Published in final edited form as:

Bioconjug Chem. 2009 January ; 20(1): 120–128. doi:10.1021/bc8003414.

Efficient Nuclear Targeting by NLS-PEG-Acridine Conjugates in the Transfection of Blood-Brain Barrier Endothelial Cells with Lipoplexes and Polyplexes

Hongwei Zhang, Anton Mitin, and Serguei V. Vinogradov *

Department of Pharmaceutical Sciences and Center for Drug Delivery and Nanomedicine, College of Pharmacy, University of Nebraska Medical Center, Omaha, United States

Abstract

Brain capillary endothelial cells of the blood brain barrier (BBB) are difficult target for non-viral transfection even for most potent transfection agents. Efficient protection and nuclear delivery of plasmid DNA are the key requirements for enhancing the transfection. We designed novel DNA intercalating conjugates of PEG-tris(acridine) with a short nuclear localization signal (NLS) peptide and investigated effect of their complexes with luciferase-encoded plasmid DNA on lipoplex- and polyplex-mediated transfection of murine brain capillary endothelial bEnd.3 cells. These intercalation complexes protected DNA from nucleolytic degradation forming a protective PEG layer around plasmid DNA and could be efficiently condensed by Lipofectamine2000 or ExGen500 into nanosized particles. Complexation of plasmid DNA with PEG-acridine/NLS-PEG-acridine mixture (9:1 w/w), taken in amount equal to 5–6 NLS peptides per DNA molecule, significantly enhanced both lipo- and polyplexes transfection efficacies and increased the number of transfected bEnd.3 endothelial cells in the presence of serum. Comparative transgene expression efficiency was significantly higher at longer PEG linker and optimal conjugate-to-DNA weight ratio, especially, at lower N/P ratio for both transfection agents, reaching 15–16-fold for lipoplexes and 10–11-fold for polyplexes. In addition, the NLS-PEG-acridine conjugates didn't increase cytotoxicity of lipoplexes and polyplexes to bEnd.3 cells. These conjugates can serve as promising components for development of systemic non-viral transfecting approach to the transfection of the BBB and temporary modulation of its drug permeability.

Introduction

Many chemotherapeutic agents have limited penetration into the brain through the BBB because brain capillary endothelial cells (BCEC), forming the BBB, express various drug efflux transporter (DET) membrane proteins that actively remove drugs from the brain (1). Transient inhibition of the selective DET expression using RNA interference or other therapeutic approaches can potentially increase drug efficacy in the treatment of CNS-related diseases (2). It is known that successful application of non-viral vectors is largely limited by their relatively low transfection efficiency in spite of some attractive features like safety and low cost. Even more challenges remain for systemic non-viral gene delivery to the BCEC that have a high intrinsic resistance to the transfection compared to other cell lines (3). Nuclear accumulation of plasmid DNA is among the major barriers of non-viral gene delivery, especially, in post-mitotic, slow dividing and quiescent cells (4–5). Actively dividing cells

*Corresponding author: 986025 Nebraska Medical Center, Omaha, NE 68198-6025, Phone: 1-402-559-9362; email: vinograd@unmc.edu.

display a regular nuclear envelope disassembly and offer more opportunity for DNA to reach the nuclei. In addition, DNA is sensitive to degradation by nucleases in the cytoplasm.

Nuclear proteins are transferred from the cytoplasm into the nuclei through active transport across nuclear pores mediated by importins recognizing a nuclear localization signal (NLS) sequence (6). Various applications of short NLS peptides to increasing the nuclear entry of plasmid DNA have been investigated, including covalent or non-covalent attachment of NLS peptides to DNA (7–10). Zanta *et al.* demonstrated that only one NLS peptide covalently linked to DNA could increase the transfection efficacy following an intracellular injection of the plasmid (11). Jeon *et al.* conjugated a NLS peptide to PLGA copolymer and demonstrated that slowly degradable PLGA nanospheres could increase the nuclear localization and transfection efficiency of foreign DNA (12). Covalent conjugation of NLS peptides to DNA appeared to be a straightforward method, but the modification could result in the loss of biological activity (13). Peptide nucleic acids (PNA) were also employed to attach NLS peptide to DNA via sequence specific non-covalent binding in order to avoid decrease in transcription activity (14). However, this method required individual PNA synthesis for each targeted DNA.

Non-specific intercalation strategy is looking promising for application to the non-viral transfection. Acridine is a well-known molecule with high affinity to DNA duplex. However, previous attempts to apply acridine-conjugates of NLS peptide resulted in controversial data about their structural requirements for efficient transfection (15–16). In the present study, we investigated effects of direct modification of plasmid DNA with NLS-PEG-acridine conjugates having various PEG linker lengths on the transfection of murine brain capillary endothelial bEnd.3 cells, an *in vitro* model of the BBB (17), by lipoplexes and polyplexes..

EXPERIMENTAL PROCEDURES

Materials

All solvents and reagents, except specially mentioned, were purchased from Sigma-Aldrich (St. Louis, MO) at highest available quality grade and used without purification. Maleimide (MAL)-PEG₅₀₀₀-N-hydroxysuccinimide (NHS), MAL-PEG₃₄₀₀-NHS and PEG₅₀₀₀-NHS linkers were from JenKem Technology USA (Allen, TX) and MAL-PEG₉₀₀-NHS linker –from Pierce (Rockford, IL). The cystein-containing NLS peptides CAPKK~~K~~RKVA-CONH₂ and mutant peptide CAPKT~~K~~RKVA-CONH₂ were synthesized by Biomer Technology (Pleasanton, CA) and purified by reverse-phase HPLC. Acridine 9-isothiocyanate was purchased from Acros Organic and purified by chromatography on silicagel before use.

Both gWIZ-Luc (6.7 kb) and gWIZ-GFP (5.7 kb) plasmids were originally purchased from Gene Therapy System (San Diego, CA), propagated in *E.coli* (DH5- α) and isolated using a Qiagen Giga plasmid purification kit (Valencia, CA). The plasmid DNA integrity and topology was analyzed by agarose gel electrophoresis.

Synthesis and characterization of NLS-PEG-Acridine conjugates

Modification of the G1.0 PA-dendrimer with PEG-linkers

MAL-PEG₅₀₀₀ (PEG₃₄₀₀ or PEG₉₀₀)-NHS linkers or PEG₅₀₀₀-NHS linker (10 μ mol) were dissolved in 0.5 mL of water and slowly added to G1.0 PA-dendrimer (9 mg) dissolved in 0.5 mL of water and adjusted to pH 8.0 by 1N HCl. The reaction continued at stirring for 30 min at 20 °C. The high-MW fraction of the PEGylated products was isolated by gel-filtration on the Sephadex G-25 column (G-10 for the PEG₉₀₀ derivative), concentrated *in vacuo* and immediately used in the next step.

Reaction with acridine 9-isothiocyanate

Acridine 9-isothiocyanate (13 mg) was dissolved in 3 mL of methanol containing 4 μ L of triethylamine and mixed with the previously obtained aqueous solution of a PEG-dendrimer conjugate. The reaction mixture was stirred for 1 h at 20°C and methanol was removed *in vacuo*. The high-MW yellow fraction of the MAL-PEG-acridine product was isolated by gel-filtration as previously, concentrated *in vacuo* and immediately used in the last step.

Reaction with cystein-containing NLS-peptides

A purified NLS-peptide (9 mg) was dissolved in 0.5 mL of DMF and mixed with degassed aqueous solution of the MAL-PEG-acridine conjugate obtained in the previous step. The reaction mixture was left overnight at 4 °C. The final product was concentrated *in vacuo*, isolated by gel-filtration as previously, purified on the semi-preparative reverse-phase HPLC column (C18, 5 μ m, 1 $\times$ 25 cm), using gradient elution by acetonitrile in 0.1% trifluoroacetic acid (v/v), and lyophilized.

Analysis of NLS conjugates

Isolated compounds were analyzed by analytical reverse phase HPLC. UV absorbance of these conjugates at 420 nm was measured to calculate the acridine content ($\epsilon_{420} 5500$). MALDI-TOF mass spectrometry in positive mode and sinapinic acid as a matrix was used to determine an average MW of these conjugates. Amino acid analysis was conducted on the ABI Protein Analyzer following acid hydrolysis (UNMC Protein Structure Core Facility).

Preparation of lipoplexes and polyplexes containing NLS conjugates

7.5 μ g of pDNA was initially incubated with different amounts of NLS-PEG-acridine conjugates or PEG-acridine conjugate (10 mg/mL) in 1 mL of 5% dextran (w/v) for 30 min. To prepare lipoplexes, 15 or 10 μ L of Lipofectamine2000 in 5% dextran (w/v) was added to these complexes or pDNA. To prepare polyplexes, 20 or 14 μ L of ExGen500 in 5% dextran (w/v) was added to these complexes or pDNA. Final formulations were incubated for 30 min at 20 °C with continuous shaking.

Particle size and the surface charge

The particle size and ζ -potential of the transfection complexes were measured using Zetasizer Nano ZS equipped with 50 mV laser (Malvern Instruments, Worcestershire, UK) in PBS at 25 °C (in triplicate).

Agarose gel mobility shift assay

Samples were loaded on 0.8% (w/v) UltraPure agarose (Invitrogen, Carlsbad, CA) and analyzed by electrophoresis at 120V for 35 min in 1 $\times$ TAE buffer. After electrophoresis, the gel was stained by ethidium bromide and subjected to image analysis.

Cell line culture

Murine brain capillary endothelial cell line bEnd.3 was purchased from American Type Culture Collection (ATCC, Manassas, VA) and cultivated in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and 2% penicillin-streptomycin in a humidified incubator containing 5% CO₂ at 37 °C.

***In vitro* transfection**

All transfection studies were performed in the presence of 10% FBS. Unless specific indicated, bEnd.3 cells were seeded in 96-well plate with an initial density of 7×10^3 cells per well 24 h prior to transfection. The transfection complexes containing 0.5 μ g of pDNA were added to each well and incubated for 4 h at 37 °C. The medium was then replaced by fresh medium and cells were incubated for additional 40 h. Luciferase expression level was determined in the Bio-Tek FLx800 microplate fluorescence reader using a firefly luciferase assay kit (Biotium, Hayward, CA). Protein content was measured by the BCA assay (Pierce Biotechnology, Rockford, IL). All experiments were performed in 5 parallels and the results were expressed as ng of luciferase per mg of protein.

DNA resistance to degradation by DNase I

Both NLS-lipoplexes and NLS-polyplexes (pDNA/NLS-conjugate = 4:1, w/w) were incubated at 37 °C with DNase I (1 U/ μ g pDNA) in DNase reaction buffer (Promega/Fisher Scientific, Pittsburgh, PA). After incubation, the samples were placed on ice and treated with 10 μ L of stop buffer (Promega) and 30 μ L of heparin sodium (5000 USP U/mL, American Pharmaceuticals Partners, Schaumburg, IL). The samples were then analyzed by 0.8% agarose gel electrophoresis as described above.

In the functional test of DNA protection, 4.5 μ g of pDNA was incubated with 1.1 μ g of PEG₅₀₀₀-acridine for 30 min at room temperature. This complex was incubated with DNase I (0.005 U/ μ g DNA) at 37 °C for 15, 30 or 60 min. The reaction was stopped by adding 1 μ L of stop buffer and cooling on ice. The solution was then incubated with 12 μ L of ExGen500 for 15 min at 25°C. Naked pDNA was used as control and treated by DNase I under identical conditions. The bEnd.3 cells were transfected by these complexes in the presence of serum as indicated above.

Flow cytometry quantification of gene expression

bEnd.3 cells were seeded in 6-well plate with an initial density of 4×10^5 cells per well 24 h prior to transfection. 3 μ g of the green fluorescent protein (GFP)-encoded pDNA was pretreated with NLS-PEG₅₀₀₀-acridine, encapsulated in lipo- or polyplexes and added to the cells in the medium containing 10% FBS. Following incubation for 4 h at 37 °C, the medium was replaced by a fresh one, and cells were incubated for additional 40 h. The transfected cells were rinsed with PBS, trypsinized with 0.3 mL trypsin-EDTA solution, and resuspended in 0.5 mL PBS with 0.1% FBS. The percentage of cells displaying GFP-associated fluorescence was quantified by flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA). The experiments were performed in triplicates.

Confocal microscopy analysis

pDNA was labeled with CX-Rhodamine by the Label IT Tracker intracellular nucleic acid localization kit (Mirus Bio Corporation, Madison, WI) according to the manufacturer's instructions. bEnd.3 cells were seeded in 8-chamber culture slides (BD Biosciences, Bedford, MA) with an initial cell density of 1.5×10^4 per chamber 24 h prior to transfection. The transfection complexes containing 0.7 μ g of the rhodamine-labeled pDNA were added to the cells as described above. After transfection, cells were washed by PBS and fixed by 4% (w/v) paraformaldehyde for 15 min on ice. Then, the SlowFade gold with DAPI solution (Invitrogen) was applied to the cells. The slides were carefully sealed and observed under an LSM 510 confocal laser scanning microscope (Carl Zeiss, Germany).

Nuclear accumulation of pDNA

To measure directly nuclear accumulation of fluorescent DNA, bEnd.3 cells were seeded in 6 well plates with an initial density of 2×10^5 cells per well 24 h prior to transfection. CX-Rhodamine labeled pDNA was used to prepare NLS-polyplexes at 4:1 (w/w) ratio as mentioned above. Cells were transfected for 4 h in the presence of 10% FBS at 37 °C, then washed by PBS 3 times and allowed to grow in complete medium for another 4 h. The transfected cells were rinsed with PBS, trypsinized with 0.3 mL trypsin-EDTA solution, and resuspended in 0.5 mL PBS-0.1% FBS. For each sample, cells were counted and 4×10^5 cells were collected by centrifugation at 600 g for 5 min at 4 °C. The genomic DNA was extracted from nuclei using the Genomic DNA Isolation Kit (Biovision Inc., Mountain View, CA) (18). Extracted DNA was resuspended in 1 mL of TE buffer and analyzed by fluorescence at λ_{ex} 576 nm/ λ_{em} 597 nm. The experiment was performed in triplicates.

Cytotoxicity assay

Cytotoxicity of NLS-conjugates and transfection complexes in bEnd.3 cell culture was evaluated by the thiazolyl blue tetrazolium bromide (MTT) assay as described previously (19). Briefly, bEnd.3 cells were seeded in 96-well plate at initial density of 7×10^3 per well 24 h prior the assay. NLS-conjugates and transfection complexes were incubated with cells using transfection conditions. Then, 20 μ L of the MTT solution (5 mg/mL) was added to each well and formazan crystals were allowed to form for 4 h. The medium was carefully removed and 200 μ L of dimethylsulfoxide (DMSO) was added to each well. The plate was then incubated for 15 min at 37 °C and the absorbance of formazan was measured at 570 nm using a Model 680 microplate reader (Bio-Rad Laboratories, Hercules, CA). The experiments were performed in 5 parallels and cell viability was calculated relatively to untreated cells.

Statistical analysis

Experiment results are presented as means $\pm$ SEM. Statistical significance was determined by the paired sample's *t*-test using SPSS 13.0 software (SPSS Inc., Chicago, IL). In all cases, *P* < 0.05 was considered as statistically significant.

RESULTS

Synthesis and characterization of NLS conjugates

Preliminary molecular modeling showed that conjugates of acridine 9-isothiocyanate with the generation 1.0 PA-dendrimer have proper dimensions and good fitting for intercalation in the double helix of DNA. To prepare NLS-PEG-acridine conjugates, we used heterofunctional maleimide-PEG-N-hydroxysuccinimide linkers of MW 900, 3400 and 5000 Da (Scheme 1). Initially, these linkers were modified with a large excess of generation 1.0 PA dendrimer. For the fast product isolation we used a significant difference between PEGylated products and reactants. All MAL-PEG-dendrimer conjugates were isolated by gel filtration, and then all three remaining primary amino groups were immediately modified with acridine 9-isothiocyanate. The MAL-PEG-acridine product was also purified by gel filtration and immediately introduced into reaction with the thiolated NLS peptide. Final NLS-PEG-acridine conjugates were purified by reverse phase HPLC using a C18 semi-preparative column and extended gradient of acetonitrile. Additional cation exchange chromatography on small TSK SAX column allowed us to remove small amounts of NLS-PEG conjugates and conjugates with lower acridine content detected in final mixtures.

The NLS-PEG-acridine conjugates were homogenous by analytical HPLC and could be easily separated from NLS peptide (peak 1, Fig.1A). A stronger retention of NLS-PEG conjugates of higher MW was also observed. However, it was difficult to completely separate the NLS-

PEG-acridine conjugates from corresponding PEG-acridine conjugates. The characteristic mass distribution in the MALDI-TOF mass spectra using a sinapinic acid as a template confirmed the presence of overlapping groups of PEG-acridine and NLS-PEG-acridine molecules in near equimolar ratio with the correct peak MWs. As an example, NLS-PEG₅₀₀₀-acridine sample showed overlaying of two PEG-containing peaks with an average MW between 5909 and 7036 Da, corresponding to PEG₅₀₀₀-acridine and NLS-PEG₅₀₀₀-acridine conjugates, respectively, presented in the final product in near equal amounts (Fig. 1B). By UV-spectrophotometric data, NLS-PEG-acridine conjugates contained the molar amount of acridine close to values calculated for the corresponding *tris*(acridine) conjugates (Fig. 1C). Finally, aminoacids microanalysis after the total acidic hydrolysis of NLS-PEG-acridine conjugates demonstrated the presence of peptide with the correct aminoacids content and provided the protein content that was used to calculate the NLS peptide derivatization degree. Based on these data, the total peptide content in the obtained NLS-conjugates was equal to 8–13% and equivalent to the NLS peptide derivatization degree of 40–50%.

Binding of NLS-conjugates to pDNA

The binding of NLS-conjugates with plasmid DNA was analyzed by the band shift assay using agarose electrophoresis. As shown in Figure 2, pDNA showed the first signs of retardation at NLS-PEG₅₀₀₀-acridine/pDNA ratio 1:4 (wt) and, then, efficiently retained in the wells at the ratio between 4:1 and 6:1. By contrast, equivalent amounts of individual NLS peptide or acridine showed no influence on the pDNA mobility. These results suggest that only the binding of NLS-PEG₅₀₀₀-acridine conjugate with pDNA resulted in the increase of total MW and steric hindrance of the intercalation complex and observed as band retardation during electrophoresis. Similar results were obtained with NLS-PEG₃₄₀₀-acridine and NLS-PEG₉₀₀-acridine, although at various for all conjugates weight ratio (data not shown).

Properties of NLS-lipoplexes and NLS-polyplexes

Intercalation complexes have been prepared by pre-mixing of NLS-conjugates with pDNA and, following the short incubation, these complexes were mixed with Lipofectamine2000 or Exgen500 at the optimized ratio 2 μ l per μ g DNA and 2.7 μ l per μ g DNA, respectively. Compared to the corresponding lipo- and polyplexes (without NLS-conjugates), NLS-lipoplexes and NLS-polyplexes demonstrated slightly increased particle size and decreased surface charge (Table 1). Introduction of NLS-conjugates in lipo- and polyplexes practically did not affect protection of encapsulated pDNA against enzymatic degradation, while the free pDNA was completely digested under the same condition (Figure S1, Supporting materials).

The intrinsic cytotoxicity of NLS-conjugates was very low ($IC_{50} = 5\text{--}7$ mg/mL at 24 h incubation) and did not enhance the cytotoxicity of lipoplexes and polyplexes incubated with bEnd.3 cells. As shown in Figure S2 (Supporting materials), the cytotoxicity of lipoplexes at the optimized ratio of transfection reagent to DNA was generally higher than that of polyplexes; NLS-lipoplexes and polyplexes followed the same trend.

In vitro transfection

bEnd.3 cells were transfected by lipoplexes or polyplexes in the presence of 10% serum. First, the capability of NLS-conjugate to improve the transgene expression was investigated. NLS-PEG₅₀₀₀-acridine significantly enhanced luciferase expression by lipoplexes (Figure 3a) and polyplexes transfection (Figure 3b) ($P < 0.01$). On the contrary, no or slight decrease in transfection was observed when pDNA was coupled with PEG₅₀₀₀-acridine or mNLS-PEG₅₀₀₀-acridine before complexation with transfection reagents. The influence of various NLS-conjugates and different pDNA/conjugate ratio on lipoplex and polyplex-mediated luciferase transgene expression is illustrated in Figure 4. At the optimized ratio of Lipofectamine2000 to pDNA (2 μ L per μ g DNA, Figure 4a), the highest luciferase expression

level was observed at the ratio of pDNA to NLS-PEG₅₀₀₀-acridine 16:1 (w/w). The NLS-PEG₃₄₀₀-acridine conjugate showed a very similar enhancing effect. However, further reducing of the PEG length resulted in lower or no enhancement as indicated for NLS-PEG₉₀₀-acridine conjugate. At the lower ratio of Lipofectamine2000 to pDNA (1.4 μ L per μ g DNA, Figure 4c), the observed comparative transfection increase for NLS-lipoplexes over plain lipoplexes was even more significant ($P < 0.0001$). The addition of NLS-PEG₅₀₀₀-acridine, NLS-PEG₃₄₀₀-acridine and NLS-PEG₉₀₀-acridine to lipoplexes resulted in 15-, 15- and 8-fold increase of the luciferase expression, respectively. In terms of polyplexes, NLS-conjugates also displayed a very strong effect on transfection (Figure 4b and 4d). At the optimized ratio of Exgen500 to pDNA (2.7 μ L per μ g DNA, Figure 4b), the highest luciferase expression level was observed at the pDNA to NLS-PEG₅₀₀₀-acridine ratio 4:1 (w/w). At the lower ratio (1.8 μ L Exgen500 per μ g DNA, Figure 4d), the improving effect of NLS-PEG-acridine conjugates also becomes more evident. Application of NLS-PEG₅₀₀₀-acridine, NLS-PEG₃₄₀₀-acridine and NLS-PEG₉₀₀-acridine resulted in 11-, 11- and 6- fold increase of the luciferase expression, respectively. The pDNA dose dependence was determined at fixed optimal ratios for both NLS-lipoplexes and polyplexes (Figure S3, Supporting materials). Our results demonstrated that the highest transfection level could be achieved at pDNA doses used in the study (0.5 μ g pDNA per well) and the transgene expression began to drop at higher doses, evidently, because of the toxicity of transfection reagents.

The protective role of bulk PEG chains on the stability of pDNA to enzymatic hydrolysis was illustrated by the functional test of luciferase-encoded pDNA/PEG₅₀₀₀-acridine complex primarily treated with DNase I and then complexed with ExGen500. Comparative results of the bEnd.3 cell transfection by the protected and unprotected DNA treated with DNase I are shown in Figure 5. Tremendous loss of transfection activity of naked pDNA was observed following the enzymatic digestion and it was almost gone after 30 min of incubation. By contrast, DNA/PEG₅₀₀₀-acridine complex was much more stable and 20% of luciferase activity still remained after 60 min of incubation.

Flow cytometry assay

Since NLS-PEG₅₀₀₀-acridine demonstrated the strongest enhancing effect, we quantitatively studied transfection efficiency and GFP expression levels for NLS-lipoplexes and polyplexes by flow cytometry. Our flow cytometry results showed that NLS-PEG₅₀₀₀-acridine could enhance both GFP transfection efficiency and GFP expression. Specifically, we observed a stronger effect on transfection efficiency (2.13- and 1.81-fold enhancement), than on the GFP expression level (1.40- and 1.31-fold enhancement) for lipoplexes and polyplexes, respectively ($P < 0.01$, Figure 6a and 6b).

Subcellular localization of pDNA

Effect of NLS-PEG₅₀₀₀-acridine on the nuclear accumulation of rhodamine-labeled pDNA in bEnd.3 cells was studied 24h post-transfection (Figure 7). Using confocal microscopy, we observed only a limited amount of rhodamine-labeled pDNA in the nuclei and perinuclear area following the transfection by lipoplexes or polyplexes (Figure 7A and 7D). In contrast, cells transfected by NLS-lipoplexes or polyplexes showed much more pDNA delivered inside the nuclei and in the perinuclear area (Figure 7B and 7E). Similar results were obtained with NLS-PEG₃₄₀₀-acridine and NLS-PEG₉₀₀-acridine conjugates for both types of transfection complexes. The lateral confocal images clearly demonstrate efficient penetration of the rhodamine-labeled DNA into the nuclei (Figure 7C and 7F).

For direct evaluation of nuclear accumulation of rhodamine-labeled pDNA following the transfection by polyplexes, the total nuclear DNA was extracted from nuclei of transfected cells and the fluorescence intensity was determined. As shown in Figure 7G, NLS-PEG₅₀₀₀-

acridine/Exgen500 polyplex enhanced nuclear accumulation of pDNA by almost 2-folds compared with plain polyplexes ($P < 0.05$), while control mNLS-polyplex showed no enhancement effect.

Discussion

Efficient nuclear delivery of plasmid DNA is one of major requirements for successful non-viral transfection. Different approaches have been explored to increase nuclear accumulation including association of DNA with hydrophobic molecules (20), engineered histone H2B (21), adenovirus hexon protein (22) but without a notable success. Application of NLS peptides and cellular machinery for transporting of nuclear proteins was shown to be a very promising approach; although, many experiments resulted only in a limited success. The major problem is the size of nuclear pores, which is restrictive to molecules larger than 23 nm in diameter. Only direct attachment of NLS peptide to pDNA, not affecting its expression activity, demonstrated positive effects, while condensing the DNA with NLS-modified carriers usually had no effect on transfection (11). The second decisive factor is stability of pDNA in cytoplasm. Fast degradation of pDNA by cytosolic nucleases, following its release from lipo- or polyplex, and lower mobility of free pDNA in cytoplasm can be additional reasons of the fast loss of transfection activity. And, finally, in slowly dividing cells pDNA has lower chance to internalize in the nuclei during mitosis, which is, probably, the major way for the carrier-associated DNA to efficiently penetrate nuclei.

In this study, we demonstrated that non-covalent modification of pDNA by PEG-*tris*(acridine) conjugates of NLS peptide could significantly enhance nuclear accumulation and transgene expression in slowly dividing cells following the lipoplex or polyplex-mediated transfection. Acridine-based intercalating agents tightly bind double-stranded DNA ($K_d < 10^{-9}$ M) and no sequence specificity is required for this process (23–24). Previously, we demonstrated that modification of oligonucleotides with acridine 9-isothiocyanate strongly enhanced their antisense activity (25). Recent data on NLS-polypeptide-acridine constructs confirmed that acridines as anchor molecules for non-covalent, strong and reversible binding of NLS peptide to pDNA could be an inexpensive alternative to PNA clamps (16). Here we constructed novel intercalating conjugates based on the generation 1.0 PA dendrimer linked to three acridine molecules and one NLS peptide via a PEG linker. This scheme allowed us to find an appropriate distance between the peptide and *tris*(acridine) anchor to achieve an efficient pDNA protection and *in vitro* transfection by lipo- and polyplexes. Previously, Boulanger *et al.* reported that when the distance between NLS peptide and anchor molecule was shorter than 20 methylene groups, NLS-conjugates showed no enhancing effect on transfection ability of lipo- and polyplexes *in vitro* (15). Our results demonstrated that the NLS-PEG-acridine conjugates with longer PEG linkers (PEG₅₀₀₀ and PEG₃₄₀₀) showed rather similar high transfection enhancement, while the effect of shorter PEG linker with 40 methylene groups was much lower. Our data showed that the optimal MW requirement for the PEG linker is close to 3,000 Da. Evidently, a sufficiently long PEG linker can facilitate the NLS peptide recognition by nuclear transport proteins, such as importins.

Since high PEG density may potentially affect the cellular binding of transfection complexes, reducing transfection efficiency, the amount of NLS conjugates used in transfection was optimized at two constant weight ratios of transfection agent to pDNA. A significant increase was observed at the higher weight ratios recommended for both used transfection agents by manufacturers (Figure 3a and 3b), although, the highest differences could be observed at the lower ratios ($P < 0.0001$, Figure 3c and 3d). Strong positive charge of transfection complexes is the major factor that determines cellular uptake and endosomal release of pDNA *in vitro*. In our conditions, at the optimized ratio of transfection agents to pDNA, the cellular capacity to internalize and express large amounts of exogenous DNA can be already at saturation. The

effect of nuclear targeting on the transgene expression can be hidden in these conditions. This conclusion is partially confirmed by the dose dependence results, where both lipoplexes and polyplexes at fixed ratios of NLS-conjugate to pDNA and transfection agents to pDNA demonstrated a bell-shaped dependence on the pDNA dose and maximum luciferase expression was observed at the dose 0.5 μ g per well used in transfection experiments (Figure S3). Thus, our results at the higher ratio are obtained at near a maximum transfection level for the used number of bEnd.3 cells. While, at the lower ratio, the number of internalized pDNA molecules was reduced and the effect of NLS-associated nuclear accumulation became more evident. Very low amounts of NLS conjugates required to obtain a significant accumulation of pDNA, 1/16 and 1/4 of the pDNA weight for lipoplexes and polyplexes, respectively. At the 10% peptide substitution rate in conjugates, the calculated optimal number of peptide molecules per pDNA molecule was equal to 4–5 for lipoplexes and 16–20 for polyplexes. Interestingly, at lower amount of ExGen500 in polyplexes the optimal pDNA to NLS conjugates ratio dropped to 12:1 or 5–7 peptides per pDNA. Evidently, an average minimal amount of five NLS peptides is sufficient to efficiently deliver one pDNA molecule into the nuclei. On the contrary, equal amounts of the PEG₅₀₀₀-acridine conjugate did not result in any enhancement and even suppressed the transfection. In control experiments with the PEG₅₀₀₀-acridine conjugate containing a mutated NLS sequence, where lysine was substituted with threonine, the luciferase expression level was even slightly lower compared to plain polyplexes (Figure 4). These results indicated that observed improvements of the transgene expression was caused by the sequence-specific NLS peptide-mediated nuclear targeting rather than by the beneficial effect of the cationic peptide sequence.

Transfection efficiency usually indicates the ability to transfect a certain number of cells, while transgene expression level typically shows how efficiently the pDNA express the transgene within the cell. Successful nuclear entry and subsequent expression of only one copy of pDNA may result in the GFP-derived fluorescence and change the cell count from negative to positive. Efficient degradation of internalized exogenous pDNA can be a cause of the observed differences. At equally effective cellular uptake of transfection complexes, pDNA protected by the PEG coating has a better chance to remain intact in cytoplasm and eventually be delivered to the nuclei. Previously, nucleolytic degradation of pDNA in cytoplasm was recognized as another barrier to the efficient non-viral transfection (26). PEGylation is one of successful approaches to protect protein therapeutics from enzymatic degradation and hydrolysis (27). Here, we investigated the protective effect of PEG coating of pDNA against DNase digestion. Evidently, sufficient amount of attached PEG molecules along with the optimal number of NLS peptide-modified PEG molecules via intercalating strategy can form a protective PEG layer that considerably shield pDNA from interaction with cytosolic nucleases following its release from lipo- and polyplexes. Our results demonstrated longer persistence of functional pDNA following the treatment with DNase I for NLS-PEG-acridine conjugate-modified pDNA vs. naked pDNA (Figure 5).

Our confocal microscopy results qualitatively demonstrated better cellular persistence and nuclear incorporation of NLS-PEG-acridine conjugate-modified fluorescent pDNA; a significant pDNA accumulation was observed also in perinuclear regions after lipo- or polyplex-mediated transfection (Figure 7B and 7D). In the nuclear incorporation studies, fluorescent pDNA was observed in form of red dots rather than in form of diffuse staining. This is well consistent with previous reports which showed the similar punctuated signals for nuclear-imported pDNA (28–29). The diffuse fluorescent signals observed in earlier experiments could be caused by degraded DNA (30). However, the confocal pictures are insufficient for quantitative evaluation of nuclear accumulation of pDNA and explanation of all these observed improvements in the expression of luciferase-encoded pDNA. The direct isolation and quantitation of fluorescent pDNA from the nuclei of transfected cells strongly suggested that NLS-conjugate could significantly ($P < 0.005$) improve nuclear transport of

exogenous pDNA in comparative polyplex transfection experiments, while mutant NLS-modified and non-modified conjugates did not affect the nuclear accumulation compared to naked DNA (Figure 7G).

Safety is another important issue in the development of gene delivery systems. The NLS-PEG-acridine conjugates showed very low cytotoxicity and at used concentrations had no effect on the summary cytotoxicity of lipoplexes and polyplexes (Figure S3). Additionally, the lower ratio of transfection agent to pDNA together with application of NLS-conjugates potentially can significantly reduce toxicity of lipo- and polyplexes without compromising the high level of the achieved non-viral transfection.

In conclusion, the present study demonstrated that the novel NLS-PEG-acridine conjugates could effectively increase nuclear accumulation of pDNA and transgene expression levels at the lipoplex- or polyplex-mediated transfection of slowly dividing cells such as BCEC in the presence of serum. Therefore, these conjugates can be used as potential components for development of non-viral gene delivery systems for systemic applications. This general approach can be also applied to the attachment of other modifying or targeting peptides to further enhance gene delivery to specific cells *in vivo* and penetration across cellular barriers hampering application of non-viral transfection strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENT

The financial support from National Institute of Neurodegenerative Diseases and Stroke (R01 NS050660 for S.V.V.) is gratefully acknowledged. The authors thank Janice Taylor and James Talaska of the Confocal Laser Scanning Microscope Core Facility (University of Nebraska Medical Center) supported by the Nebraska Research Initiative and the Eppley Cancer Center for providing assistance with confocal microscopy. The authors also thank Victoria B. Smith and Linda M. Vilkie of the Cell Analysis Core Facility at UNMC for their help in flow cytometry assay.

LITERATURE CITED

- Ohtsuki S, Terasaki T. Contribution of carrier-mediated transport systems to the blood-brain barrier as a supporting and protecting interface for the brain: importance for CNS drug discovery and development. *Pharm Res* 2007;24:1745–1758. [PubMed: 17619998]
- Calcagno AM, Kim IW, Wu CP, Shukla S, Ambudkar SV. ABC drug transporters as molecular targets for the prevention of multidrug resistance and drug-drug interactions. *Curr Drug Deliv* 2007;4:324–333. [PubMed: 17979652]
- Suh W, Han SO, Yu L, Kim SW. An angiogenic, endothelial-cell-targeted polymeric gene carrier. *Mol. Ther* 2002;6:664–672. [PubMed: 12409265]
- Zabner J, Fasbender AJ, Moninger T, Poellinger KA, Welsh MJ. Cellular and molecular barriers to gene transfer by a cationic lipid. *J Biol Chem* 1995;270:18997–19007. [PubMed: 7642560]
- Brunner S, Sauer T, Carotta S, Cotton M, Saltik M, Wagner E. Cell cycle dependence of gene transfer by lipoplex, polyplex and recombinant adenovirus. *Gene Ther* 2000;7:401–407. [PubMed: 10694822]
- Paschal BM. Translocation through the nuclear pore complex. *Trends Biochem Sci* 2002;27:593–596. [PubMed: 12468221]
- Ciolina C, Byk G, Blanche F, Thuillier V, Scherman D, Wils P. Coupling of nuclear localization signals to plasmid DNA and specific interaction of the conjugates with importin alpha. *Bioconjugate Chem* 1999;10:49–55.
- Nagasaki T, Myohoji T, Tachibana T, Futaki S, Tamagaki S. Can nuclear localization signals enhance nuclear localization of plasmid DNA? *Bioconjugate Chem* 2003;14:282–286.
- Bremner KH, Seymour LW, Logan A, Read ML. Factors influencing the ability of nuclear localization sequence peptides to enhance nonviral gene delivery. *Bioconjugate Chem* 2004;15:152–161.

10. Pouton CW, Wagstaff KM, Roth DM, Moseley GW, Jans DA. Targeted delivery to the nucleus. *Adv Drug Deliv Rev* 2007;59:698–717. [PubMed: 17681634]
11. Zanta MA, Belguise-Valladier P, Behr JP. Gene delivery: a single nuclear localization signal peptide is sufficient to carry DNA to the cell nucleus. *Proc. Natl. Acad. Sci. USA* 1999;96:91–96. [PubMed: 9874777]
12. Jeon O, Lim HW, Lee M, Song SJ, Kim BS. Poly(l-lactide-co-glycolide) nanospheres conjugated with a nuclear localization signal for delivery of plasmid DNA. *J Drug Target* 2007;15:190–198. [PubMed: 17454356]
13. Sebestyen MG, Ludtke JJ, Bassik MC, Zhang G, Budker V, Lukhtanov EA, Hagstrom JE, Wolff JA. DNA vector chemistry: the covalent attachment of signal peptides to plasmid DNA. *Nat. Biotechnol* 1998;16:80–85. [PubMed: 9447599]
14. Branden LJ, Mohamed AJ, Smith CI. A peptide nucleic acid nuclear localization signal fusion that mediates nuclear transport of DNA. *Nat. Biotechnol* 1999;17:784–787. [PubMed: 10429244]
15. Boulanger C, Giorgio CD, Vierling P. Synthesis of acridine-nuclear localization signal (NLS) conjugates and evaluation of their impact on lipoplex and polyplex-based transfection. *Eur J Med Chem* 2005;40:1295–1306. [PubMed: 16225967]
16. Shiraishi T, Hamzavi R, Nielsen PE. Targeted delivery of plasmid DNA into the nucleus of cells via nuclear localization signal peptide conjugated to DNA intercalating bisand trisacridines. *Bioconjug Chem* 2005;16:1112–1116. [PubMed: 16173787]
17. Omid Y, Campbell L, Barar J, Connell D, Akhtar S, Gumbleton M. Evaluation of the immortalized mouse brain capillary endothelial cell line, b.End3, as an in vitro blood-brain barrier model for drug uptake and transport studies. *Brain Research* 2003;990:95–112. [PubMed: 14568334]
18. Kim Y, Yoon JW, Xiao X, Dean NM, Monia BP, Marcusson EG. Selective down-regulation of glioma-associated oncogene 2 inhibits the proliferation of hepatocellular carcinoma cells. *Cancer Res* 2007;67:3583–3593. [PubMed: 17440069]
19. Zhang HW, Zhang L, Sun X, Zhang ZR. Successful transfection of hepatoma cells after encapsulation of plasmid DNA into negatively charged liposomes. *Biotech Bioeng* 2007;96:118–124.
20. Vandenbroucke RE, Lucas B, Demeester J, De Smedt SC, Sanders NN. Nuclear accumulation of plasmid DNA can be enhanced by non-selective gating of the nuclear pore. *Nucleic Acids Res* 2007;35:e86. [PubMed: 17584788]
21. Wagstaff KM, Glover DJ, Tremethick DJ, Jans DA. Histone-mediated transduction as an efficient means for gene delivery. *Mol Ther* 2007;15:721–731. [PubMed: 17327830]
22. Carlisle RC, Bettinger T, Ogris M, Hale S, Mautner V, Seymour LW. Adenovirus hexon protein enhances nuclear delivery and increases transgene expression of polyethylenimine/plasmid DNA vectors. *Mol Ther* 2001;4:473–483. [PubMed: 11708884]
23. Wirth M, Buchardt O, Koch T, Nielsen PE, Norden B. Interactions between DNA and mono-, bis-, tris-, tetrakis-, and hexakis(aminoacridines). A linear and circular dichroism, electric orientation relaxation, viscometry, and equilibrium study. *J. Am. Chem. Soc* 1988;110:932–939.
24. Nielsen PE, Zhen WP, Henriksen U, Buchardt O. Sequence-influenced interactions of oligoacridines with DNA detected by retarded gel electrophoretic migrations. *Biochemistry* 1988;27:67–73. [PubMed: 2831963]
25. Cho-Chung YS, Nesterova MV, Severin ES, Vinogradov SV. Chemical modification enhances the inhibitory effect of regulatory subunit antisense oligodeoxynucleotide of cAMP-dependent protein kinase type I on cell proliferation. *Biochemistry Internat* 1991;25:767–773.
26. Wagstaff KM, Jans DA. Nucleocytoplasmic transport of DNA: enhancing non-viral gene transfer. *Biochem J* 2007;406:185–202. [PubMed: 17680778]
27. Harris JM, Chess RB. Effect of pegylation on pharmaceuticals. *Nat Rev Drug Discov* 2003;2:214–221. [PubMed: 12612647]
28. Rebuffat A, Bernasconi A, Ceppi M, Wehrli H, Verca SB, Ibrahim M, Frey BM, Frey FJ, Rusconi S. Selective enhancement of gene transfer by steroid-mediated gene delivery. *Nat Biotechnol* 2001;19:1155–1161. [PubMed: 11731785]
29. Hama S, Akita H, Iida S, Mizuguchi H, Harashima H. Quantitative and mechanism-based investigation of post-nuclear delivery events between adenovirus and lipoplex. *Nucleic Acids Res* 2007;35:1533–1543. [PubMed: 17287293]

30. Zelphati O, Liang X, Hobart P, Felgner PL. Gene chemistry: functionally and conformationally intact fluorescent plasmid DNA. *Hum Gene Ther* 1999;10:15–24. [PubMed: 10022527]

Figure 1.

(A) Analytical reverse phase HPLC (UV detector: 220 nm) of isolated NLS-peptide (**1**) and NLS-PEG-acridine conjugates (**2**: PEG₉₀₀; **3**: PEG₃₄₀₀; **4**: PEG₅₀₀₀). (B) UV spectrum of the NLS-PEG₅₀₀₀-acridine. Acridine absorbance: 420 nm (50% acetonitrile-water, ϵ 5500). (C) Aminoacids analysis of the NLS-PEG₅₀₀₀-acridine conjugate. An expected aminoacids ratio in the NLS peptide is shown in the third row. (D) MALDI-TOF spectrum of the NLS-PEG₅₀₀₀-acridine. The MW range of 5700–5900 belongs to PEG₅₀₀₀-*bis* and *tris*(acridine) conjugates and the MW range of 6800–7000 corresponds to NLS-PEG₅₀₀₀-*bis* and *tris*(acridine) conjugates; superposition of two near equal fractions of both conjugates results in the observed maximum of 6240.

Figure 2.

Agarose electrophoresis mobility assay following the pDNA binding with NLS-PEG₅₀₀₀-acridine. 0.5 µg of pDNA was incubated with the NLS-conjugate for 30 min at 25 °C at different weight ratios and analyzed by 0.8% (w/v) agarose gel electrophoresis. Lanes 1–5, pDNA with NLS-PEG₅₀₀₀-acridine at weight ratios of 1:6, 1:4, 1:2, 1:1 and 2:1; lane 6, pDNA with NLS peptide alone; lane 7, pDNA with PEG₅₀₀₀ alone; lane 8, pDNA with acridine alone; lane 9, pDNA with the mixture of NLS peptide, PEG₅₀₀₀ and acridine; lane 10, pDNA alone as control.

Figure 3.

Sequence-specific enhancement of luciferase expression levels in bEnd.3 cells transfected by NLS-lipo- and polyplexes. bEnd.3 cells were transfected with different complexes at pDNA/conjugate ratio 6:1 containing 0.5 μ g pDNA/well for 4 h at 37 °C in the presence of 10% FBS. The luciferase expression level and protein content were determined 40 h post-transfection. Results are presented as mean $\pm$ SEM (n=5). **, $P < 0.01$; n.s., no significant difference.

Figure 4.

Optimization of the effect of NLS-conjugates on the luciferase expression in bEnd.3 cells transfected by lipo- and polyplexes. NLS-lipo- and polyplexes were prepared at optimized (*a* and *b*) and 1/3 lower (*c* and *d*) ratios of transfection reagents to pDNA. bEnd.3 cells were incubated with complexes containing 0.5 μ g pDNA/well for 4 h at 37°C in the presence of 10% FBS. The luciferase expression level and protein content were determined 40 h post-transfection. Results are presented as mean $\pm$ SEM ($n=5$). **, $P < 0.01$; ***, $P < 0.005$; ****, $P < 0.0001$.

Figure 5.

Protective effect of PEG molecules on the luciferase pDNA activity. Naked pDNA or pDNA complexed with PEG₅₀₀₀-acridine conjugate at the ratio 6:1 was treated by DNase I at 37°C for different time periods. ExGen500 was then added to both mixtures and they were used to transfect bEnd.3 cells for 4h at 37°C in the presence of 10% FBS. Results are presented as mean $\pm$ SEM (n=5). (**, $P < 0.01$)

Figure 6.

Enhancement of the GFP expression level and transfection efficiency in the presence of NLS-PEG₅₀₀₀-acridine conjugate. bEnd.3 cells were treated in 6-well plates with NLS-lipo- and polyplexes containing 3 μ g of green fluorescent protein pDNA for 4 h at 37°C. 40h later cells were trypsinized and analyzed by flow cytometry. Results are presented as mean $\pm$ SEM (n=3). *, $P < 0.05$; **, $P < 0.01$.

Figure 7.

Enhanced intracellular and nuclear accumulation of rhodamine-labeled pDNA following the transfection of bEnd.3 cells with NLS-lipo- and polyplexes. Confocal microscopy images of bEnd.3 cells 24 h post-transfection by pDNA complexed with NLS-PEG₅₀₀₀-acridine conjugate (6:1) at optimal transfection agent ratios in the presence of 10% FBS. After fixation, the cells were imaged using laser scanning confocal microscope. Panes (A, D) show lipo- and polyplexes without NLS-conjugates, respectively. NLS-poly- and lipopolyplexes are shown in panes (B, E) in regular mode, respectively, and in panes (C, F) in lateral mode, respectively. (G), Accumulation of rhodamine-labeled pDNA in nuclei 24 h following transfection of bEnd.3 cells by plain or NLS-polyplexes. The fluorescence of isolated nuclear DNA following transfection with plain polyplex was taken for 100%. ***, $P < 0.005$.

Scheme 1. Synthesis of NLS-PEG-acridine conjugates

MAL-PEG-NHS: bifunctional maleimide-PEG-N-hydroxysuccinimide derivatives.

Table 1
Physicochemical properties of NLS-lipo- and polyplexes

DNA/conju-gate (w/w)		Diameter (nm)	ζ-potential (mV)
Lipoplexes		180.6 ±1.3	37.8 ±1.1
+NLS-PEG ₅₀₀₀ -acridine-	1:1	204.7 ±1.3	32.4 ±0.9
	4:1	176.2 ±0.9	35.9 ±0.8
	16:1	177.9 ±0.7	37.1 ±1.2
+NLS-PEG ₃₄₀₀ -acridine-	1:1	189.2 ±1.2	31.8 ±0.9
	4:1	168.1 ±1.1	35.2 ±1.4
	16:1	177.8 ±0.9	36.6 ±1.2
+NLS-PEG ₉₀₀ -acridine-	1:1	178.2 ±0.8	33.1 ±1.2
	4:1	176.4 ±1.3	33.5 ±1.3
	16:1	177.4 ±1.2	34.8 ±0.9
Polyplexes		68.4 ±1.1	35.6 ±0.9
+NLS-PEG ₅₀₀₀ -Acridine-	1:1	71.9 ±1.1	30.1 ±0.8
	4:1	70.6 ±0.8	33.4 ±1.2
	16:1	70.4 ±0.8	35.1 ±1.1
+NLS-PEG ₃₄₀₀ -Acridine-	1:1	75.1 ±1.1	29.8 ±1.2
	4:1	71.4 ±0.8	35.7 ±1.1
	16:1	71.5 ±1.2	35.2 ±0.9
+NLS-PEG ₉₀₀ -Acridine-	1:1	76.2 ±1.2	29.5 ±1.2
	4:1	71.2 ±1.1	33.9 ±1.2
	16:1	72.8 ±0.9	34.6 ±1.1