Application of Novel Solid Lipid Nanoparticle (SLN)-Gene Vector Formulations Based on a Dimeric HIV-1 TAT-Peptide *in Vitro* and *in Vivo*

Carsten Rudolph,[1,4] Ulrike Schillinger,[2] Aurora Ortiz,[1] Kerstin Tabatt,[3] Christian Plank,[2] Rainer H. Müller,[3] and Joseph Rosenecker[1]

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Purpose. To optimize gene delivery of SLN-based gene vectors by incorporation of a dimeric HIV-1 TAT peptide (TAT_2) into SLN gene vectors.

Methods. Plasmid DNA was complexed with two SLN preparations either with or without pre-compaction of DNA by TAT_2, poly-L-arginine, or the mutant TAT_2-M1. DNA complexed with polyethylenimine (PEI) served as a standard. Gene expression was analyzed upon transfection of bronchial epithelial cells *in vitro* and after intratracheal instillation or aerosol application to the lungs of mice *in vivo*. Stability of DNA was analyzed by agarose gel electrophoresis.

Results. Incorporation of TAT_2 into SLN gene vectors induced an up to 100-fold sequence-dependent increase of gene expression as compared with the mutant TAT_2-M1 and was 4- to 8-times higher as compared with PEI *in vitro*. *In vivo* application of TAT_2-SLN gene vectors via jet nebulization increased SLN-based gene expression but was accompanied with DNA degradation. DNA degradation was not observed when an innovative device operating on the principle of a perforated vibrating membrane was used.

Conclusions. Incorporation of TAT_2 into SLN gene vectors is suitable to optimize gene transfer *in vitro*. The use of a mild nebulization technology avoids DNA degradation and offers the opportunity for further studies in large animal models.

KEY WORDS: aerosol; gene delivery; plasmid DNA; polyethylenimine; solid lipid nanoparticles; TAT.

INTRODUCTION

Solid lipid nanoparticles (SLN) have been shown to condense DNA into nanometric colloidal particles capable of transfecting mammalian cells *in vitro* (1,2). Compared with standard DNA carriers such as cationic lipids (3) or cationic polymers (4), SLN offer several technological advantages such as a relative ease of production without any organic solvent (5), the possibility of large scale production with qualified production lines (6,7), good storage stabilities (8), the possibility of steam sterilization (9), and lyophilization (10). In particular, the use of substances that are generally accepted as safe (GRAS) (11) leads to an advantageous toxicity profile (1) when compared with, for example, polyethylenimine (PEI), a highly efficient cationic polymer (12,13) that can lead to liver necrosis and animal death after intravenous application (14) and induces neutrophil infiltration and reduced lung function upon intratracheal application (15,16). However, in the absence of endosomolytic agents such as chloroquine, gene transfer efficiency mediated by SLN derived gene vectors consisting of optimized lipid composition remains lower as compared with standard transfection agents such as PEI 25 kDa (1,2). Recent work of our group showed that pre-compaction of DNA with oligomers of the HIV-1 TAT peptide for the formulation of gene vector complexes led to an increase of up to two orders of magnitude in gene transfer efficiency (17). The dimeric TAT peptide was found to be most efficient. This effect was related to the unique features of the HIV-1 TAT peptide which represents a protein transduction domain (PTD) (18,19) and a nuclear localization sequence (NLS) (20). The PTD could improve cellular uptake due to its cell penetrating properties. In addition, the NLS function could facilitate nuclear transport of the DNA due to interaction with the endogenous cytoplasmic-nuclear transport machinery. In this study we attempted to apply this formulation technique (i.e., the formulation of ternary gene vector complexes consisting of DNA precompacted with a dimeric TAT peptide (TAT_2) which were completed by the addition of a cationic gene carrier to SLN formulation. In order to further investigate whether the TAT_2 peptide mediates a sequence-dependent effect, gene vectors were formulated by pre-compaction of DNA with either poly-L-arginine (pLa) or the dimeric peptide TAT_2-M1 of the nuclear transport-deficient TAT-M1 mutant (20). Transfection experiments were performed on a bronchial epithelial cell line (16HBE14o-) *in vitro*. For *in vivo* application two methods were tested i) direct intratracheal instillation into the mouse lung or ii) whole body nebulization of the gene vectors.

MATERIALS AND METHODS

Materials

Cetylpalmitate (Cutina CP) was a gift from Henkel (Düsseldorf, Germany), Tween 80 and Span 85 were a gift of ICI Surfactants (Eversberg, Belgium). The cationic lipid *N,N*-di-(β-stearoylethyl)-*N,N*-dimethyl-ammonium chloride (Esterquat 1, EQ 1) was provided by Gerbu Biotechnik (Gaiberg, Germany). 1,2-dioleyl-sn-glycero-3-trimethylammoniumpropane (DOTAP), chloroquine phosphate (CQ), and polyethylenimine (pEI, 25,000 kDa) were purchased from Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany).

SLN Production

The SLN were produced by hot high pressure homogenization as described previously (11). Briefly, the solid lipids were heated at around 10°C above their melting points. The mixture of molten lipid (4% [wt/wt] when referred to the final product) and the hot aqueous solution of surfactant and cationic lipid (2% [wt/wt] Tween 80 and Span 85 in a 7:3-ratio and 1% [wt/wt] cationic lipid) formed a pre-emulsion after stirring for 1 min.

A 1.5 kg batch of SLN containing the cationic lipid EQ1 (SLN_Eq) was produced using a specially modified LAB 60 homogenizer (APV-Gaulin, Lübeck, Germany) (6,7), which

[1] Department of Pediatrics, Ludwig-Maximilians University, 80337 Munich, Germany.
[2] Institute of Experimental Oncology and Therapy Research, Technical University of Munich, 81675 Munich, Germany.
[3] Department of Pharmaceutical Technology, Biopharmacy and Biotechnology, Free University of Berlin, 12169 Berlin, Germany.
[4] To whom correspondence should be addressed.

worked continuously. Formation of the pre-emulsion took place in the feeding vessel of the homogenizer using a built-in stirring unit. The dispersions were processed for 30 min at 85°C, applying 50 bars as second pressure and 500 bars as the main homogenization pressure.

Batches of SLN of 3.5 g containing the cationic lipid DOTAP (SLN_DOTAP) were homogenized using the heatable EmulsiFlex-B3 (Avestin Inc., Ottawa, Canada), which worked discontinuously. The pre-emulsion was obtained using a high-speed stirrer (Ultra Turrax T25, Jahnke & Kunkel, Germany) and homogenized at 85°C while applying a pressure of 480 bar and four homogenization cycles.

Peptide Synthesis

Peptides were synthesized on an Applied Biosystems 431 A automatic synthesizer according to a standard Fmoc protocol, purified by reversed phase HPLC and analyzed by mass spectroscopy. The following peptides were synthesized: Peptide $C(YGRKKRRQRRRG)_2$ (TAT_2) containing the arginine-rich motif of the HIV-1 TAT protein. Peptide $C(YGRKERRQERRG)_2$ (TAT_2-M1) was synthesized by the Department of Medicine (Charité), Institute of Biochemistry, Humboldt-University, Berlin. The free sulfhydryl groups were modified by dithiodipyridin reaction (21).

Plasmid

The plasmid pCMV-Luc containing firefly luciferase cDNA under the control of the cytomegalo virus (CMV) promoter was generously provided by E. Wagner (Department of Pharmacy, University of Munich, Germany) and used as a reporter gene. The plasmid was propagated in *Escherichia coli* and purified by the PlasmidFactory (Bielefeld, Germany). The purity (LPS) of this plasmid is $\leq$0.1 E.U./μg DNA, the amount of supercoiled DNA $\geq$ 90% ccc.

Cell Culture

Human bronchoepithelial cells (16HBE14o-) were provided by Dieter C. Gruenert (University of Vermont, Burlington, USA). Cells were grown in FCS (10%) supplemented MEM (GIBCO,BRL) at 37°C in a 5% CO_2 humidified air atmosphere.

Preparation of Gene Vector Complexes

Binary gene vector complexes for one well were formulated as follows: 1 μg of DNA and the corresponding amount of vector, that is, peptide, SLN, or PEI were diluted in HBS (150 mM NaCl, 10 mM HEPES, pH = 7.4) to 75 μl, respectively, if not stated otherwise in the text. The DNA solution was pipetted to the vector solution and mixed vigorously by pipetting up and down. The complexes were incubated for 20 min at ambient temperature before use. Ternary gene vector complexes for one well were generated in the same manner but DNA, TAT_2 (or pLa, TAT_2-M1), and standard cationic transfection agent (PEI, average MW = 25 kDa, dialyzed against water, 12–14 kDa MW cutoff, and adjusted to pH = 7; SLN) were diluted in HBS to 50 μl, respectively. 50 μl of the DNA solution containing 1 μg DNA was pipetted to the TAT_2, pLa (Sigma, P 4663, MW 5,000–15,000), or TAT_2-M1 solution, respectively, mixed vigorously, incubated at ambient temperature for 10 min and then 50 μl of the SLN solution was added, followed by incubation at ambient temperature for 10 min. Alternatively, the TAT_2 or TAT_2-M1 solution was first pipetted to the SLN_DOTAP vector solution and then added to the DNA solution as stated in the text.

In Vitro Transfection Procedure and Luciferase Activity Measurement

16HBE14o- cells were seeded in 24-well plates at a density of 100,000 cells per well the day prior transfection. Immediately before transfection, the medium was replaced with 850 μl of serum-free medium. One hundred fifty microliters of gene vector complex solution, corresponding to 1 μg of DNA, were added to the cells. After 4 h of incubation at 5% CO_2 and 37°C, the medium was replaced by 10% FCS containing medium supplemented with penicilline/streptomycine 0.1% (vol/vol) and gentamycine 0.5% (vol/vol) (#15140-122, #15710-049, Gibco, Invitrogen GmbH, Karlsruhe, Germany). Twenty-four hours later, cells were washed with PBS and lysed by the addition of 100 μl lysis buffer (250 mM Tris, 0.1% Triton X-100, pH = 7.8) per well. Luciferase activity was measured upon addition of 100 μl luciferase substrate solution (470 μM D-luciferin; 270 μM coenzyme A; 33.3 mM DTT; 530 μM ATP; 1.07 mM $(MgCO_3)_4Mg(OH)_2 \times 5\ H_2O$; 2.67 mM $MgSO_4$; 20 mM tricine; 0.1 mM EDTA) to 50 μl of cell lysate using a Lumat LB 9507 instrument (Berthold, Bad Wildbach, Germany). Protein content was determined by a standard BioRad protein assay (Bradford-method). The results are shown as relative light units (RLU) per mg of protein.

Animals and Delivery of Gene Vectors to the Lung

Gene vector complexes used for *in vivo* experiments were generated as described above but for direct intratracheal application each component was diluted in double distilled water (Delta Pharma, Boehringer Ingelheim, Germany), respectively. For one mouse, the appropriate amounts of SLN or the mixture of SLN and peptides were diluted to 25 μl and added to 25 μl of water containing 50 μg of pCMVLuc and incubated for ten minutes. Gene vector application was performed as described by Rudolph *et al.* (15). When gene vectors were administered via aerosol application, the complexes were prepared in the same manner but different volumes and amounts of DNA were used. For the formation of binary gene vector complexes, SLN (2.5 mg), PEI (1.3 mg), and DNA (1 mg) were each diluted to 4 ml with double distilled water. The DNA solution (1 mg) was pipetted to the solutions containing the SLN or PEI and mixed vigorously and incubated for 10 min before nebulization. When ternary gene vector complexes were formulated, the same amounts of DNA, peptide, and SLN were each diluted to 2.67 ml with water, respectively. The peptide solution was mixed with the SLN solution and pipetted to the DNA solution. In all cases, the final volume of the gene vector solution was 8 ml containing 1 mg of pCMVLuc. For the nebulization procedure four mice were placed in a sealed 9.8 × 13.2 × 21.5 cm acrylic glass box which was connected at one end with a 45 × 7.7 cm plastic cylinder (diameter of the connecting piece 2.1 cm) which in turn was connected to the jet nebulizer (PARI BOY LC plus, PARI GmbH, Starnber, Germany). At the opposite side of the plastic box four small holes were inserted to allow aerosol flow

through the plastic box. The bottom of the connecting plastic cylinder was homogeneously covered with 150 g of silica gel (1–3 mm, #85330, Fluka, Switzerland). The gene vector solution was split into two 4 ml fractions and each of the fractions was nebulized to the mice during approximately 30 min. The nebulizer was operated with synthetic air containing 5% CO_2 (Westfalen AG, Münster, Germany). The PARI BOY jet nebulizer generates an aerosol with an median mass diameter (MMAD) of 3.4 μm. 24 h post transfection mice were anaesthetized intraperitoneally with pentobarbital and the peritonea were opened by midline incisions. In order to purge blood from the lungs and to avoid interference with the subsequent luciferase assay, a posterior vena cava exit was cut, and 10-15 ml of an heparanized isotonic sodium chloride solution (25000 I.E. heparin/1000 ml) were slowly perfused into the mice right cardiac ventricle. The lungs were dissected from animals and washed in PBS. After the addition of 750 μl lysis buffer (Promega) the organs were weighed and homogenized in a minibeadbeater (Biospec Products, Bartlesville, USA). Luciferase activity was measured using a topcount microplate scintillation and luminescence counter (Canberra-Packard, Dreieich, Germany). Recombinant luciferase (Roche Diagnostics GmbH, Mannheim, Germany) was used as standard to calculate the amount of luciferase expressed in the lung tissue. Eight- to 10-week-old female BALB/c mice were used (Charles River Laboraties, Sulzfeld, Germany). All animal procedures were approved and controlled by the local ethics committee and carried out according to the guidelines of the German law of protection of animal life.

Nebulization of Gene Vector Complexes and Agarose Gel Electrophoresis

Three milliliters of the gene vector complex solution formulated as described above ($c_{DNA} = 10$ μg/ml) were nebulized for 10 min using either a PARI BOY jet nebulizer (PARI BOY LC plus) or a perforated vibrating membrane device (e-Flow, PARI GmbH, Starnberg, Germany). The aerosol stream was concentrated by a syringe connected to the outlet of the nebulizer reservoir and samples were collected in a 1.5 ml tube.

To determine DNA integrity, 30 μl of the nebulized complex solutions were loaded onto a 0.6% agarose gel and run in 0.05 M NaOH with 1mM EDTA for 30 min at 60 V.

Statistical Analysis

Results are reported as means ± SD. The statistical analysis between different groups has been determined with a nonpaired *t* test. Probability (p) ≤ 0.05 was considered significant. All statistical analyses were performed using the program StatView 5.0. (SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

Effect of TAT_2 on the *in Vitro* Gene Transfer Efficiency Mediated by SLN Consisting of Different Lipid Compositions

In a first set of experiments, we investigated whether the TAT_2 peptide would be capable of enhancing gene transfer efficiency of SLN gene vectors and whether transfection efficiency was dependent on the SLN lipid composition. Ternary gene vector complexes consisting of plasmid DNA (pCMVLuc, encoding for the firefly luciferase), TAT_2, and SLN were formulated as described for other gene vectors previously (17). The DNA was pre-compacted with the cationic TAT_2 peptide at a charge ratio of +/− = 1 which resulted in negatively charged complexes (17). Gene vector formation was completed through the addition of positively charged SLN. Two SLN formulations resulting from previous systematic optimization of cationic lipid and matrix lipid composition were selected for transfection experiments. Both formulations showed minimal toxicity *in vitro* (2). SLN_DOTAP which was formulated from cetylpalmitate, Tween, Span and the cationic lipid DOTAP showed maximal gene transfer, SLN_Eq (also formulated from cetylpalmitate but with the cationic lipid Eq1) was ten times less effective (2).

In the absence of endosomolytic agents such as chloroquine, *in vitro* gene transfer efficiency mediated either by SLN_DOTAP- or SLN_Eq gene vectors was 37- and 500-fold less efficient as compared with PEI (Fig. 1). In the presence of chloroquine gene expression of SLN_DOTAP gene vectors increased 23-fold resulting in 60% of the level of gene expression mediated by PEI. Gene transfer efficiency of SLN_Eq based gene vectors was not affected by the presence of chloroquine. These results show that SLN-based gene vectors mediated efficient gene transfer under optimized conditions. However, the addition of the endosomolytic agent chloroquine was required for high gene expression. This suggests that the DNA-SLN complexes are internalized via some endo-lysosomal pathway (22,23).

Pre-compaction of the DNA with TAT_2 for gene vector formulation led to a 101- and 145-fold increase of gene expression as compared with the binary SLN vectors from SLN_DOTAP and SLN_Eq, respectively (Fig. 1). Whereas gene expression mediated by the ternary gene vectors based on SLN_Eq resulted in 30% of gene expression level achieved with PEI, the SLN_DOTAP based ternary gene vectors mediated a 3-fold higher gene expression as compared with PEI. These results demonstrate that the TAT_2 peptide is capable of potentiating gene transfer efficiency of SLN gene vectors.

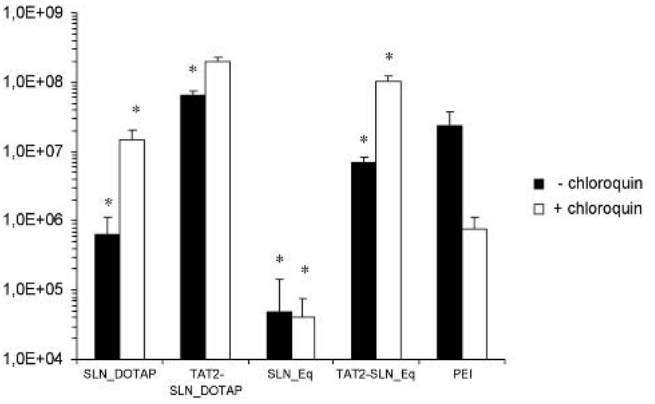

Fig. 1. Transfection efficiency of SLN-based gene vectors on 16HBE14o- cells. DNA was either complexed with SLN_DOTAP or SLN_Eq (wt/wt = 2.5 and 2.0, referring to DOTAP or Eq 1) or pre-compacted with TAT_2 (+/− = 1, wt/wt = 0.65) in the absence or presence (100 μM) of chloroquine. PEI gene vectors were formulated with an N/P-ratio of 10. Data show a representative experiment, n = 4, ± SD; transfection rates significantly different from PEI in the absence of chloroquine are indicated by * ($p < 0.05$).

Further, we examined whether the addition of chloroquine which should lead to further gene vector accumulation in the cytoplasm due to enhanced endosomal release would also improve gene expression of ternary SLN-TAT_2 gene vectors. Indeed, the presence of chloroquine further increased gene expression 3- and 15-fold for SLN_DOTAP- and SLN_Eq-based ternary gene vectors, respectively. These results suggest that the endosomolytic effect of chloroquine resulted in a further increase of the gene vector concentration in the cytoplasm which could be associated with a facilitated nuclear uptake of the DNA via the NLS function of the TAT peptide. Consequently, rather the NLS function of the TAT peptide than the function of the protein transduction domain is involved (17,24,25). Gene expression mediated by the ternary gene vectors in the presence of chloroquine was found to be 8- and 4-fold higher as compared with PEI gene vectors (Fig. 1). To gain further insights into the underlying gene transfer mechanism of the ternary SLN gene vectors, we analyzed the role of each gene vector component in more detail.

Examination of the Influence of Each of the Ternary Gene Vector Components on the Gene Transfer Efficiency

First we tested to which extent the SLN might contribute to the gene transfer efficiency mediated by the ternary TAT_2-based SLN gene vectors. Since SLN_DOTAP was shown to be more efficient than with SLN_Eq, further experiments were performed using SLN_DOTAP. As shown in Fig. 2, gene expression mediated by TAT_2 at a ratio of +/– = 1 without the addition of SLN resulted in 66- and 113-fold lower gene expression as compared with binary SLN_DOTAP gene vectors formulated at wt/wt = 2.0 or 5.0, respectively. In contrast, when SLN were added to the preformed TAT_2-DNA complexes to form ternary gene vectors, gene transfer efficiency increased 60-fold as compared with SLN gene vector complexes at wt/wt = 2.5. These results demonstrate that the high gene transfer efficiency mediated by the ternary SLN gene vectors is not only due to the TAT_2 peptide but that the SLN are required for complex formation to improve gene expression.

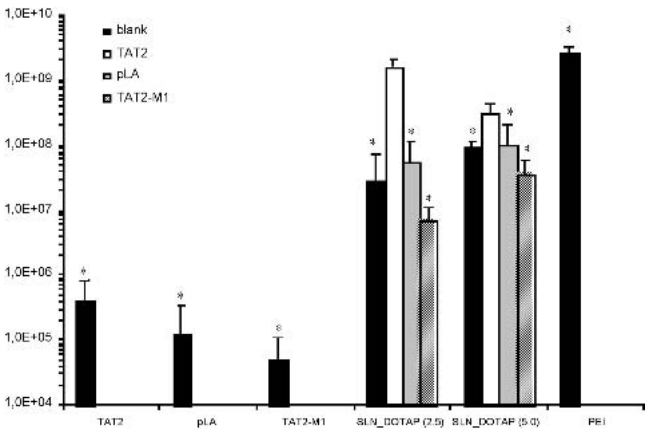

Fig. 2. Transfection efficiency of SLN-based gene vectors on 16HBE14o- cells. DNA was either complexed with SLN_DOTAP (wt/wt = 2.5 and 5.0, referring to DOTAP), TAT_2, pLa, or TAT_2-M1 (+/– = 1) or first pre-compacted with TAT_2, pLa, or TAT_2-M1 (+/– = 1). PEI gene vectors were formulated with an N/P-ratio of 10. Data show a representative experiment, n = 4, ± SD; transfection rates significantly different from TAT_2-DNA-SLN_DOTAP are indicated by * ($p < 0.01$).

To test whether the effect of the TAT_2 peptide is sequence-dependent or whether only pre-compaction of DNA is required to increase gene expression, analogous experiments were performed with peptides of similar but not identical sequences. Since 6 of 12 amino acids of the monomeric TAT peptide were arginines, we used a poly-L-arginine (pLa) as control. In addition, we used the dimeric sequence of the TAT-M1 sequence as another control. The TAT-M1 sequence was shown to be a nuclear transport deficient mutant of the TAT peptide (20). Within this sequence two arginines were substituted by glutamic acids. As observed for the TAT_2 peptide gene expression of both pLa-DNA- and TAT_2-M1-DNA complexes at ratio of +/– = 1 was two orders of magnitude lower as compared with binary SLN gene vectors. In contrast to the strong increase of gene expression observed for TAT_2-based ternary SLN gene vectors, neither pLa nor TAT_2-M1 showed such a pronounced effect on gene expression. Gene expression of pLa-based and TAT_2-M1-based SLN gene vectors were not significantly different as compared to the binary SLN gene vectors at w/w = 2.5. These results indicate that enhancement of gene expression of the ternary TAT_2-based SLN gene vectors is sequence-dependent and can not be explained by simple pre-compaction of DNA with cationic peptides.

Interestingly, the strong increase of gene expression induced through incorporation of the TAT_2 peptide when formulating SLN gene vectors was only observed at SLN/DNA ratio of wt/wt = 2.5. When the SLN/DNA ratio was increased to wt/wt = 5.0 gene expression of the binary SLN-DNA complexes increased slightly (3-fold) but gene expression of the ternary TAT_2-SLN-DNA complexes decreased 5-fold as compared with ternary TAT_2-based SLN gene vectors formulated at a SLN/DNA ratio of wt/wt = 2.5. As a result, the ternary TAT_2-based SLN gene vectors require accurate formulation for optimal outcome with lower SLN amounts resulting in superior gene expression. We suggest that the ternary gene vectors require an optimal balanced charge ratio to form efficient transfection complexes.

To assess the potential of SLN derived gene vectors for *in vivo* application it is important to compare their gene transfer efficiency with a known standard. PEI was shown to efficiently deliver DNA to several tissues *in vivo* either when administered intravenously (13,26,27) or topically to the lung (13,15,28). For this reason, PEI was chosen as standard gene vector to evaluate the transfection efficiency of the ternary TAT_2-based SLN gene vectors *in vitro*. The gene transfer efficiency mediated by the ternary TAT_2-based SLN gene vectors reached the level of gene expression mediated by PEI gene vectors under optimized conditions (N/p = 10) on a human bronchial epithelial cell line *in vitro* (Fig. 2). As a consequence, ternary TAT_2-based SLN gene vectors could be suitable for gene transfer *in vivo*, in particular to the lung.

Examination of the *in Vitro* Gene Transfer Efficiency of Stable Formulated Ternary Gene Vectors Suitable for *in Vivo* Application

A prerequisite for the delivery of gene vectors *in vivo* is the preparation of stable formulations suitable for the desired route of application. Our focus is the delivery of gene vector

complexes to the lung for treating hereditary diseases such as cystic fibrosis. For this purpose, presumably relatively high DNA doses will be required in order to achieve a therapeutic effect. This assumption is based on previous observations by us and other researchers which document the relative inefficiency of synthetic constructs for gene delivery *in vivo*. For experimentation in mouse models, particularly in models of gene delivery to the lungs via the airways, high DNA doses require high vector concentrations (100–200 μg DNA/ml) due to the limited volumes of the target organs. It will be documented below that this requirement imposes severe restrictions on vector formulation and instrumentation used for gene delivery and that these restrictions limit the suitability of mouse models for aerosol gene delivery to the lungs. For example, such gene vector concentrations exclude the use of isotonic saline solutions for gene vector formulation as it is used in cell culture experiments because of salt-induced aggregation of the polyelectrolyte complexes leading to precipitation (26,29). To avoid gene vector precipitation, polyelectrolyte complexes can either be stabilized by modification with e.g., PEG- or HPMA-based protective copolymers (30,31), or by reduction of the ionic strength of the gene vector solution. For this reason, we examined whether ternary TAT_2-based SLN gene vectors formulated in distilled water remained as efficient as observed for gene vectors formulated in HBS. As shown in Fig. 3 gene transfer mediated by the ternary TAT_2-based SLN gene vectors increased dose-dependently when raising the +/– ratio from +/– = 0.5 to +/– = 1.0 independent of the chronological order of gene vector formulation. This is surprising since it was shown previously that the chronological order of gene vector formulation, that is, if the DNA was first pre-complexed with the TAT_2 peptide and the gene carrier was added afterwards or *vice versa*, had a strong effect on the gene transfer efficiency (17). However, in this study different gene carriers such as PEI and fractured dendrimers were examined. In contrast to cationic polymers which tightly condense DNA into spherical or rod-like structures, the DNA is rather adsorbed to the SLN surface (1,2). This structure apparently allows peptide binding to the DNA even after the DNA is complexed to the SLN. In agreement with the results mentioned above when ternary TAT_2-M1-based gene vectors were formulated in HBS, the level of gene expression remained on the level mediated by the binary SLN gene vectors independent of the order of formulation. The level of gene expression mediated by the ternary TAT_2-based SLN gene vectors formulated in distilled water reached 50% as compared with gene expression mediated by PEI. As a consequence, ternary TAT_2-based SLN gene vectors showed promising results *in vitro* when formulated in a manner acceptable for *in vivo* application.

Gene Transfer Efficiency Mediated by SLN-Based Gene Vectors *in Vivo* upon Intratracheal Instillation

SLN-based gene vectors which were directly instilled through the trachea into the mouse lungs did not show consistent gene expression neither when formulated without nor when formulated with the TAT_2- and TAT_2-M1 peptide. Only in one out of three mice luciferase gene expression was observed for the SLN- (1.6 pg luciferase/g lung tissue) and SLN-TAT_2-M1 (1.1 pg/g) gene vector formulations and no gene expression was observed for the SLN-TAT_2 gene vectors. In contrast, PEI-based gene vectors mediated higher gene expression but also only in two out of three treated animals (0.7 pg/g and 27.3 pg/g). A possible reason for this observation could be complex instability. For each of the gene vector formulations precipitation was observed which was stronger for the SLN gene vectors as compared with the PEI-

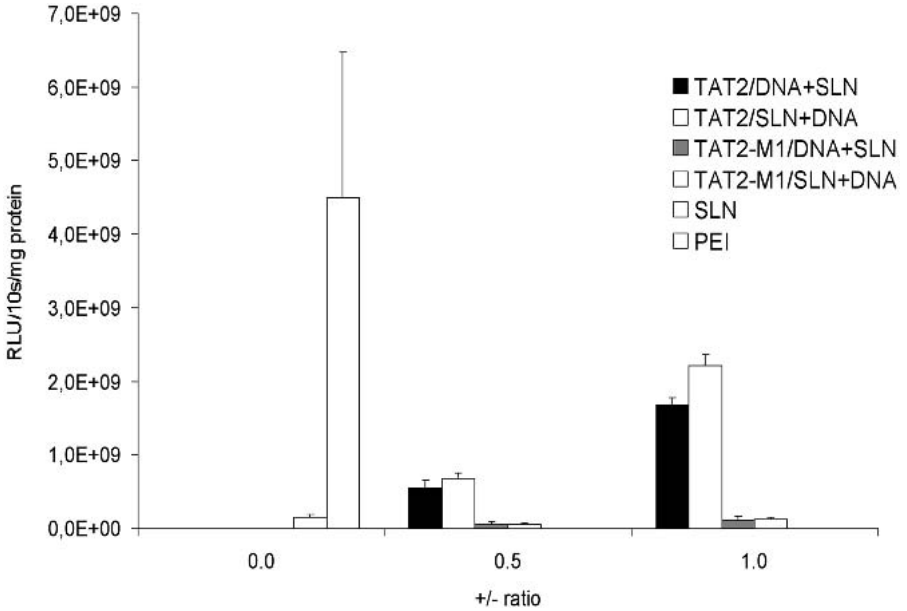

Fig. 3. Influence of the formulation of ternary SLN gene vectors on transfection efficiency on 16HBE14o- cells. Gene vectors were formulated either through pre-compaction of DNA with increasing charge ratios of TAT_2 or TAT_2-M1 followed by the addition of SLN_DOTAP (wt/wt = 2.5), or TAT_2 and TAT_2-M1 have been first mixed with SLN_DOTAP and then complexed with DNA. PEI gene vectors were formulated with an N/P-ratio of 10. All gene vector complexes were generated in water. Data show a representative experiment, n = 4, ± SD.

gene vectors. These results indicate that direct intratracheal application is not a suitable method to test gene vectors *in vivo* if relatively high vector concentrations are required. In order to avoid formulation instability we further performed experiments using a nebulization procedure for gene vector application.

Gene Transfer Efficiency Mediated by SLN-Based Gene Vectors *in Vivo* upon Nebulization

Very recently it was demonstrated by Densmore *et al.* that jet nebulization of gene vector complexes in a whole body chamber experimental setup can be used to efficiently deliver gene vector complexes to the mouse lungs (28). Since this application method is better tolerated by the mice than direct intratracheal instillation of gene vector complexes (15) and lower gene vector concentrations can be used, we chose a whole body chamber nebulization method for application. Size measurements of the gene vector formulations revealed stable but polydisperse particles (Table I). As shown in Fig. 4, gene expression mediated by the ternary TAT_2-based SLN gene vectors was 1.6-fold higher as compared with binary SLN gene vectors. The TAT_2-M1-based SLN gene vectors mediated 1.2-fold higher gene expression as compared with SLN gene vectors. However, the differences did not reach statistical significance. When compared to the efficiency mediated by the PEI gene vectors the level of gene expression was 80- to 100-fold lower. These data demonstrate that SLN-based gene vector formulations are capable to deliver DNA *in vivo* to the mouse lungs. Although an increase of gene expression was observed through incorporation of the TAT_2 peptide in the SLN gene vector complex, gene transfer efficiency remains low. These observations did not correlate with results obtained from cell culture experiments. One possible explanation to this could be the effect of the nebulization process on SLN gene vector stability. Unexpectedly, neither SLN nor its combination with the TAT_2 or the TAT_2-M1 peptide sufficiently protected DNA from degradation during the nebulization procedure as indicated by a smear of small DNA fragments in agarose gel electrophoresis (Fig. 5A). Such degradation was not observed when DNA was complexed with PEI (32). As a result, only low amounts of intact DNA are expected to be delivered to the mouse lungs when DNA is complexed with SLN.

To circumvent this problem we examined a novel nebulizer which operates by the principle of a perforated vibrating membrane driven by a piezocrystal (e-Flow, PARI GmbH, Starnberg, Germany). This nebulizer represents an innovative nebulization technology. In contrast to the jet nebulizer, no degradation of DNA formulated as ternary gene vectors was observed after nebulization. DNA degradation was largely reduced for binary SLN gene vectors and even naked DNA was only moderately degraded (Fig. 5B). In fact, these results indicate that this novel nebulizer represents a very mild nebulization technology. At higher gene vector concentrations as used within these experiments the perforated membrane clogged up which did not allow nebulization of the gene vectors. At 10-fold lower concentrations (12.5 μg DNA/ml; a total dose of 100 μg of DNA) the SLN-based gene vectors could be successfully nebulized but no gene expression was detected in the mouse lungs (data not shown). This is probably due to the low doses of DNA which had to be used in these experiments. Apparently such low doses were not sufficient to deliver effective DNA doses to the lungs in this animal model. Even when PEI was used at comparable low concentrations (25 μg DNA/ml, a total dose of 250 μg of DNA) no gene expression was measured in the lungs of mice by using a similar aerosol device (33).

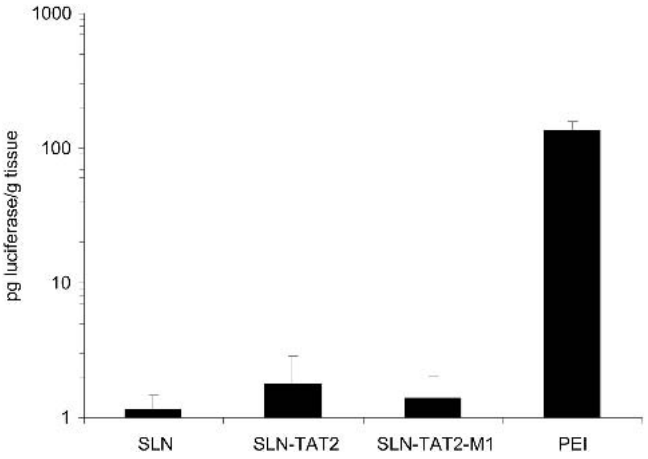

Fig. 4. Gene transfer efficiency mediated by SLN-based gene vectors *in vivo* upon jet nebulization. DNA (1 mg) was either complexed with SLN_DOTAP (wt/wt = 2.5, referring to DOTAP) or SLN_DOTAP first supplemented with TAT_2, or TAT_2-M1 (+/− = 1). PEI gene vectors were formulated with an N/P-ratio of 10. All gene vector complexes were generated in water (n = 4, ± SD).

Table I. Size of Gene Vectors

Gene vector	Size (nm)	PDI
SLN	400 ± 15	0.835 ± 0.096
SLN-TAT_2	204 ± 4	0.351 ± 0.020
SLN-TAT_2-M1	198 ± 3	0.336 ± 0.014
PEI	91 ± 2	0.500 ± 0.031

These observations indicate that, although no gene expression was detected in this mouse model, aerosol delivery of physically intact plasmid DNA of fragile gene delivery systems is successful by using a mild nebulization technology. This is an important finding for future studies. Testing this gene delivery system in future studies will require large animal models for the following reasons: i) in contrast to the whole body nebulization method, which we used in this study, a large animal model will allow the controlled application of the aerosol directly into the trachea after intubation or by a mask device, ii) this will allow to increase the ratio of inhaled to nebulized DNA, iii) which will result in the deposition of effective DNA doses in the lungs for successful gene expression.

CONCLUSIONS

In this study, it was shown that a dimeric TAT peptide derived from the arginine-rich motif of the HIV-1 TAT protein that functions as nuclear localization sequence and as a protein transduction domain could be used to substantially enhance gene transfer efficiency of SLN-based gene vectors leading to gene expression levels even higher as observed for PEI gene vectors. The enhancement of gene transfer effi-

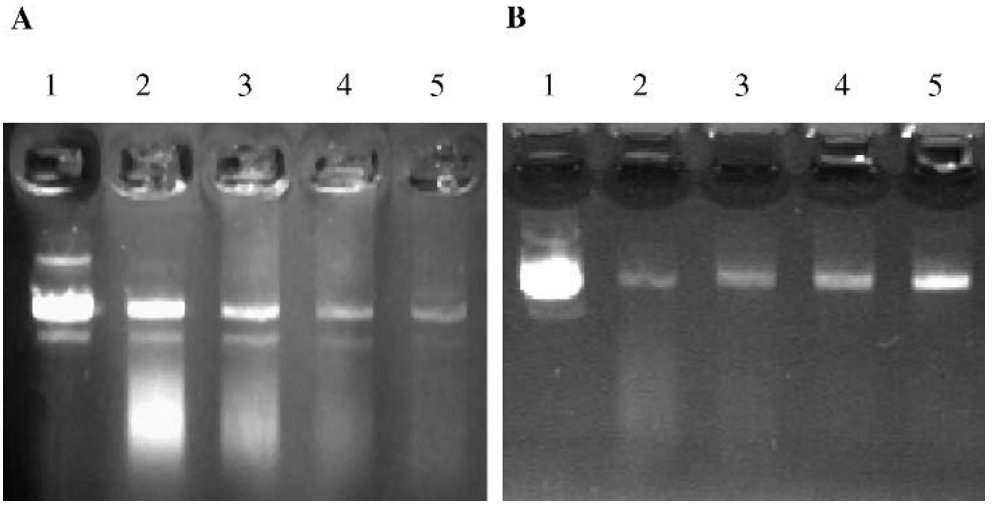

Fig. 5. DNA integrity after nebulization of SLN gene vectors. SLN gene vectors were examined by alkaline gel electrophoresis to separate DNA from the SLN and TAT_2 or TAT_2-M1. The DNA was extensively degraded by the nebulization using the jet nebulizer as indicated by the short DNA fragments found on the gel (A). In contrast, DNA was less degraded when using the e-Flow (B). (1, control DNA not nebulized; 2, naked DNA nebulized; 3, SLN-DNA nebulized; 4, TAT_2-DNA-SLN nebulized; 5, TAT_2-M1-DNA-SLN nebulized.)

ciency was not due to simple pre-compaction of DNA with cationic peptides but showed sequence-dependency as compared to pLa and TAT_2-M1. SLN gene vectors mediated gene expression in the mouse lungs upon aerosol application which was increased by the TAT_2 peptide. Gene transfer efficiency was lower as compared to PEI gene vectors which apparently resulted from DNA destruction during the nebulization process. Such DNA destruction could successfully be avoided when gene vectors were nebulized by a mild nebulization technology based on a novel perforated membrane technology. These are important findings which will allow aerosol application of fragile gene delivery systems to the lungs in future studies.

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