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Research paper

Nuclear targeting of non-viral gene carriers using psoralen-nuclear localization signal (NLS) conjugates

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Abstract

A nuclear localization signal was non-covalently attached to DNA for the purpose of enhancing transfection efficiencies of non-viral gene carriers. Psoralen, a nucleic acid-intercalating agent, was chemically attached to a signal peptide. The conjugate spontaneously intercalated into DNA and then poly(ethyleneimine) [PEI] was added to prepare a DNA/PEI complex containing the signal peptide moieties. The existence of the conjugate did not alter the complexation process between DNA and PEI, which was confirmed by dynamic light scattering. The conjugate was slowly released from the DNA/PEI complex for 24 h, while a burst release was examined when the conjugated was added to DNA without PEI. The complex containing a signal peptide moiety increased transfection efficiencies on COS-1 cells, compared to a mutant signal peptide or a control. Cytotoxicity of the conjugate slowly increased as the amount of the conjugate increased, however, the cytotoxic effect of the conjugate was not significant at the effective concentration of the conjugate for transfections. Therefore, the psoralen-nuclear localization signal is expected to be a potent transfection enhancing agent without a covalent modification of transgenes.

Keywords: Nuclear localization signal; Transfection; PEI; Psoralen

1. Introduction

Since non-viral gene carriers were introduced, lower transfection efficiencies of non-viral gene carriers have been a major obstacle to be circumvented despite their superior safety over viral carriers [1,2]. Many studies therefore have been focused on increasing transfection efficiencies by mimicking viral intruding mechanism to cells [3]. In target cells, carrier/DNA should pass through two rate-determining steps to access to a nuclear region, where transgene expression should occur [4]. First, carrier/DNA complex or dissociated DNA should escape from endosomes to be released into cytoplasmic spaces. Second, they should enter the nucleus where their gene expressions occur. Nuclear pores are about 10 nm in an effective diameter, therefore, a nuclear entry of foreign DNA molecules is not spontaneously obtained [4,5].

A nuclear localization signal was originally employed by a virus to efficiently integrate their nucleic acids to host DNA [6,7]. When non-viral gene carriers are delivered to cells, DNA/carrier complexes endocytosed by cells undergo an endosomal pathway and then an endosomal disruption makes foreign DNA be released out to a cytosolic space. However, the cytosolic DNA cannot easily access to nuclear regions surrounded by nuclear membranes, where its expression should occur. Generally, DNA can gain an access to the nucleus only when cells undergo a cell division process, which is regarded as one of the rate-limiting steps in non-viral gene delivery [5]. Therefore, many NLSs have been conjugated to

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DNA or gene carriers in an attempt to facilitate nuclear localization process of foreign DNA, subsequently increasing transgene expressions in target tissues [7,8]. In those studies, NLS peptide was frequently conjugated to the adenine bases in double-stranded DNA, making 60–100 NLS associate with one kilobase pair of DNA, which increased uptake of DNA only in digitonin-permeablized cells. Zanta et al. synthesized a loop-forming oligodeoxyribonucleic acid (ODN)–NLS peptide conjugate and it was ligated with a lineralized double-stranded DNA [9]. ODN–NLS conjugate complexed with PEI showed increased transfection efficiencies in vitro, however, the nuclear entry effects of the NLS peptide attached to DNA are still under controversy [10,11]. Furthermore, although many researchers chemically conjugated NLS to DNA or carriers to guide their carriers or DNA to nuclear membranes, transfection efficiencies were not so significant or diminished in many cases because chemical modification of DNA reduced the transcription efficiency stability in the nucleus [10,12].

In this study, a nuclear localization signal was chemically conjugated to psoralen, a DNA intercalating reagent, in order to non-covalently modify DNA molecules to non-covalently attach NLS moieties along the DNA molecules. Fully functional protein can be successfully expressed from unmodified DNA without producing non-functional mutant protein because DNA molecules were not chemically modified by NLS peptide. Therefore, we investigated that the intercalated NLS sequences can successfully guide DNA to the nucleus, resulting in enhanced transfection efficiencies compared to a mutant nuclear localization signal sequence.

2. Materials and methods

2.1. Materials

Succinimidyl-[4-(psoralen-8-yloxy)]-butyrate (SPB) was purchased from Pierce (Rockford, IL). Dulbecco's modified Eagle's medium (DMEM) with low glucose and fetal bovine serum (FBS) were purchased from Invitrogen Life Technologies (Carlsbad, CA). A Simian kidney cell transformed with SV40 (COS-1) was obtained from the Korea Cell Line Bank (Seoul, South Korea). Dimethylsulfoxide (DMSO), PEI (MW 25 kDa), and 3-(4,5-dimethylthiazol-3-yl)-2,5-diphenyltetrazolum bromide (MTT) were from Sigma. All other chemicals and reagents were of analytical grade.

2.2. Psoralen-NLS conjugate

The psoralen-NLS or psoralen-mutant NLS (mNLS) conjugates were synthesized by Peptron, Inc. (Daejeon, South Korea) (Fig. 1). Amine-reactive psoralen (SPB) was used during the peptide synthesis of NLS or mNLS to obtain psoralen-terminated NLS or mNLS conjugates. *N*-Hydroxysuccimide group of SPB was released by conjugating the α-amine group of the first amino acid to SPB and then the other amino acids were subsequently conjugated according to an peptide synthesis protocol by the company. Therefore, psoralen-NLS and psoralen-mNLS sequences are psoralen-GGGPK**K**KRKV and psoralen-GGGPK**T**KRKV, respectively. The purification was performed by a reversed-phase chromatography and high pure grades of conjugates were selected with 95% or above of

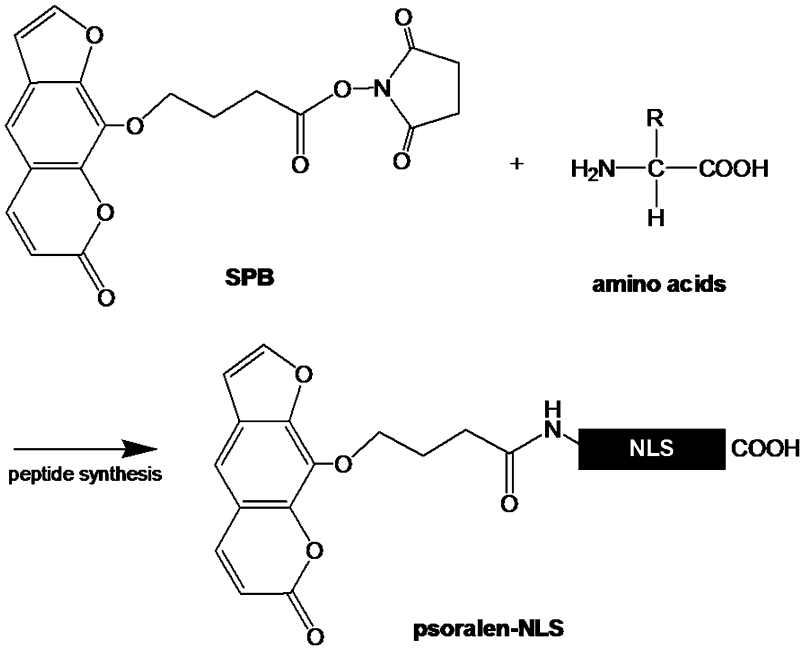

Fig. 1. Schematic conjugation of SPB to NLS peptides.

HPLC purity according to the manufacturer's own quality control.

2.3. Preparation of DNA/PEI complex with psoralen-NLS conjugates

Polyelectrolyte DNA/PEI complexes containing psoralen-NLS conjugates were prepared (N/P ratio: 0.1–8) as previously described with a minor modification [13]. Briefly, psoralen-NLS conjugates of were slowly added to DNA in 20 mM NaCl solution (50 μg/ml) and then stirred for 3 h. PEI solution (5 mg/ml) was subsequently added to the mixture and stirred for another 1 h. The size distribution and zeta-potential of the complex were measured by dynamic light scattering (Zetasizer 3000, Malvern Instruments, UK).

2.4. Release experiment of psoralen-NLS from DNA/PEI complex

In vitro release of psoralen-NLS conjugates from DNA/PEI complex was investigated by a dialysis method. DNA/NLS/PEI complex solution (1 ml) containing 100 μg of DNA was dialyzed against 10 ml of PBS (pH 7.4) (Spectra-Por 6, MW cutoff = 10,000). Released amount of psoralen-NLS was quantitated by a spectrofluorimetric method. The fluorescence intensity of released psoralen-NLS was measured at an excitation wavelength of 365 nm and an emission wavelength of 450 nm. The known amount of psoralen-NLS was used as a standard. All experiments were performed in a triplicate manner.

2.5. In vitro transfection

Transgene expression levels of DNA/NLS/PEI were measured by a luminometric assay as described previously [13,14]. Briefly, COS-1 cells at a logarithmic phase were seeded on a 6-well plate cell culture dish and cultivated 24 h in DMEM supplemented with 10% FBS. The transfection was performed in DMEM for 3 h, then fresh DMEM with 10% FBS was fed. After 48 h, the cells were harvested and the luciferase activities were measured by a Luciferase Assay System (Promega, WI) according to the manufacturer's protocol. Five micrograms of DNA was used per well in all transfection studies.

2.6. Cytotoxicity assay

Cytotoxicity of DNA/PEI complex containing NLS or mNLS was evaluated by a MTT-based cytotoxicity assay as described previously with a minor modification [15]. COS-1 cells at a logarithmic phase were plated on a 96-well culture plate (1×10^3 cell/well). After 24 h, DNA/PEI complex containing NLS or mNLS was added to each well and further incubated for 48 h. At the end of incubation time, 20 μl of MTT dye (5 mg/ml) was added to each well. After 4 h of further incubation at 37 °C, the percentage of cell viability was determined at 570 nm relative to non-treated cells.

2.7. Confocal microscopy

For a confocal microscopic observation, a plasmid DNA and PEI was separately labeled with psoralen and fluorescein isothiocyanate (FITC), repectively. Briefly, PEI and FITC mixture at a molar ratio of 100 (FITC/DNA) dissolved in PBS (pH 6.5) was incubated for 3 h and then washed with distilled water three times [14]. DNA was mixed with psoralen at the molar ratio of 10 (psoralen-NLS/DNA) and then a shortwave UV was irradiated for 5 min to crosslink between the bases of DNA and psoralen. Unreacted psoralen was completely removed by an extensive dialysis. FITC-conjugated PEI and psoralen-conjugated DNA were used for DNA/PEI and NLS/DNA/PEI complex. COS-1 cell at a logarithm phase was plated on a slide glass and incubated for 24 h. DNA/PEI complex or NLS/DNA/PEI complex was added to COS-1 cells and incubated at 37 °C. After 3 h, COS-1 cells were washed three times with PBS and examined under a confocal microscope (CarlZeiss 510, Germany).

2.8. Statistical analysis

All data were processed and analyzed by Sigma Plot 8.0 software (SPSS, IL). The statistical significances were evaluated by *t*-test of the software and $p < 0.05$ was considered significant.

3. Results and discussion

NLS sequence was chosen according to the literature and mNLS sequence was made to replace a critical amino acid, Lys, with Thr as shown in Table 1 [6,7]. Psoralen, a DNA-intercalating agent, is conventionally used to stain nucleic acids. In this study, SPB, *N*-hydroxy succinimide form of psoralen was directly conjugated to N-terminal of NLS sequences during a peptide synthesis. After the manufacturer's purification, the purity of the conjugate was confirmed by a reversed-phase chromatography (data not shown).

The complexation between DNA and PEI was monitored by dynamic light scattering as shown in Fig. 2. The size of those complexes decreased as the amount of PEI increased, ranging from 150 to 200 nm. It should be noted that the size of DNA/PEI complex did not change at the same N/P ratio although the amount of psoralen-NLS

Table 1
Psoralen-conjugated NLS sequence and mutant NLS (mNLS) sequence

Conjugate	Peptide sequence
Psoralen-NLS	Psoralen-GGGPKKKRKV
Psoralen-mNLS	Psoralen-GGGPK**T**KRKV

Psoralen is conjugated at the N-terminal of peptides.

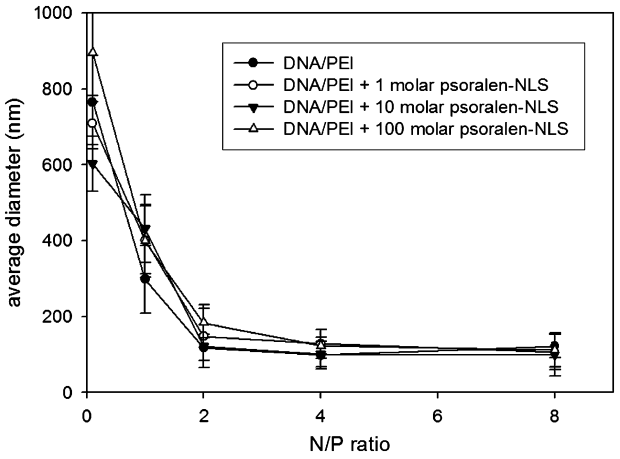

Fig. 2. Average diameters of DNA/PEI complex with or without psoralen-NLS. Molar ratios between psoralen-NLS and DNA were 1, 10, and 100 (psoralen-NLS/DNA).

increased, confirming that the association of psoralen-NLS with DNA did not inhibit a complexation process between PEI and DNA. Many studies employing cationic polymers as gene carriers confirmed that size of gene carriers was significant in terms of endocytotic uptakes by cells. Therefore, this result confirmed that DNA/PEI complex with psoralen-NLS conjugates could be endocytosed by cells to the same degree as DNA/PEI complex without psoralen-NLS.

Since psoralen-NLS sequences are physically associated with DNA molecules, it should be confirmed how fast they are released out from DNA/PEI complexes before examining the effects of NLS for nuclear targeting. Fig. 3 shows a release profile of psoralen-NLS from DNA/PEI complex or DNA only for 24 h. As expected, psoralen-NLS physically associated to naked DNA without PEI showed an initial burst in a short period of time. However, psoralen-NLS was slowly released out from the DNA/PEI complex, which confirmed that released amount of psoralen-NLS was below 40% compared to initially loaded psoralen-NLS in the complex after 24 h incubation. In case of naked DNA without PEI, intercalated psoralen-NLS was easily released out by a simple diffusion because no other forces can keep psoralen-NLS inside DNA. On the other hand, psoralen-NLS molecules cannot be easily diffused out into the outer medium because DNA is tightly entangled with PEI, making them physically unable to escape out from the complex. Therefore, this type of physical encapsulation of NLS can be a great advantage to render nuclear localizing signals to a gene carrier without a covalent modification of genes or their carriers to be delivered. However, there was no difference when the molar ratio of NLS conjugates to DNA molecules was varied. Therefore, it could be concluded that NLS conjugates tightly intercalated between nucleotides of DNA irrespective of the amount of NLS conjugates added to DNA/PEI complex.

Transfection efficiency of DNA/PEI complex with psoralen-NLS was investigated as shown in Fig. 4. When we compared the transfection efficiency of the complexes with psoralen-NLS or those without psoralen-NLS, a significant difference was observed among those groups with a statistical significance ($p < 0.05$). Furthermore, gene carriers with mutant NLS showed the same transfection efficiency to DNA/PEI, confirming that mutant NLS did not enhance transfection efficiency at all. Therefore, psoralen-NLS in those complexes played a significant role in increasing transfection efficiencies of DNA/PEI complex, presumably by localizing DNA or DNA carrier to be delivered to nucleus. This is however of interest because at a molar ratio of 5 (psoralen-NLS/DNA) or above, the transfection efficiencies did not increase significantly. Therefore, it could be inferred from the result that one to five NLS per a DNA molecule is enough to deliver the DNA carrier to the nucleus. Previously, Ciolina et al. showed that microinjected DNA with covalently attached NLS increased transfection efficiency by 60% of β-galactosidase activities compared to unmodified DNA [6]. However,

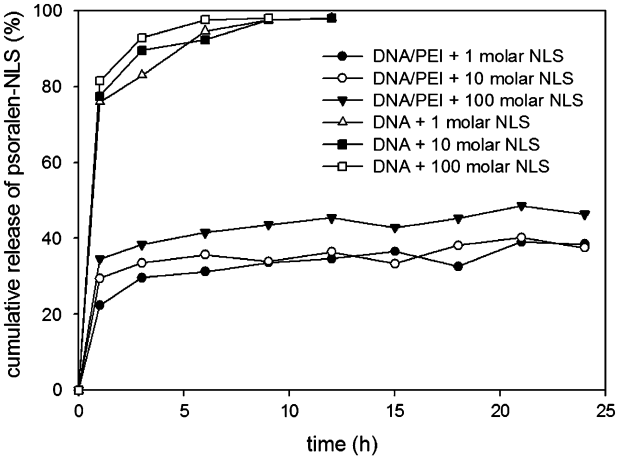

Fig. 3. In vitro release of psoralen-NLS from DNA or DNA/PEI complex. The amount of psoralen-NLS ranged from 1 to 100 at the molar ratios of psoralen-NLS to DNA.

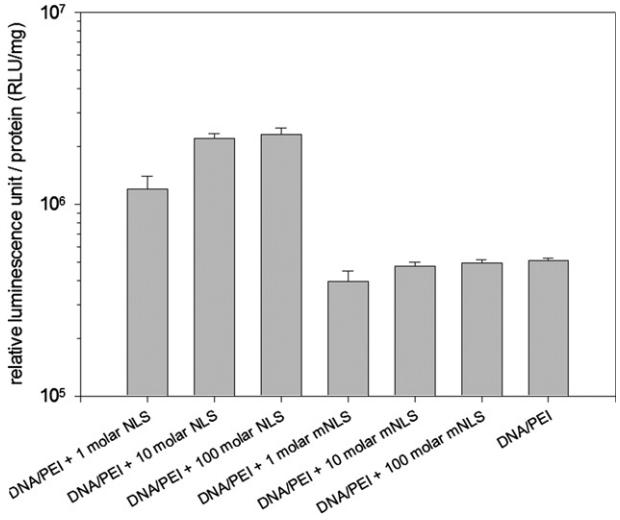

Fig. 4. In vitro transfection efficiency of DNA/PEI complex with or without psoralen-NLS and psoralen-mNLS in COS-1 cells.

the expression level decreased by 60% with plasmids bearing 43 NLS peptides, suggesting covalent coupling of NLS to the DNA could cause transcriptional inactivation. On the other hand, Leahy et al. showed that a biotinylated plasmid prepared by photo-crosslinking lost 60% biological activities when a plasmid contained 40 biotins [16]. In the present study, however, the transfection efficiency did not decrease when a single DNA molecule was associated with 1000 molecules of psoralen-NLS. Therefore, non-covalent modifications of DNA with NLS can be a potent alternative because transfection efficiency can be enhanced without modifying target molecules by covalent modifications [17–21].

Cytotoxicity of DNA/PEI complex was measured as increasing the amount of psoralen-NLS as shown in Fig. 5. After 48 h incubation, the viability of COS-1 cell slowly decreased as the number of incorporated psoralen-NLS molecules slowly increased. This can be attributed that intercalated psoralen molecules prohibited essential genes from being transcribed. However, these cytotoxic effects can be neglected because the number of affected base pairs is so small considering the plasmid is 8.5 kbp. Furthermore, it should be noted that transfection efficiency increased at a low molar ratio of psoralen-NLS to DNA, where relatively non-cytotoxicity of psoralen-NLS was confirmed.

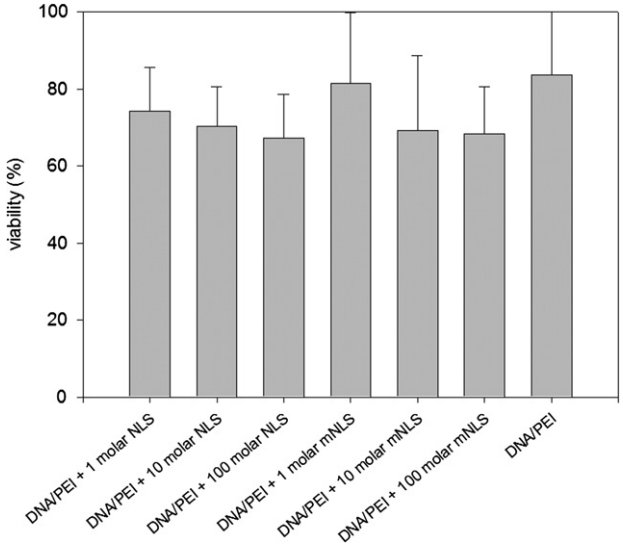

Fig. 5. Cytotoxicity of DNA/PEI complex with or without psoralen-NLS conjugates in COS-1 cells.

Fig. 6 shows confocal microscopy of DNA/PEI complex containing increasing amount of psoralen-NLS conjugates. As the amount of NLS increased, the nuclear localization of foreign DNA was increased as well, indicating NLS indeed increased nuclear localization of foreign DNA without chemical modifications. This result agrees with the previous results showing that cytosolic microinjected DNA showed increased nuclear localization when NLS signal was employed. However, the nuclear localization of foreign DNA within the nuclear region did not lead to increased expression of foreign DNA in the previous study because transgene expression was prevented by a chemical modification of DNA with NLS [5,18,21].

4. Conclusion

Psoralen-NLS conjugates physically associated to DNA molecules showed relatively strong association to DNA in the presence of PEI. A physical association of psoralen-NLS to DNA significantly increased transgene expression compared to mutant NLS conjugates. Therefore, this novel conjugate can be a potent additive for in vitro gene transfection without needs for chemically modifying DNA.

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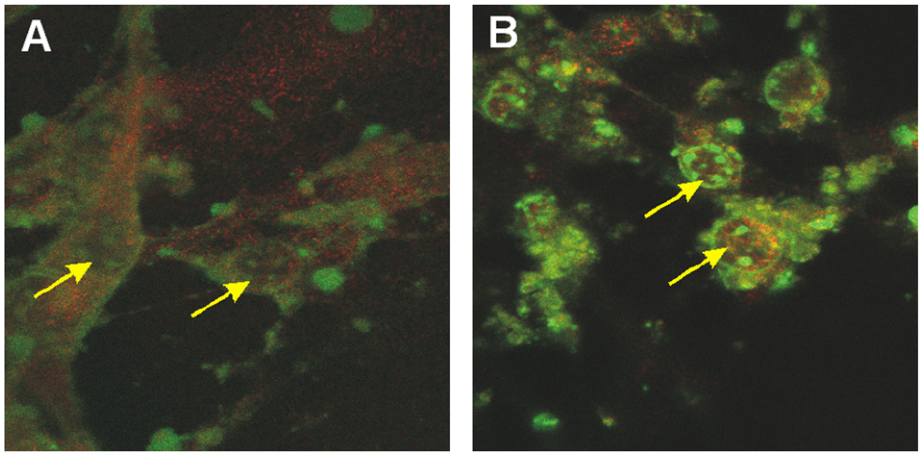

Fig. 6. Confocal microscopy of COS-1 cells transfected with DNA/PEI complex containing psoralen-NLS conjugates. FITC-conjugated PEI is shown in green and psoralen-conjugated of plasmid DNA is shown in red. Yellow arrows indicate distributions of PEI and plasmid DNA in the cytosol and the nucleus. (A) DNA/PEI complex (B) DNA/PEI complex containing psoralen-NLS conjugates. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

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