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Cellular entry pathway and gene transfer capacity of TAT-modified lipoplexes

Roosmarijn E. Vandenbroucke *, Stefaan C. De Smedt *, Joseph Demeester, Niek N. Sanders

Laboratory of General Biochemistry and Physical Pharmacy, Ghent University, Harelbekestraat 72, B-9000 Ghent, Belgium

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Abstract

Several reports have shown a fast and efficient translocation of TAT-modified lipoplexes and particles into the cell cytoplasm. However, neither the uptake mechanism nor the biological effect of TAT-modified lipoplexes has been studied in detail. In this report we show that the increase in gene transfer of TAT-modified lipoplexes depends on the amount of cationic lipid in the lipoplexes and on the way TAT was coupled to the lipoplexes. We demonstrate that the cellular uptake of both TAT-modified and unmodified lipoplexes is very fast and, in contrast to previous publications, temperature-dependent. Additionally, after internalization TAT-modified as well as unmodified lipoplexes end up in lysosomal vesicles, indicating the involvement of clathrin-mediated endocytosis. Furthermore, chlorpromazine, a specific inhibitor of clathrin-dependent endocytosis, strongly inhibits the cellular uptake and biological activity of both the TAT-modified and unmodified lipoplexes. We also found that the uptake and biological activity of these lipoplexes are diminished when cholesterol in the cell membrane was bound by filipin, an inhibitor of the lipid-raft mediated pathway. Considering these data, we conclude that TAT-modified and unmodified lipoplexes are mainly internalized via a cholesterol-dependent clathrin-mediated pathway.

Keywords: Gene therapy; Endocytosis; Clathrin; Lipid rafts; Lysosome; TAT

1. Introduction

Gene therapy may become an important strategy in the treatment of various diseases. However, the clinical applications of gene therapy depend largely on the development of suitable gene transfer vehicles. Although generally not as efficient as viral vectors, non-viral systems, such as lipids, have the advantages of being less toxic, safer, non restrictive in DNA size, potentially targetable, and easy to produce in relatively large amounts. However, poor transfection efficiencies presently limit their usefulness for gene therapy applications, caused by critical steps such as endosomal release and nuclear uptake [1].

Viruses, such as influenza viruses and adenoviruses, have developed peptides that warrant their escape from endosomes by membrane fusion or disruption upon the acidification of the endosomes. Such viral peptides and also synthetic polymers capable to disrupt endosomes upon acidification have been applied to enhance non-viral gene delivery (reviewed by [1–4]). However, it has been shown that an excessive disruption of endosomes by endosomolytic agents may be harmful to the cell [5]. Alternatively, to circumvent the endosomal escape barrier, it has been proposed to bypass the endosomal pathway by using cell penetrating peptides (CPPs) that rapidly translocate cargoes across the cell membrane [6–8]. Peptides derived from the V-1 transactivator of transcription protein or 'TAT', have been shown to facilitate both *in vitro* and *in vivo* the intracellular delivery of cargoes of various sizes and physicochemical properties (recently reviewed by [8–10]. These TAT peptides, typically having a positive charge at physiological pH, consist of a short stretch of basic amino acids (GRKKRRQRRR), which is responsible for the translocation across cellular mem-

* Corresponding authors. Tel.: +32 9 2648047; fax: +32 9 2648189.
E-mail addresses: Roosmarijn.Vandenbroucke@UGent.be (R.E. Vandenbroucke), Stefaan.DeSmedt@UGent.be (S.C. De Smedt).

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branes and is called the protein transduction domain (PTD) sequence [11]. Additionally, this sequence also functions as a nuclear localization signal [12].

Initially it was believed that the uptake of TAT peptide or TAT peptide conjugated cargoes occurs in a receptor-independent [13,14] and temperature-independent manner [11,15–17]. However, this is currently questioned by the increasing evidence that the original data might originate from artefacts in the experimental procedure for fixation and removal of membrane-bound material [18]. Until now, the precise entry mechanism of TAT peptides and TAT peptide conjugated cargoes is still controversial (reviewed by [19]). Both clathrin-mediated endocytosis [20–23], caveolae-mediated endocytosis [24–27] and macropinocytosis [14,28] have been suggested. Possibly the mechanism of cellular entry depends on the cell type, the TAT sequence, physicochemical properties of the cargo, the amount of TAT peptides on the surface of the cargo, and the way TAT peptides are bound to the cargo [9].

The ability of TAT peptides to translocate cargoes across cell membranes is an attractive feature that could bring significant advantages to the cellular delivery of proteins, therapeutic DNA or small interfering RNA (siRNA) molecules [24,29,30]. However, in contrast to TAT peptides conjugated to proteins, the biological effect and the cellular entry mechanism of TAT/plasmid DNA (TAT/pDNA) conjugates and in particular TAT-modified lipoplexes [30–33] have not been studied in detail. Therefore, in this work we aimed to determine (1) whether the attachment of TAT peptides to lipoplexes could enhance their transfection efficiency, (2) whether the uptake of the TAT-modified lipoplexes is energy dependent, and (3) the cellular entry mechanism and intracellular routing of the TAT-modified lipoplexes.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), L-glutamine, heat-inactivated fetal bovine serum (FBS), and penicillin–streptomycin were supplied by GibcoBRL (Merelbeke, Belgium). The TAT peptide AA 37–72 (CFITKALGISYGRKKRRQRRRAPQGSQTHQVSLSKQ) was obtained from the Centralised Facility for AIDS Reagents, EU Programme EVA (European Vaccine Against AIDS). The secreted alkaline phosphatase (SEAP) expression plasmid (pMet7 hβ_c SEAP) was a kind gift from Prof. Tavernier (Ghent University, Belgium). 1,2-Dioleolyl-3-trimethylammonium-propane (chloride salt) (DOTAP), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-[maleimide(polyethyleneglycol)2000] (DSPE-PEG_{2000}-MAL) were purchased from Avanti Polar Lipids (AL, USA).

2.2. Cell culture

Cos-7 cells (African green monkey kidney cells; ATCC number CRL-1651) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 2 mM glutamine, 10% heat-inactivated FBS, 100 U/ml penicillin/streptomycin and grown at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. Preparation of cationic liposomes

Cationic liposomes composed of DOTAP:DOPE:DSPE-PEG_{2000}-MAL with molar ratios of 5:5:0.2, 3:7:0.2, and 1:9:0.2 (containing 50 mol%, 30 mol% and 10 mol% DOTAP, respectively) were prepared as described previously [34]. Briefly, appropriate amounts of lipids were dissolved in chloroform and mixed. To obtain FITC labeled liposomes 0.01 mol% FITC-DOPE was added to the mixture. The chloroform was subsequently removed by rotary evaporation at 37 °C followed by flushing the obtained lipid film with nitrogen during 30 min at room temperature. The dried lipids were then hydrated by adding HEPES buffer (20 mM, pH 7.4) till a final lipid concentration of 10.2 mM. After mixing in the presence of glass beads, liposome formation was allowed overnight at 4C. Thereafter, the formed liposomes were extruded 11 times through two stacked 100 nm polycarbonate membrane filters (Whatman, Brentfort, UK) at room temperature using an Avanti Mini-Extruder (Avanti Polar Lipids).

2.4. Preparation of TAT-modified and unmodified lipoplexes

To prepare 'post TAT-lipoplexes', the liposomes, with or without FITC-DOPE, were mixed with the appropriate amount of pDNA, in a +/− charge ratio of 4:1 and incubated at room temperature for 30 min. The final concentration of pDNA in the lipoplex dispersion was 0.126 μg/μl. Subsequently, TAT peptides were coupled to the MAL-group, present at the end of the PEG chains in DSPE-PEG_{2000}-MAL. Therefore, the TAT AA 37–72 peptide was first incubated under a nitrogen flow for 2 h with a 10 fold molar excess of TCEP (tris-2 (carboxyethyl)phosphine) to reduce the disulfide bonds. Again under a nitrogen flow, the 'activated' TAT AA 37–72 peptide was added to the lipoplexes in a 4 fold molar excess compared to the DSPE-PEG_{2000}-MAL and incubated overnight at 4 °C on a shaker.

To prepare 'pre TAT-lipoplexes', the same protocol was used, except that the TAT peptide was coupled to the liposomes before they were complexed with the pDNA.

The unmodified lipoplexes (i.e. lipoplexes without TAT) were prepared as the 'pre or post TAT-lipoplexes', but without the addition of TAT AA 37–72 peptide.

2.5. Size and zeta potential measurements

The average particle size and the zeta potential (ζ) of the liposomes and lipoplexes were measured by photon correlation spectroscopy (PCS) (Autosizer 4700, Malvern, Worcestershire, UK) and by particle electrophoresis (Zetasizer 2000, Malvern, Worcestershire, UK), respectively. The liposome and lipoplex dispersions were diluted 40 fold in 20 mM HEPES buffer pH 7.4 before the particle size and zeta potential were measured.

2.6. Agarose gel electrophoresis

To determine the extent of pDNA association to the liposomes and the release of the pDNA from the liposomes after incubation with SDS, agarose gel electrophoresis was used. 18 μl of the TAT-modified and unmodified lipoplexes, prepared as described above, were mixed with 2 μl 20 μg/μl SDS or 2 μl dH_2O and loaded together with 5 μl 5× loading solution (30% glycerol, 0.02% bromphenol blue) on a 1% agarose gel prepared in TBE buffer (10.8 g/l Tris base, 5.5 g/l boric acid and 0.58 g/l EDTA). The samples were subjected to electrophoresis at 80 V during 45 min and the DNA was visualized by UV transillumination using ethidium bromide (0.5 μl/ml) staining prior to photography.

2.7. Cytotoxicity assay

The cytotoxicity of the lipoplexes was determined using the tetrazolium salt based colorimetric MTT assay (EZ4U, Biomedica, Vienna, Austria) according to the manufacturer's instructions. Briefly, 2.5×10^4 cells/cm^2 were seeded in a 96-well plate and allowed to adhere. After 24 h, cells were washed twice with serum free medium and incubated with lipoplexes, containing 0.18 μg pDNA per cm^2 in serum free medium. After 2 h, the complexes were removed and cells were washed with culture medium. Finally, to each well, 20 μl substrate was added together with 180 μl culture medium and after 4 h incubation at 37 °C the concentration of reduced formazan was measured at 450 nm and 630 nm on a Wallac Victor2 absorbance plate reader (Perkin Elmer-Cetus Life Sciences, Boston, MA).

2.8. Transfection experiments

Cells were seeded into 24-well plates (2.5×10^4 cells/cm^2) and allowed to attach overnight. The culture medium was removed from the cells and they were washed twice with serum free medium. Subsequently, the lipoplexes (with and without bound TAT peptide) were diluted in serum free medium and the diluted lipoplexes, containing 0.18 μg pDNA per cm^2, were added to the 24-well plates. Cells were incubated at 37 °C for 2 h and, after a washing step with culture medium, the medium was replaced by 1 ml culture medium, and further incubated at 37 °C.

For the transfection experiments at 4 °C the cells, grown overnight as described above, were cooled down to 4 °C during 30 min. Subsequently, pre-cooled solutions for washing and for the transfection were used. After addition of the complexes, the cells were incubated at 4 °C and after 2 h, cells were washed with culture medium.

For treatment with uptake inhibitors, cells were pre-incubated for 1 h at 37 °C in DMEM with chlorpromazine (10 mg/ml) [35], filipin III (1 mg/ml) [36] before the complexes were added. In a recent study we have demonstrated that these inhibitors, at their respective concentrations, predominately block clathrin or raft mediated pathways without causing drastic cytotoxic effects [37]. After removal of the lipoplexes and addition of culture medium, cells were further incubated in an incubator. After 48 h the SEAP activity in the culture medium and the total protein concentration in the cells was measured.

2.9. Gene expression analysis

To determine the SEAP activity in the medium, 100 μl of the culture medium above the cells was taken and incubated at 65 °C for 30 min. Subsequently 100 μl dilution buffer (0.1 M glycine, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, pH 10.4) and 15 μl 4-methylumbelliferyl phosphate (4-MUP, 5.1 μg/μl in distilled water) was added to the culture medium in a 96-well plate. The obtained mixtures were then incubated at 37 °C and the fluorescence ($\lambda_{ex}/\lambda_{em}$=360/449 nm) was followed during 45 min on a Wallac Victor2 fluorescence plate reader (Perkin Elmer-Cetus Life Sciences, Boston, MA). The fluorescence obtained after 30 min was used for comparing the gene transfer capacity of the different lipoplexes.

Finally, after removing the culture medium, the cells were washed twice with PBS to remove dead cells and serum proteins. Thereafter, cells were lysed with lysis buffer (10% glycerol, 2.3% w/v SDS, 0.125 M Tris–HCl, pH 6.8), sonicated for 30 min and the protein concentration was determined by BioRad DC protein assay.

2.10. Cell binding and cellular uptake experiments

2.5×10^4 cells/cm^2 were seeded onto sterile glass bottom culture dishes (MatTek Corporation, MA, USA) and allowed to adhere for 1 day. For lysosome staining, the cells were incubated during 30 min at 37 °C with 50 pM of the lysosomal marker LysoTracker® Red (Molecular Probes, Eugene, OR, U.S.A.). For 4 °C experiments, cells were cooled in the refrigerator during 30 min, washed with pre-cooled DMEM and incubated with the lipoplexes at 4 °C. At different time points samples were analyzed.

In the experiments with cellular uptake inhibitors, the cells were pre-incubated for 1 h at 37 °C in DMEM with chlorpromazine (10 mg/ml) [35], filipin III (1 mg/ml) [36]. Subsequently, the cells were washed twice with DMEM before the appropriate amount of fluorescent TAT-modified or unmodified lipoplexes and inhibitor-containing serum free medium was added. Immediately after adding lipoplexes, the cells were visualized on different time points by confocal laser scanning microscopy (CLSM) (BioRad MRC 1024; Hemel Hempstadt, UK). After capturing the image with a 60× water immersion objective and a krypton/argon laser (488 nm and 547 nm) for the excitation of the FITC label and the LysoTracker® Red, pseudocoloring was performed by using the digital imaging system Confocal Assistant (CAS; BioRad; Hemel Hempstadt, UK).

2.11. Statistical analysis data

The experimental data in this report (zeta potential, DLS, cytotoxicity and transfection) are expressed as mean ± standard deviation (SD). One way ANOVA was used to determine whether data groups differed significantly from each other. A p-value lower than 0.05 was considered statistically significant.

3. Results and discussion

3.1. Preparation and physicochemical characterization of unmodified and TAT-modified lipoplexes

Pegylated DOTAP:DOPE:DSPE-PEG_{2000}-MAL lipoplexes bearing covalently bound TAT peptides at the end of their PEG chains and containing increasing amounts of DOTAP (10, 30 and 50 mol%) were obtained via two strategies: TAT peptides were either coupled to the PEG chains of the pegylated liposomes before complexing them with pDNA ('pre TAT-lipoplexes') or after complexing them with pDNA ('post TAT-lipoplexes'). The latter approach ensured that the TAT peptides were presented on the surface of the lipoplexes, a prerequisite for TAT peptides to promote the internalization of cargoes [38]. Moreover, to prevent that steric hindrance could abolish the interaction of TAT with the cell membrane [30], we attached the TAT peptides via a 2 kDa PEG spacer to the lipoplexes by the aid of DSPE-PEG_{2000}-MAL moieties. The maleimide (MAL) groups on these PEG chains are known to react specifically with sulfhydryl groups at neutral pH creating a stable thioether bond with the N-terminal cysteine of the TAT peptide. A schematic representation of the TAT-modified lipoplexes is shown in Fig. 1.

To assess the DNA binding capacity of the cationic liposomes and the ability of SDS to release the pDNA from the lipoplexes agarose gel electrophoresis experiments were performed (Fig. 2). Lipoplexes containing 30 and 50 mol% DOTAP showed, both in the presence and absence of TAT, a complete complexation of the pDNA. 10 mol% DOTAP lipoplexes lacking the TAT peptide failed to bind all the pDNA. Coupling of TAT peptides to these 10 mol% DOTAP lipoplexes retarded the pDNA on the agarose gel, but did not result in a full complexation. Finally, the release properties in the presence of SDS of the different types of lipoplexes were not altered by TAT peptides.

The effect of the attachment of TAT peptides to the lipoplexes was further evaluated by comparing the particle size and surface charge of TAT-modified and unmodified lipoplexes. Fig. 3A and B show that the attachment of TAT-peptides to the lipoplexes causes, in the case of both the 'pre TAT-lipoplexes' (pre TAT-LPX) and the 'post TAT-lipoplexes' (post TAT-LPX), a significant ($p<0.05$; ANOVA) increase in particle size and surface charge. The latter is an indication that cationic TAT peptides were indeed attached to the surface of the pegylated lipoplexes. The particle size and surface charge of the 10 mol% DOTAP lipoplexes could not be determined accurately, due to large aggregates and/or a too high polydispersity. This is not surprising as huge amounts of free pDNA were present in the 10 mol% DOTAP lipoplexes (Fig. 2).

3.2. Cytotoxicity

Several groups have reported a significant cytotoxicity of free TAT peptides [9,39,40]. Therefore, we characterized the cytotoxicity of our lipoplexes by measuring the mitochondrial activity. Linking of TAT peptides to the lipoplexes slightly

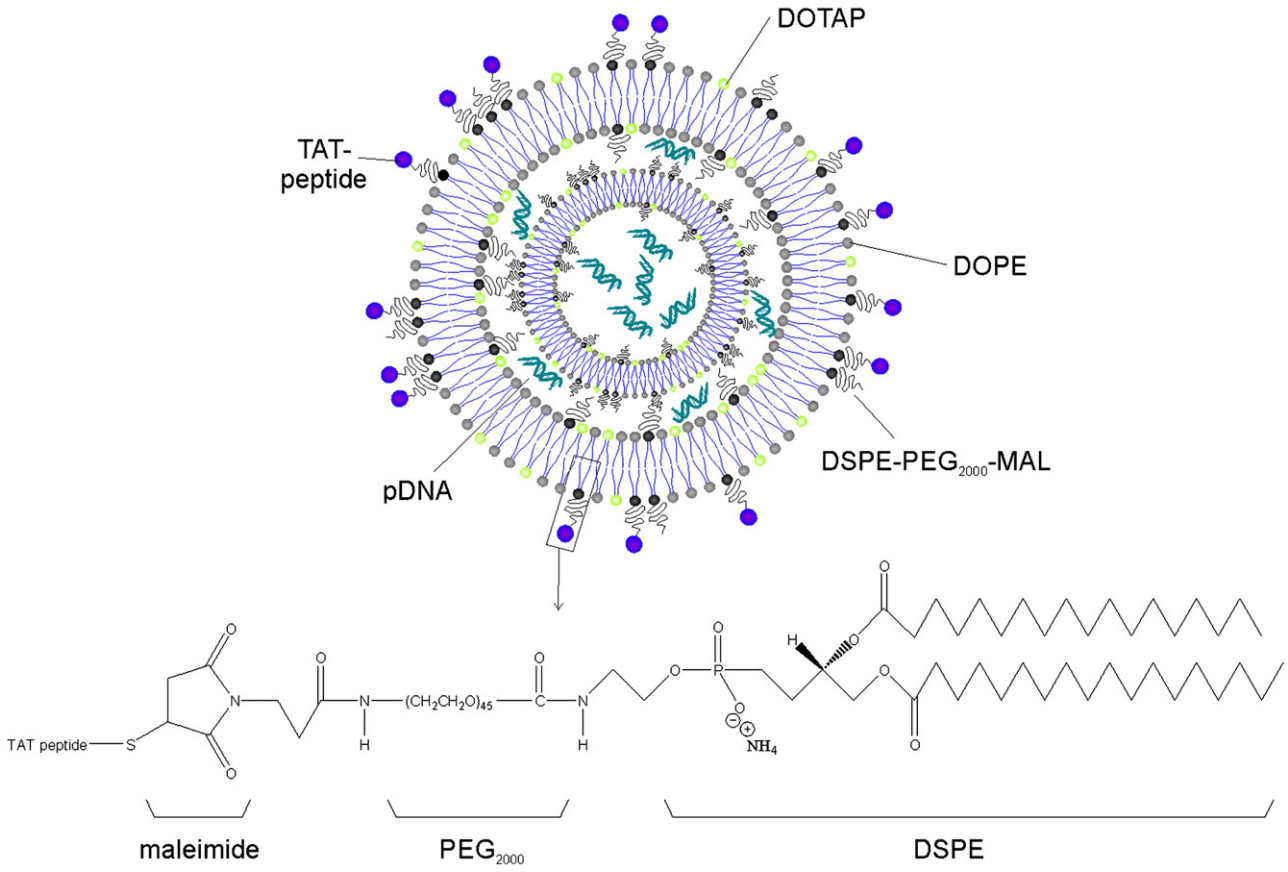

Fig. 1. Schematic representation of the TAT-modified DOTAP:DOPE:DSPE-PEG_{2000}-MAL lipoplexes.

increased their cytotoxicity (Fig. 4). It is currently unclear via which mechanism TAT causes cell toxicity, however, it has been shown in HeLa cells that the α-helix structure, present in the TAT AA 37–72 peptide, plays an important role [11].

Furthermore, except for the pre TAT-LPX containing 10 and 30 mol% DOTAP, the cytotoxicity of the lipoplexes slightly increased when they contain lower amounts of DOTAP and thus higher amounts of DOPE. DOPE is a fusiogenic lipid that disrupts endosomal membranes during their acidification [41]. A higher DOPE content in the lipoplexes probably results in an increased endosomal breakdown, which, as previously been suggested for polyethyleneimine [5], may explain the increased cytotoxicity.

3.3. Transfection capacity of unmodified and TAT-modified lipoplexes

The transfection efficiency of the unmodified lipoplexes, the 'pre TAT-LPX' and the 'post TAT-LPX' were subsequently compared. To correct for the above observed cytotoxicity of the

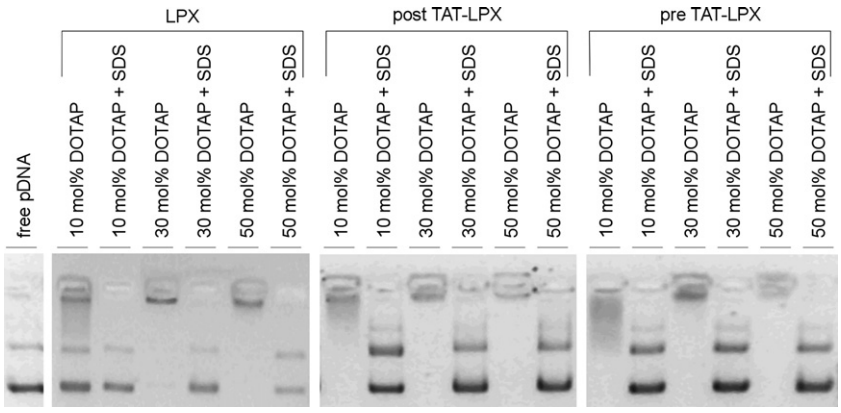

Fig. 2. Agarose gelelectrophoresis of unmodified, pre and post TAT-LPX containing 10, 30 and 50 mol% DOTAP before and after addition of SDS.

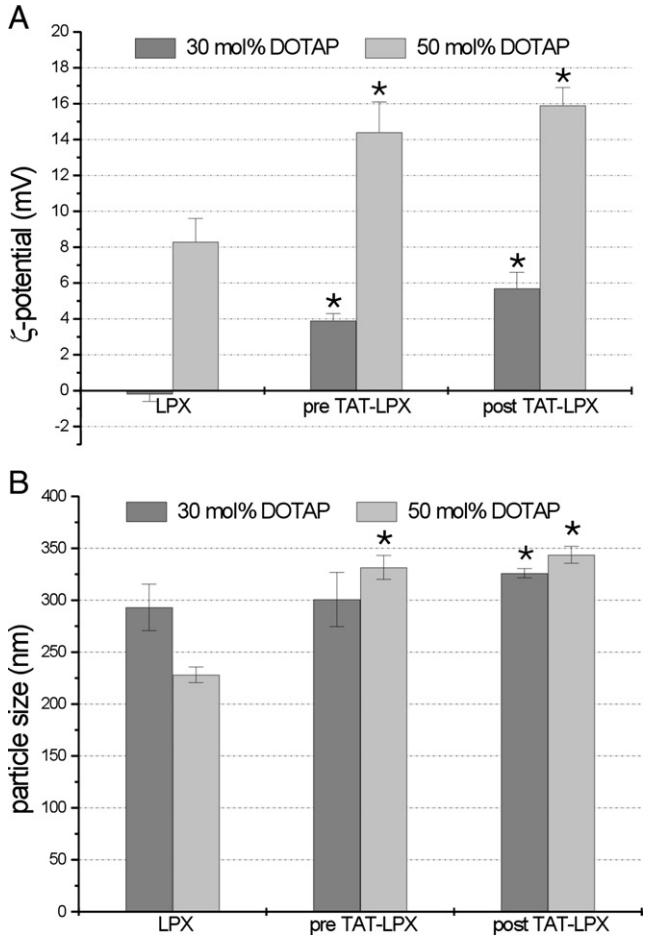

Fig. 3. Zeta potential (A) and size (B) measurement of the unmodified and TAT-modified lipoplexes containing 30 mol% (dark grey) and 50 mol% (light grey) DOTAP. The size and zeta potential of the 10 mol% DOTAP lipoplexes could not be measured accurately due to the presence of large aggregates and/or a too high polydispersity. All data are shown as mean±SD with $n=4$ for DLS and $n=5$ for zetapotential data. The asterisk (*) represents data points that significantly differ ($p<0.05$; ANOVA) from the data of unmodified lipoplexes.

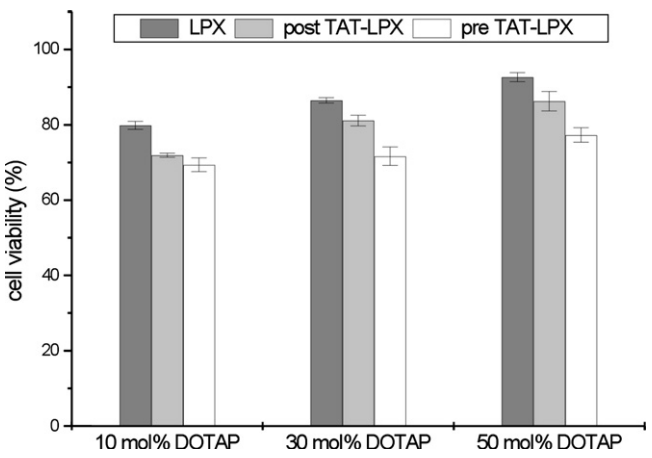

Fig. 4. Cytotoxicity of unmodified and TAT-modified lipoplexes containing 10, 30 or 50 mol% DOTAP relatively to untreated cells. Data are shown as mean± SD with $n=3$.

lipoplexes, the results of the transfection experiments were expressed relatively to the total cell protein content.

Fig. 5 reveals the following trends. First, the transfection capacity of the lipoplexes increased with increasing DOTAP content: the gene expression of the 50 mol% DOTAP lipoplexes without TAT peptide was 3.3 fold and with TAT peptide 1.4 fold higher than the gene expression of the corresponding 30 mol% DOTAP lipoplexes. Second, TAT peptide enhanced the gene expression of the 30 mol% DOTAP lipoplexes. 'Post TAT' modification of these lipoplexes gave the best results and led to a doubling of the transfection efficacy. This effect of TAT is slightly smaller than the effect of TAT seen on the gene expression of the lipoplexes used in the work of Torchilin et al. [42]. This may be explained by the lower initial gene expression of the lipoplexes in the latter work, which made it more likely that TAT could increase their gene expression. Additionally, the fact that in Torchilin's work a shorter TAT peptide was used may also explain the slight difference in gene expression. Third, Fig. 5 shows that the TAT peptide could not further enhance the gene transfer capacity of the (well transfecting) 50 mol% DOTAP lipoplexes. The 'post modification' of the 30 mol% DOTAP lipoplexes with TAT gave also slightly higher transfection efficiencies compared to the 'pre modification'. This may indicate that the preceding attachment of TAT to the liposomes complicated an efficient complexation of the pDNA. Finally, the unmodified 10 mol% DOTAP lipoplexes did not transfect the cells, which is explained by their poor DNA binding capacity (see Fig. 2). Also TAT-modified 10 mol% lipoplexes showed low transfection efficiency despite the reduced amount of free DNA caused by electrostatic binding of the TAT peptides to the free pDNA. However, we (unpublished data) and others have shown [43,44] that such TAT/pDNA complexes do not posses significant gene transfer capacities. Consequently, this explains why addition of TAT peptides to the 10 mol% DOTAP lipoplexes did not enhance their gene expression. Considering the low gene expression of the 10 mol% lipoplexes we decided to exclude them in our further work.

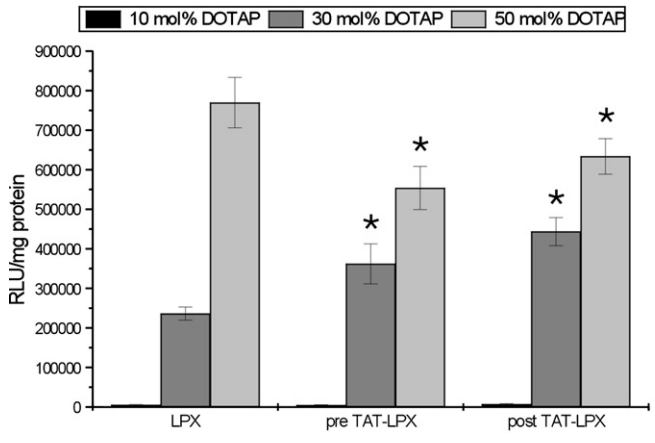

Fig. 5. Transfection efficiency of the unmodified, pre and post TAT-LPX containing 10 (black), 30 (dark grey) or 50 mol% (light grey) DOTAP. Data are shown as mean±SD and $n=3$. The asterisk (*) represents data points that significantly differ ($p<0.05$; ANOVA) from the data of unmodified lipoplexes.

3.4. TAT peptide modified lipoplexes enter Cos-7 cells in an energy dependent manner and turn up in acidic vesicles

The ability of TAT peptides to enhance the uptake of liposomes and other types of nanoparticles was demonstrated by several groups at the beginning of this millennium [29,30,43,45]. Later on, Torchilin et al. [42] showed that the cellular uptake and gene expression of lipoplexes was also enhanced when TAT peptides were coupled to their surface. In all these reports it was proposed that the TAT-modified particles penetrate the cell membrane in an energy independent manner. In contrast, Fretz et al. [31] showed that OVCAR cells internalize TAT peptide modified liposomes by energy dependent endocytosis. To determine whether the TAT-modified lipoplexes under investigation in this study follow an energy dependent or independent pathway, we focused on the cellular uptake of FITC-labeled lipoplexes at 4 °C and 37 °C by confocal scanning fluorescence microscope (Fig. 6). To elucidate whether the lipoplexes end-up in acidic vesicles, the cells were pre-incubated with LysoTracker® Red, a compound known to localize primarily in the acidic compartments of cells [46]. The experiments were performed using living cells, as previous reports have shown that cell fixation may lead to an artificial uptake of TAT-modified proteins [18]. Fig. 6A, B, E and F show that after incubation at 37 °C, both the unmodified and TAT peptide modified lipoplexes were rapidly (even within 5 min) taken up by the cells. However, at 4 °C the uptake was negligible for both types of lipoplexes (Fig. 6C, D, G and H). In contrast to previous reports this clearly demonstrates that the uptake of the TAT-modified lipoplexes occurs, like for unmodified lipoplexes, via an energy-dependent process. To further confirm the energy dependency of the cellular uptake of the lipoplexes, we performed transfection experiments at 4 °C. As expected from the data above, gene expression of both the TAT-modified and unmodified lipoplexes was reduced to about 3% of the gene expression measured at 37 °C (data not shown).

Both the TAT-modified and the unmodified lipoplexes appeared in the cells in a punctuated pattern and strongly co-localized with LysoTracker®, a marker of acidic organelles (Fig. 6A, B, E and F). This implies that the lipoplexes were most likely internalized via a clathrin-mediated pathway as lysosomes are the end point of a clathrin-mediated rather than a lipid raft-mediated internalization pathway. Additionally, the very fast internalization observed for the lipoplexes, is also typical for a clathrin-mediated uptake mechanism [47–49]. To further strengthen this conclusion we subsequently studied the effect of specific inhibitors of clathrin- and lipid raft-mediated endocytosis on the cellular uptake and gene expression of the lipoplexes. The fact that at least a portion of our lipoplexes turns up in lysosomes may be surprising as they contain DOPE, a helper lipid that is known to promote the endosomal escape of DNA and hence to avoid degradation of the pDNA by lysosomal enzymes [50–52].

3.5. Cholesterol-dependent clathrin-mediated endocytosis is the productive route for gene expression of TAT-modified and unmodified lipoplexes

Cells can internalize extracellular material via several mechanisms. The best characterized is the clathrin-coated pits pathway, which leads to intracellular vesicles that can either recycle back to the cell membrane or move, while they undergo a gradual acidification, along the microtubili to the perinuclear region [53]. Less defined are the lipid raft-mediated internalization mechanisms, among which the caveolae-mediated pathway is the best described (reviewed by [54]). Lipid rafts are dynamic regions of the plasma membrane which are enriched in cholesterol, sphingomyelin,

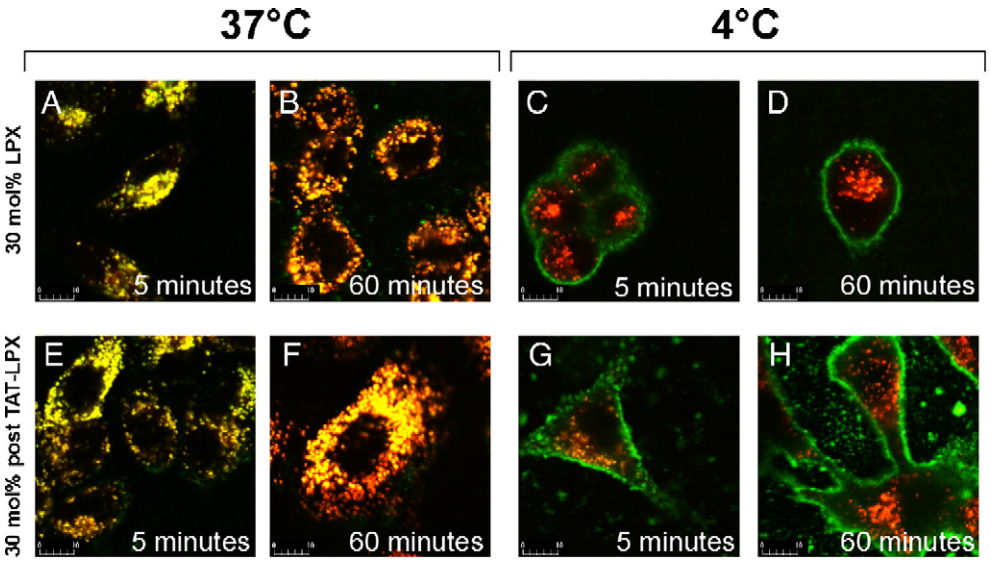

Fig. 6. Confocal laser scanning microscopy images of FITC-labeled unmodified and TAT-modified lipoplexes (containing 30 mol% DOTAP) incubated with the cells at 37 °C (A, B, E and F) and at 4 °C (C, D, G and H). The red fluorescence in the cells originates from LysoTracker® Red. Yellow indicates co-localization of the green fluorescent from the lipoplexes with the red fluorescence from the Lysotracker® Red. Scale bar represents 10 μm. Similar results were obtained with the 50 mol% DOTAP lipoplexes.

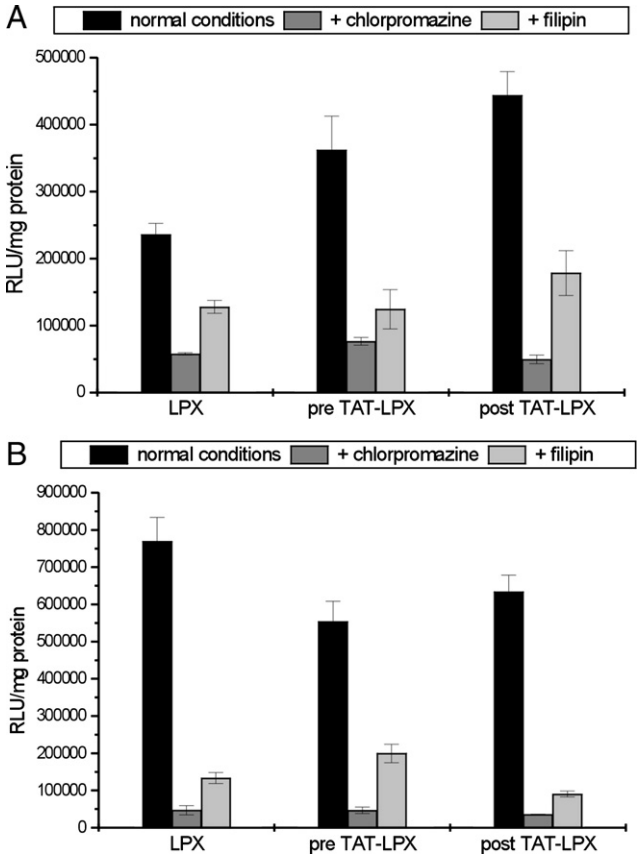

Fig. 7. Transfection efficiency of the unmodified, pre TAT- and post TAT-LPX containing 30 mol% (A) or 50 mol% (B) DOTAP in the absence or presence of uptake inhibitors chlorpromazine and filipin. Data are shown as mean ± SD and $n=3$.

glycolipids, GPI-anchored proteins and other membrane proteins. For caveolae-mediated uptake it is known that this pathway leads to neutral vesicles which stay after their formation near the cell surface before they are transported via the Golgi apparatus to the endoplasmatic reticulum. It is obvious that the distinctive intracellular routing profile and properties of the vesicles occurring from the different pathways may either favour or disfavour non-viral gene expression. Nevertheless, the cellular pathways by which non-viral gene complexes enter mammalian cells have gained the attention of only a few groups [48,49,55]. Therefore, elucidating the entry mechanism is of interest for both the TAT-modified and unmodified lipoplexes.

To study the role of the clathrin- and lipid raft-mediated endocytosis in the uptake and gene expression of TAT-modified and unmodified lipoplexes, we performed transfection experiments in the presence of a specific inhibitor of respectively clathrin-mediated (i.e. chlorpromazine) [35] and lipid raft-mediated endocytosis (i.e. filipin III) [36]. Chlorpromazine is an amphiphilic drug that prevents the recycling of clathrin proteins from formed endosomes back to the cell membrane and inhibits in this manner the formation of new clathrin-coated pits. On the other hand, filipin binds strongly to cholesterol which is especially present in the lipid raft domains of the cell membrane, which will prevent lipid raft-mediated endocytosis. However, also other entry pathways may be inhibited as cholesterol is present all over the cell membrane.

The gene expression of the TAT-modified and unmodified lipoplexes in the presence of chlorpromazine and filipin is shown in Fig. 7. Chlorpromazine significantly ($p<0.05$; ANOVA) inhibits the gene expression of the 30 mol% (>86% inhibition) and the 50 mol% (>92% inhibition) DOTAP lipoplexes. Surprisingly, also filipin lowered the gene expression of both the TAT-modified and unmodified lipoplexes significantly ($p<0.05$; ANOVA): the inhibition ranged between 46% (for the unmodified lipoplexes) and 66% (for the TAT-modified lipoplexes). The effect of filipin on the gene expression was significant lower ($p<0.05$; ANOVA) than the effect of chlorpromazine. In the light of the clear indications for a clathrin-mediated uptake mechanism of our lipoplexes (i.e. fast internalization and co-localization with acidic organelles), we suspect that the effect of filipin is due to its limited selectivity. Indeed, as cholesterol is required for the formation of curved coated pits [56,57] the binding of filipin to cholesterol might consequently also inhibit clathrin-mediated endocytosis. This hypothesis is in agreement with the work of Zuhorn et al. [49] who showed that depletion of cell membrane cholesterol by methyl-β-cyclodextrin, a compound previously proposed to selectively inhibit caveolae-mediated endocytosis [58,59], strongly inhibited cholesterol-dependent clathrin-mediated

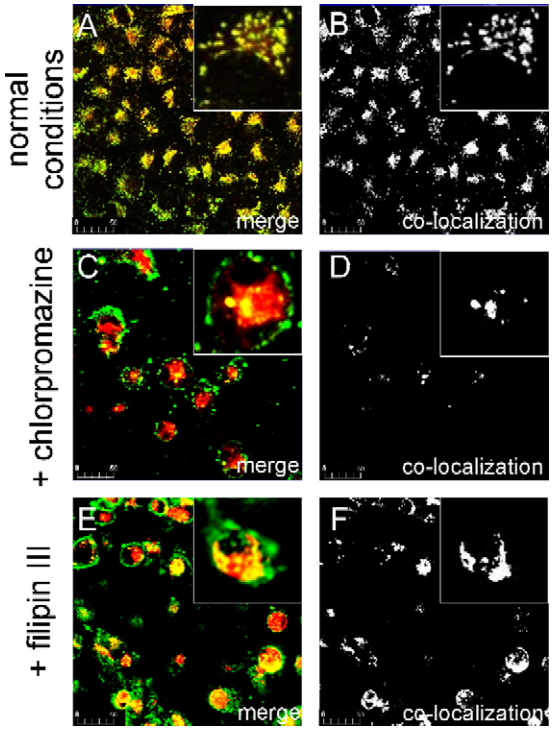

Fig. 8. Confocal laser scanning microscopy images of Cos-7 cells incubated with FITC-labeled unmodified and TAT-modified 30 mol% DOTAP lipoplexes in normal conditions (A and B), in the presence of chlorpromazine (C and D) and in the presence of filipin (E and F). As marker of acidic organelles, LysoRed® tracker was used. The left panel (A, C and E) shows the merged images with the complexes represented as green and the lysosomes as red and the right panel (B, D, F) shows the co-localization of red and green signals. Scale bar represents 50 μm. The inserts show, at a higher magnification, the co-localization of LysoRed® tracker and lipoplexes in one cell.

endocytosis of lipoplexes by Cos-7 cells. Therefore, we speculate here that our lipoplexes are internalized via cholesterol-dependent clathrin-mediated endocytosis and that this entry mechanism is also sensitive to filipin. However, we have to remark that we cannot totally exclude from our data that a small portion of the lipoplexes are also taken up via caveolae-mediated endocytosis.

It has been shown that the entry mechanism may depend on the size of the nanoparticles. Indeed, polystyrene nanoparticles with a diameter less than 200 nm were exclusively internalized via clathrin-coated pits, whereas those of 500 nm entered the cells via caveolae [47]. The average diameter of our lipoplexes, as determined by DLS, was around 300 nm. However, the size distribution of these lipoplexes typically ranged between 100 and 350 nm, with more than 70% of the lipoplexes having a diameter smaller than 200 nm. Therefore, from the size of the lipoplexes a clathrin-mediated endocytosis pathway and to a lesser extend a caveolae-mediated pathway could be expected.

Finally, fluorescence confocal microscopy experiments showed a nice correlation between the decrease in gene expression caused by chlorpromazine or filipin and the amount of internalized lipoplexes in the presence of the respective uptake inhibitors (Fig. 8). In normal conditions (Fig. 8A and B), the 30 mol% lipoplexes clearly co-localize with the lysosomes, while the presence of chlorpromazine (Fig. 8C and D) almost completely blocks the co-localization. In contrast, filipin (Fig. 8E and F) partially inhibits the uptake of the complexes. Similar results were obtained with the 50 mol% lipoplexes.

In conclusion, our study shows that the gene transfer of lipoplexes containing 30 mol% DOTAP can be enhanced by coupling TAT peptides to their surface. Post modification of pre-formed lipoplexes with TAT gives the best results. In contrast, addition of TAT to lipoplexes containing 50 mol% DOTAP does not improve their gene transfer. In contrast to previous reports [30] we clearly demonstrate that the uptake of both TAT-modified and unmodified lipoplexes is energy dependent. Furthermore, by using cellular uptake inhibitors we suppose that the TAT-modified and unmodified lipoplexes enter the cytoplasm mainly via cholesterol-dependent clathrin-mediated endocytosis, a pathway previously postulated by Zuhorn et al. [49] for the cellular uptake of cationic lipoplexes.

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