig. 2 Transfection level in MDA-MB 435 cells, 48 h post transfection using (**A**) Peptide LK15α compared with LK15β molar charge ratio peptide/DNA X = 3. (**B**) Peptides LK19α and LK15α. Molar charge ratio peptide/DNA (pD) X varying from 0.5 to 5. For both peptides LK19α and LK15α the optimal levels were observed with X = 3 and were compared. (**C**) Peptide LK19α compared with protamine (P). Protamine/DNA (PD) w/w ratio r = 2. Other experimental conditions as described in the text. Bars correspond to ± SD of 3 or 6 experiments.

Comparison of transfection levels by LK19α and protamine

Now we consider a natural basic peptide, protamine. That is an arginine-rich peptide. When complexed with plasmid pCMV-β following the protocol as with LK19α, in varying the protamine/DNA weight ratio r up to 5, any of these complexes did not give a noticeable transfection level in

MDA-MB cells after 48 h (**Fig. 2C**). This indicated that protamine can not used alone as a carrier for DNA.

As discussed below, this difference which is similar to that between levels of LK15α and LK15β suggested us to investigate the structures of protamine and LK19α by circular dichroism spectroscopy. In the conditions used for transfection in this work, results as indicated in **Fig. 1** showed that LK19α has an α-helical structure, whereas protamine did not show any bands characteristic of an α-helical structure. Such an α-helical conformation of LK19α seems to be the key structural factor for the uptake of the DNA/peptide complex (Rittner *et al*. 2002; Kerkis *et al*. 2006).

Protamine enhanced the transfection level of cationic liposomes

Although the complex of protamine alone with DNA did not deliver the latter in MDA-MB 435 cells, this complexation prior to mixing with cationic liposomes enhanced their transfection level as indicated in **Fig. 3A**. For this study, cationic liposomes DMHAPC-Chol/DOPE 1:1 were used and the transfection levels were measured 48 h after incubation. As can be seen in this Figure, where the molar charge ratio X' (cationic lipid/DNA) was kept constant and equal to 2, complexes with various w/w ratios r (protamine/DNA) prior to be mixed with cationic liposomes gave levels much higher than that of liposomes. This enhancement was in accordance with the improvement by protamine in the lipid-protamine-DNA complex, known as LPD (Li and Huang 1997) and investigated by other workers.

Protamine did not enhance the transfection level of LK19α

The same experiment was carried out with peptide LK19α instead of cationic liposomes DMHAPC-Chol. As indicated in **Fig. 3B**, the complexation of protamine with DNA prior to the mix with peptide LK19α did not enhance the transfection level compared to peptide. This Figure also showed that complexes with increasing w/w ratios of protamine/DNA prior to mixing with LK19α gave levels even lower than that obtained with LK19α. One of the reasons may be the competition of protamine with LK19α – since both of them are positively charged – in the association with negative DNA.

Effect of LK19α on the transfection level of cationic liposomes

The last question was to examine whether the enhanced effect on cationic liposomes DMHAPC-Chol/DOPE was also observed when using LK19α in place of protamine. For this, LK19α was complexed with DNA (charge molar ratio X) prior to being mixed with liposomes (charge ratio cationic liposome/DNA X' = 2) before transfection of MDA-MB 435 cells. Results in **Fig. 3C** showed that, compared with cationic liposomes, at small ratios of peptide LK19α /DNA (X = 1), the level was enhanced but at higher ratios, the level was decreased to a level lower than that of liposomes. This decrease was not observed for protamine. It may be that a competition of two cationic species, DMHAPC-Chol and LK19α, occurred. Thus, although both LK19α and protamine are cationic peptides, there was a difference in the behaviour of LK19α compared with protamine.

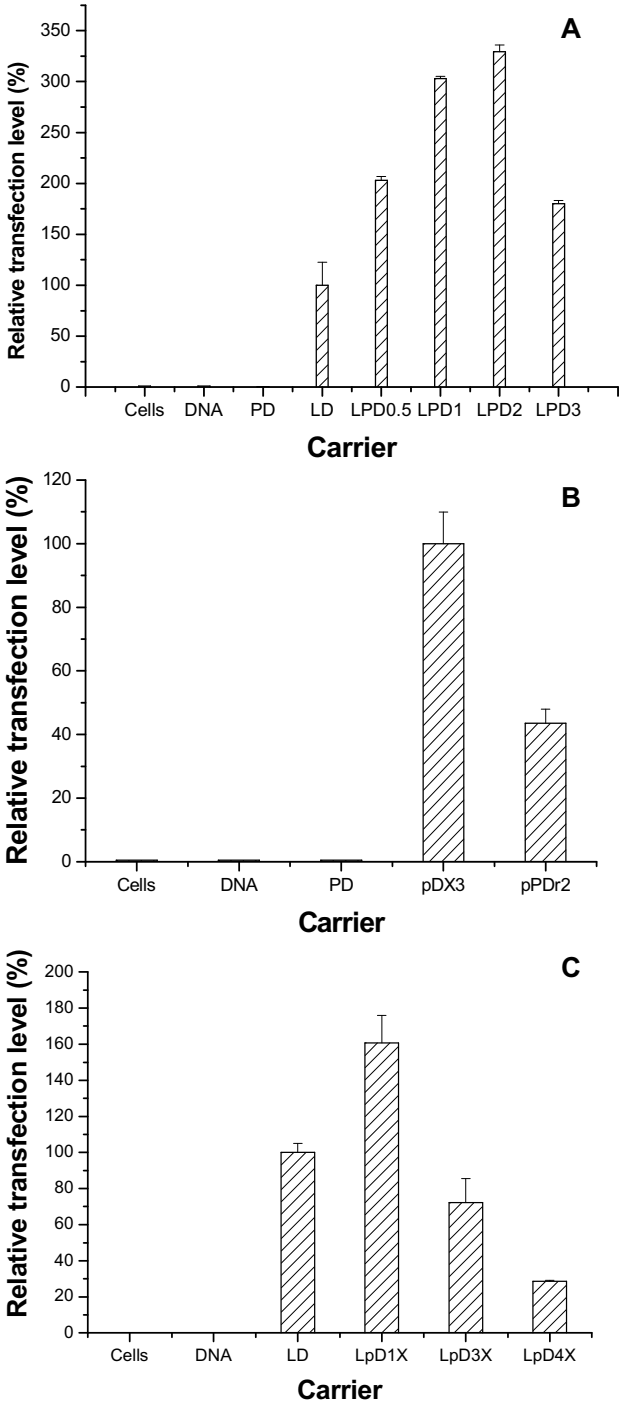

Fig. 3 Effect of precondensation of DNA (D) by protamine (P) on the transfection level in MD-MB 435 cells 48 h post transfection by (**A**) cationic liposomes DMHAPC-Chol/DOPE 1:1 (L) and (**B**) cationic peptide LK19α. (**C**) Effect of precondensation of DNA by cationic peptide LK19α (p) on the transfection level using cationic liposomes DMHAPC-Chol/DOPE 1:1 (L). Molar charge ratio Lipid/DNA (LD) X' = 2. Molar charge ratio peptide/DNA (pD) X = 3; w/w ratio protamine/DNA (PD): r = 2. Other experimental conditions as described in the text. Experiments were in duplicate.

DISCUSSION AND CONCLUSION

The above results proved that peptides containing basic amino acids such as lysine or arginine are potentially able to be used for the delivery of plasmid DNA into cells. The main observation was that a sequence allowing an α-helical structure was necessary for a peptide to be an efficient carrier. That was the case of LK15α and LK19α having a longer chain and giving a 2.5-fold level better than LK15α.

Concerning protamine, which was not in an α-helical structure, although its high potential to condense DNA (Herskovits and Brahms 1976), this condensation alone is not sufficient for an efficient delivery into MDA-MB 435 cells. This may be explained by the fact that in the complex with DNA, protamine preserved the B-form structure of DNA but was not in an α-helical structure (Herskovits *et al*. 1976). Moreover, as was mentioned by Tsuchiya (2006) for HeLa and A549 cells, the low uptake may also probably be due to the retention in the endosomes after internalization in the cell.

Although protamine alone can not deliver plasmid DNA,

this peptide improved the transfection of cationic liposomes. The improvement observed can be explained by a synergistic effect contributed by protamine and cationic lipids. Protamine precondensed the plasmid and then cationic liposomes, besides their own ability to carry DNA to cross the plasma membrane, promoted the release of DNA from the endosomes (Zelphati and Szoka 1996). This improvement may also be attributed to the presence of nuclear localization sequence of arginine in the molecule and the protection against DNAse degradation (Tsuchiya *et al*. 2006).

It is also important to note that cationic peptides were not used at a stochiometric ratio with DNA but in excess. It may be that in the case of cationic peptide LK19α, the molecules in excess disturbed the plasma membranes and prepared the way for the internalization of complexes with DNA. This argument conveniently explains why LK19α, with an α-helical structure can be used for an efficient transfection while protamine, although in excess, but without an α-helical structure, can not.

In conclusion, for cell-penetrating peptides or cationic liposomes, the ability to cross the plasma membrane is obviously essential for the cellular internalization of DNA. However, we think that cationic peptides such as LK19α or protamine can also be used for DNA delivery in cells, but each peptide has its ability, the former directly acting as a carrier, the latter indirectly contributing by a precondensation prior to the association with cationic liposomes.

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