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Long-term efficient gene delivery using polyethylenimine with modified Tat peptide

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ABSTRACT

Polyethylenimine (PEI), a cationic polymer, has been widely studied and shown great promise as an efficient gene delivery vehicle. Likewise, the HIV-1 Tat peptide, a cell-permeable peptide, has been successfully used for intracellular gene delivery. To improve the favorable properties of these two vectors, we combine PEI with the modified Tat peptide sequence bearing histidine and cysteine residues (mTat). *In vitro* mTat/PEI-mediated transfection was evaluated by luciferase expression plasmid in two cell types. mTat/PEI produced significant improvement ($\approx$5-fold) in transfection efficiency of both cell lines with little cytotoxicity when compared to mTat alone, PEI alone, or four commercial reagents. The particle size of mTat/PEI/DNA complex was significantly smaller than mTat or PEI alone, and it was correlated with higher transfection efficiency. Filipin III, an inhibitor of caveolae-mediated endocytosis, significantly inhibited mTat/PEI transfection. In contrast, chlorpromazine, an inhibitor of clathrin-mediated endocytosis, did not. This suggested caveolae-mediated endocytosis as the transfection mechanism. Furthermore, the results of *in vivo* studies showed that animals administered mTat/PEI/DNA intramuscularly had significantly higher and longer luciferase expression ($\approx$7 months) than those with mTat/DNA, PEI/DNA, or DNA alone, without any associated toxicity. The combination of mTat with PEI could significantly improve transfection efficiency, expanding the potential use as a non-viral gene vector both *in vitro* and *in vivo*.

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1. Introduction

Non-viral gene delivery systems are widely used in basic research and in pre-clinical gene therapy applications. Despite extensive testing of alternatives such as lipoplexes and polyplexes, transfection efficiency is generally low, except for cells transformed *in vitro*. The cationic polymer, polyethylenimine (PEI), is a useful delivery vehicle for oligonucleotides and ensures effective oligonucleotides delivery with low toxicity in spite of relatively short duration of gene expression [1]. Because PEI forms stable complexes with oligonucleotides, the positively charged particles are able to interact with anionic proteoglycans at the cell surface and enter cells by endocytosis [2]. PEI possesses the unique property of acting as a "proton sponge" that buffers the endosomal pH [3] and protects oligonucleotides from degradation [4]. The continuous proton influx also induces endosome osmotic swelling and rupture, which provides an escape mechanism for oligonucleotides to the cytoplasm [5]. A previous study by our group showed that, compared to other non-viral vectors, a cationic polymer-based vector is the most efficient across a wide range of cell lines [6].

The cell membrane has been recognized as a major barrier to efficient transfection [7]. In order to overcome this barrier, peptides that are capable of penetrating the plasma membrane have been employed. Cell-permeable peptides (CPPs) or protein transduction domains are derived from naturally occurring proteins such as the Tat protein of human immunodeficiency virus type 1 (HIV-1) [8,9], the structural protein VP22 of herpes simplex virus 1 [10], and the DNA binding domain (homeodomain) of Drosophila transcription

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factor Antennapedia [11]. CPPs are commonly amphipathic, rich in arginine and positively charged. The most frequently used CPP is the HIV-1 Tat peptide. A number of Tat peptides have been successfully used to deliver drugs, protein and DNA into cells [12–14]. Lo and Wang [15] demonstrated significant improvement in gene transfection efficiency using a modified Tat peptide covalently fused with ten histidine and two cysteine residues when compared to unmodified Tat. Gene transfection was improved because interpeptide disulfide bonds formed by air oxidation upon binding to DNA, led to enhanced stability of peptide/DNA complexes. Several interesting reports have shown the ability of CPPs to enhance polymer-mediated transfection in cell cultures and lung *in vivo* [16–19]. Recently, we demonstrated that modified Tat incorporated with histidine and cysteine residues (mTat) combined with a cationic lipid transfection reagent results in very efficient gene transfer across a range of cell lines [20]. Mechanistic aspects of the intracellular delivery of mTat with the cationic lipid revealed that internalization is mediated predominantly via a temperature-dependent and caveolae-mediated endocytic pathway [20].

To improve non-viral gene delivery *in vitro* as well as *in vivo*, we hypothesized that an mTat combined with PEI would enhance transfection efficiency. In this study, we conducted transfections in two different cell types, supplemented with different serum concentrations, using stable preparations of mTat mixed with PEI and plasmid DNA encoding for luciferase. The mTat/PEI preparations were also compared to four commercial transfection reagents. In addition, we investigated the mechanism of intracellular delivery of mTat/PEI/DNA. Specifically, we explored: (1) the size and surface charge of the mTat, PEI and DNA complexes and (2) whether the mode of delivery was via clathrin- or caveolae-mediated endocytosis. We studied the ability of mTat/PEI to facilitate efficient gene delivery *in vivo* via intramuscular administration and evaluated the animals for any associated toxicity or induction of an immune response.

2. Materials and methods

2.1. Cell culture

Two cell lines, MC3T3-E1 mouse preosteoblasts and C2C12 mouse myoblasts were used. MC3T3-E1 cells (gift from Dr. Mani Alikhani, New York University College of Dentistry) were cultured in Gibco alpha minimal essential medium (αMEM; Invitrogen, Carlsbad, CA) with 10% Gibco fetal bovine serum (FBS; Invitrogen). C2C12 cells (gift from Dr. Xi Huang, New York University School of Medicine) were cultured in Gibco Dulbecco's modified Eagle medium (DMEM; Mediatech, Manassas, MA) with 10% FBS. All cells were cultured with 5000 U/mL penicillin (Invitrogen) and 5000 μg/mL streptomycin (Invitrogen) at 37 °C in a humidified atmosphere with 5% CO_2. The same media were used for transfection studies.

2.2. Plasmid DNA

Plasmid DNAs, encoding for luciferase (gWIZ luciferase) under the control of the cytomegalovirus promoter/enhancer was obtained from Genlantics (San Diego, CA). Vectors were propagated in competent *Escherichia coli* DH5α cells (Invitrogen). Ultrapure endotoxin-free plasmid DNA was prepared using the QIAfilter kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Plasmid DNA was diluted in sterile water to a final concentration of 1 μg/μL DNA.

2.3. Transfection vectors for in vitro

For Tat transfection studies, the peptides were prepared using Lo and Wang's protocol [9]. The HIV-1 Tat (RKKRRQRRRR) covalently fused with ten histidine and two cysteine residues synthesized (C-5H-Tat-5H-C) was obtained from Biomatik Corporation (Cambridge, Canada). To prepare mTat/DNA complexes, the peptide solution (1 mM) and the plasmid DNA were mixed in 5% glucose solution at pH 7 (final volume 10 μL/well), and followed by quick vortex for about 5 s. After the mixture was incubated at room temperature for 30 min, the cap of the sample tube was opened to expose the solution to air. The sample was shaken vigorously for 90 min. The cap was opened intermittently to allow air replenishment. For control PEI transfection studies, jetPEI (Polyplus-transfection, New York, NY), a linear polyethylenimine, was used. Also, Arrest-In (Thermo Scientific, Huntsville, AL), ExpressFect (Denville Scientific, Metuchen, NJ), Lipofectamine 2000 (Invitrogen, Carlsbad, CA), and SuperFect (Qiagen, Valencia, CA) were used. Plasmid DNA (0.2 μg/well) encoding a luciferase gene was mixed with serum-free medium (for a final volume 10 μL/well), added to PEI, and then incubated at room temperature for 15 min. To each well, 10 μL complex (containing 0.2 μg DNA and PEI) was added. Lastly, to prepare the mTat/PEI/DNA complex, PEI was mixed with the mTat/DNA complex (described above) and incubated for 15 min at room temperature. A total of three vector/DNA groups were used: Group 1, mTat/DNA; Group 2, PEI/DNA; and Group 3, mTat/PEI/DNA.

2.4. DNA transfection

Cells were plated in a 96-well cluster dish at a density of 2×10^5 cells/mL, cultivated in the appropriate growth medium with 10% FBS. To each well, 100 μL of media with 10% FBS was added. After 24 h in culture, the cells were washed with phosphate-buffered saline (PBS), and 100 μL fresh growth medium with or without 10% FBS was added to the cells. Usually, highly toxic reagents require cells to be washed, but low toxic reagents do not. We followed several published and company protocols, which did not recommend a wash for the cells prior to the addition of the media. The method we used is also a widely accepted protocol as a serum-free condition [15,21]. The reagent/DNA complexes were then added to each well and incubated with the cells for 4 h at 37 °C for the without-FBS condition group. Then, 100 μL of appropriate media with 20% FBS was added to each well which did not contain FBS. The cells were cultured for 48 h at 37 °C in 5% (v/v) CO_2 after transfection. All transfection assays were carried out in quadruplicate simultaneously. Also, plasmid-only was tested as a control using the cells listed and the results showed the same levels of transfection as untreated cell controls (data not shown).

Also, optimal mixture sequences of mTat, PEI, and DNA with different ratio were investigated. In the 5mTat/DNA+2PEIcondition 2, PEI was added subsequent to 5 mTat/1 DNA (w/w) complexes. In the10mTat/DNA+2PEIcondition 2, PEI was added subsequent to 10 mTat/1 DNA (w/w) complexes. In the 20mTat/DNA+2PEIcondition 2, PEI was added subsequent to 20 mTat/1 DNA (w/w) complexes. In the 2PEI/DNA+10mTatcondition 10, mTat was added subsequent to 2 PEI/1 DNA (w/w) complexes. In the 10mTat/2PEI + DNAcondition 1, DNA was added subsequent to 10 mTat/2 PEI (w/w) mixture. The control group represented expression in the cells without DNA and vectors.

2.5. Detection of transgene expression

Transgene expression was detected at a standardized representative time point of 48 h after transfection. Luciferase expression level was measured with Bright-Glo™ Luciferase Assay System (Promega, Madison, WI) using a multi-detection microplate reader, Synergy™ HT (BioTek Instruments Inc, Winooski, VT). Relative light units (RLU) were recorded in duplicates with 10-s integration as their expression levels. This detection system is designed to measure directly the expression level using 96-well plates. The use of RLU/well as the unit of measure allowed for accurate detection of expression levels and is considered a well accepted method in this field [6,20,22].

2.6. Cytotoxicity evaluation

Cytotoxicity of the transfection reagents was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide, MTT assay. MC3T3-E1 cells (1×10^6/mL) in 100 μL of αMEM (Invitrogen) supplemented with 10% FBS were seeded in 96-well plates and incubated overnight. The 5 mg/mL MTT reagent in 1× PBS (10 μL/well) was added into the plates and incubated for 4 h. After incubation, the medium was aspirated and dimethyl sulfoxide (100 μL/well) was added to stop the reaction. The optical density was quantified in a multi-detection microplate reader, Synergy™ HT at 570 nm wavelength. The percentage of cell viability was calculated by comparing the appropriate optical density to the control cells, which did not contain the transfection reagents.

2.7. Zeta potential

Zeta potentials of DNA and the vector complex with and without 10% FBS were measured at 25 °C by a Zetasizer Nano ZS90 (Malvern Instruments Ltd, UK). This instrument is equipped with a red laser of wavelength 630 nm and measures the electrophoretic mobility of the particles using phase analysis of scattered light in an experimental set up similar to Laser Doppler Velocimetry (M3PALS technique, Malvern Instruments Ltd). Zeta potential was derived from the electrophoretic mobility using the Smoluchowski model since the measurements were performed in aqueous solutions of moderate ionic strength (i.e. electrical double layer thickness << the particle size). Samples that were prepared in the absence of PEI required higher concentrations of DNA and mTat, (12.1 ng/μL and 6.16 ng/μL, respectively) necessary to yield light scattering properties. DNA sample by itself required a concentration of 81 ng/μL for adequate measurement of zeta potential. Each sample was observed with 20 repeated measurements across 3 trials.

2.8. Dynamic light scattering

The size distribution of DNA and the vector complex was measured by dynamic light scattering, using a Zetasizer Nano ZS90 equipped with a red laser of wavelength

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