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Neuron-specific delivery of nucleic acids mediated by Tet1-modified poly(ethylenimine)

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Abstract

Background—The development of minimally invasive, non-viral gene delivery vehicles for the central nervous system (CNS) is an important technology goal in the advancement of molecular therapies for neurological diseases. One approach is to deliver materials peripherally that are recognized and retrogradely transported by motor neurons toward the CNS. Tet1 is a peptide identified by Boulis and coworkers to possess the binding characteristics of tetanus toxin, which interacts specifically with motor neurons and undergoes fast, retrograde delivery to cell soma. In this work, Tet1-poly(ethylenimine) (Tet1-PEI) was synthesized and evaluated as a neuron-targeted delivery vehicle.

Methods—Tet1-PEI and NT-PEI (neurotensin-PEI) were synthesized and complexed with plasmid DNA to form polyplexes. Polyplexes were assessed for binding and uptake in differentiated neuron-like PC-12 cells by flow cytometry and confocal microscopy. In order to determine gene delivery efficiency, polyplexes were exposed to PC-12 cells at various stages of differentiation. Targeted binding of polyplexes with primary neurons was studied using dorsal root ganglion cells.

Results—Tet1-PEI and NT-PEI polyplexes bound specifically to differentiated PC-12 cells. The specificity of the interaction was confirmed by delivery to non-neuronal cells and by competition studies with free ligands. Tet1-PEI polyplexes preferentially transfected PC-12 cells undergoing NGF-induced differentiation. Finally, neuron-specific binding of Tet1-PEI polyplexes was confirmed in primary neurons.

Conclusions—These studies demonstrate the potential of Tet1-PEI as a neuron-targeted material for non-invasive CNS delivery. Tet1-PEI binds specifically and is internalized by neuron-like PC-12 cells and primary dorsal root ganglion. Future work will include evaluation of siRNA delivery with these vectors.

Keywords

neurons; non-viral gene delivery; PEI; targeting; CNS delivery

Introduction

Gene delivery technology provides a potential means of treating medical conditions that have been difficult to address by conventional therapies. Impairments of the central nervous system (CNS), including genetic disorders as well as spinal cord injury, could be significantly impacted

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by gene therapy. To this end, neurotrophin gene delivery has ameliorated symptoms of diseases such as amyotrophic lateral sclerosis, Huntington's disease and Parkinson's disease in animal models [1–3]. Gene therapy can also be utilized to regenerate damaged neurons after nerve injuries and offers advantages including minimizing invasive treatments by enhancing therapeutic efficacy and prolonging healing responses at the injury site [4]. In order for these developments to be realized in a human clinical setting, a practical and efficient nucleic acid delivery system is required.

Viral delivery vectors such as herpes simplex virus and adeno-associated virus have been predominantly used to deliver desirable therapeutic genes into target neurons with high transduction efficiency [5]. However, several limitations of viral-mediated delivery have hindered its practical application, including toxicity, restricted targeting of specific cell types, limited DNA carrying capacity, production and packaging problems, recombination, and high cost [6]. Many viral vectors also require modification of the capsid protein for neuron-specific gene transfer [7]. As a result, non-viral vehicles provide an attractive alternative for gene delivery. Non-viral gene delivery to the CNS using polymer/DNA complexes has been reported [8–10]. Although these materials are internalized indiscriminately by cells, the encouraging results demonstrate the feasibility of non-viral gene transfer to the CNS. The key drawback of current non-viral gene delivery technology is low *in vivo* delivery efficiency [11,12]. The goal of our work is to develop neuron-specific, non-viral gene delivery vehicles that can be used for *in vivo* applications with minimal invasiveness. The ultimate system will likely require a multi-component, integrated design. In this work, the synthesis and assessment of a neuron-specific platform are described.

Neuron-targeting ligands used for gene delivery vehicles have included neuropeptides, nerve growth factors, and neuron-specific toxin fragments [13–16]. A desirable attribute for non-invasive neuron delivery is a vehicle that is administered intramuscularly, recognized and internalized specifically by motor neurons, and transported back to the neuronal cell body. Tetanus toxin (TeNT), a bacterial protein, is composed of a heavy chain and light chain linked through a disulfide bond. The heavy chain (TeNT H_c) mediates neuron recognition, while the light chain (TeNT L_c) is a metalloprotease that interferes with neurotransmitter activity. Recombinant TeNT H_c has been shown to be internalized by motor neurons and to undergo rapid retrograde transport [17]. Conjugation of TeNT H_c to polycations and complexation with nucleic acid results in neuron-specific gene delivery *in vitro* [15,18]. Thus neuron targeting through the TeNT pathway is a promising approach. Recently, a peptide that competes effectively with TeNT for receptor binding was identified using phage display [19]. This peptide, 'Tet1', demonstrated high binding affinity to differentiated PC-12, dorsal root ganglion, and primary motor neurons. In this work, we synthesized Tet1-modified poly(ethylenimine) (Tet1-PEI) for evaluation as a neuron-specific gene carrier. Tet1-PEI was compared to neurotensin (NT)-modified poly(ethylenimine) (NT-PEI) as a positive control and to unmodified poly(ethylenimine). NT is a 13-amino-acid neuropeptide that has been conjugated to poly(L-lysine) for neuron delivery [20]. In this work, Tet1-PEI-, NT-PEI-, and PEI-based polyplexes were formulated and characterized. Neuron-specific delivery was assessed in differentiated PC-12 cells and primary dorsal root ganglion cells.

Materials and methods

Materials

Neurotensin (NT, sequence: Pyr-LYENKPRRPYL), branched PEI (Mw 25 kDa, bPEI), poly(L-lysine) (0.1 mg/mL in water, Mw 70–150 kDa) (PLL) and disposable PD-10 desalting columns were purchased from Sigma (St. Louis, MO, USA). Cysteine-terminated Tet1 sequence (HLNILSTLWKYRC) was synthesized and purified by high-performance liquid chromatography (HPLC) by Pepton (Daejeon, South Korea). *N*-Succinimidyl 3-(2-

pyridyldithio)propionate (SPDP) and Traut's reagent were obtained from Pierce (Rockford, IL, USA). pEGFPC1 (enhanced green fluorescent protein gene under the cytomegalovirus (CMV) promoter) and pGL3-CV (luciferase reporter gene under the SV40 promoter) plasmids were purchased from Clontech (Mountain View, CA, USA) and Promega Corp. (Madison, WI, USA), respectively, and amplified under endotoxin-free conditions by Elim Biopharmaceuticals (Hayward, CA, USA). The LabelIT® Cy3 nucleic acid labeling kit and YOYO-1 were purchased from Mirus Bio (Madison, WI, USA) and Invitrogen (Carlsbad, CA, USA), respectively. Rat pheochromocytoma PC-12 cells, NIH-3T3 embryonic fibroblast and F-12K culturing medium were purchased from ATCC (Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and antibiotics were purchased from Mediatech (Herndon, VA, USA). Horse serum was purchased from Cambrex Bio Science (Walkersville, MD, USA). CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS) was purchased from Promega Corp. for determination of cell viability.

Synthesis of NT-conjugated PEI (NTPEI) and Tet1-conjugated PEI (Tet1-PEI)

Conjugation of NT to bPEI was performed as described previously for NT-poly(L-lysine) synthesis with minor modifications [20]. Briefly, NT (4.0 μmol) was pre-activated with SPDP (8.0 μmol) in dimethylformamide (DMF) and purified by collecting eluent through a PD-10 column pre-equilibrated with PBS-EDTA (100 mM phosphate, 150 mM NaCl, 2 mM EDTA, pH 7.5). The fractions containing NT were pooled and concentrated to 2 mL. The concentration of activated NT was determined by comparing the absorbance to those of NT standards. PEI (0.2 μmol) was added to NT solution, and the conjugation of NT to PEI was initiated by adding Traut's reagent (2 μmol). The reaction was monitored by measuring the absorbance at 343 nm of pyridine-2-thione (extinction coefficient at 343 nm: $8.08 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$) released during reaction. A PD-10 column was used to purify the reactant NT-PEI by removing unreacted NT and Traut's reagent. The concentration of PEI was determined by measuring the cuprammonium complex formed between PEI and copper(II) at 630 nm using UV/Vis spectrophotometry [21,22]. This method has been used previously to determine concentrations of modified PEI polymers [23]. Contributions of amino acids such as lysine and arginine to Cu(II) complexation has been shown to be comparatively low [24]. NT conjugation efficiency was determined by comparing the NT absorbance to a standard curve and found to be ~8 NTs per PEI.

For synthesis of Tet1-PEI, SPDP (2.5 μmol) was reacted with PEI (1.25 μmol) at room temperature (RT) for 4 h in DMF. SPDP-activated PEI was purified using a PD-10 column and added to 3.0 μmol of cysteine-terminated Tet1 and stirred for 12 h at RT. Tet1-PEI was isolated using a PD-10 column and peptide conjugation efficiency determined as described previously for NT-PEI and found to be ~0.6 Tet1 per PEI. NT-PEI and Tet1-PEI solutions were kept frozen at -80°C in aliquots until use.

Nanoparticle formation and characterization

Nanoparticles were formed by self-assembly of polymer with DNA. Polymer solutions were added to equal volumes of plasmid DNA (pEGFP-C1, 0.1 mg/mL in water) at the desired polymer-to-DNA charge ratio. The charge ratio is expressed as the ratio of moles of polymer nitrogen to moles of DNA phosphate (N/P). After allowing 15 min for complexation, nanoparticles were diluted to a final DNA concentration of 25 $\mu\text{g/mL}$ by addition of dH_2O . Complex formation was confirmed by electrophoresis on a 1.0% agarose gel containing 0.4 $\mu\text{g/mL}$ ethidium bromide with Tris-acetate (TAE) running buffer. Size and surface charge of the nanoparticles were measured using a ZetaPALS dynamic light scattering detector (Brookhaven Instruments Corp., Holtsville, NY, USA) in quadruplicate. Particle sizing measurements were performed at a wavelength of 659.0 nm with a detection angle of 90° at RT.

Cell culture

PC-12 cells were maintained in F-12K medium supplemented with 15% heat-inactivated horse serum and 2.5% FBS at 37 C under 5%CO₂. Every other day 75% of the culture media was replaced with fresh growth medium. The cells were passaged by exposure to 0.25% trypsin-EDTA for 2 min, followed by trituration through fire-polished glass Pasteur pipettes when they reached 50% confluence. For differentiation, cell culture plates were coated with PLL 1 day before cell seeding and cells were cultured in F-12K medium containing 1% horse serum and mouse nerve growth factor (2.5S active subunit, NGF, 100 ng/mL) was used. All binding experiments on differentiated cells were performed after 6 days of differentiation. NIH-3T3 fibroblasts were maintained in DMEM containing 10% FBS at 37 C under 5% CO₂; 100 units/mL penicillin, 100 µg/mL streptomycin and 250 ng/mL amphotericin were included in all the cell culture media.

Flow cytometry

Neuron-specific binding of NT-PEI- and Tet1-PEI-based nanoparticles was investigated by flow cytometry. pGL3-CV was labeled with YOYO-1 at one dye per 100 base pairs according to the manufacturer's instructions. PC-12 cells were seeded at a density of 1.5×10^5 cells/well in 6-well plates and differentiated. Nanoparticles of bPEI, NT-PEI and Tet1-PEI were formulated at N/P of 2 with 2.5 µg of DNA. Nanoparticles, diluted with 1 mL serum-free media, were added to each well and then incubated for 1 h. The cells were collected and washed with phosphate-buffered saline (PBS) containing 1% horse serum. Dead cells were stained with propidium iodide (PI) (2 µg/mL) for 5 min. After washing twice, the cells were resuspended in PBS containing 1% horse serum. Flow cytometry was performed with BD FACScan (BD Biosciences, Franklin Lakes, NJ, USA). Populations of live cells were identified by first gating by forward and side scattering profiles to exclude cell debris and then by selection of PI-negative cells. Nanoparticle uptake was then assessed in 10 000 live cells per sample by analyzing for YOYO-1 fluorescence using CellQuest Pro software. Each formulation was delivered to four wells for quadruplicate assessment. Delivery of nanoparticles to non-neuronal 3T3 fibroblasts was also assessed following a similar protocol.

Competition assay

Competition assay was performed by co-incubating NT-PEI- and Tet1-PEI-targeted nanoparticles with 1000 molar times excess of free peptide compared with peptide conjugated to delivery vehicles. The differentiated PC-12 cells were pre-incubated with NT and Tet1 peptides at 4 C for 30 min to block the receptors on the cell surface and then co-incubated with NT-PEI and Tet1-PEI nanoparticles and 1000-fold excess free ligands at 37 C for 1 h. The cells were collected and analyzed by flow cytometry as above.

Confocal microscopy

Neuron-specific binding of targeted nanoparticles was also examined by confocal microscopy. pEGFP-C1 plasmid was fluorescently labeled using a Cy3-labeling kit according to the manufacturer's protocol. Lab-Tek II chamber slides (Nalge Nunc, Naperville, IL, USA) were pre-coated with PLL. PC-12 cells were seeded at a density of 4×10^4 cells/chamber and differentiated with 2.5S NGF. PEI-, NT-PEI-, and Tet1-PEI-targeted nanoparticles were formulated at N/P of 2 with 1 µg of Cy3-labeled pEGFP C1 plasmid and added to duplicate chambers. After 1 h incubation, the cells were washed with PBS and fixed with 4% paraformaldehyde for 7 min. The cell nuclei were stained with DAPI. The fixed cells were sealed with antifade reagents and then mounted with a coverslip. Images of mounted slides were obtained using a Zeiss LSM 510 confocal microscope.

Transfection study

Transfection experiments were performed in triplicate. PC-12 cells were seeded at 4×10^4 cells/mL per well on a 24-well plate for transfection of undifferentiated cells or 1.5×10^4 cells/mL per well for transfection of differentiated cells. For transfection of differentiated cells, cells were cultured in the presence of NGF for either 2 days or 7 days. NT-PEI and Tet1-PEI nanoparticles were formulated at N/P of 2 with 1 μ g of pGL3-CV plasmid by using water and then diluted with Gibco Opti-MEM medium (Invitrogen). The nanoparticles were added to the culture plate, and cells incubated at 37 °C for 5 h under a 5% CO₂ atmosphere. Subsequently, the transfection medium was replaced with serum-containing medium, and the plate was incubated at 37 °C for an additional 24 h under a 5% CO₂ atmosphere before the gene expression was assessed. Gene delivery efficiency of nanoparticles was confirmed by measuring enzyme-dependent light production using a luciferase assay kit (Promega Corp.). The cells were washed twice with PBS, lysed with 100 μ L of cell culture lysis reagent (Promega Corp.), frozen at -80 °C for 1 day, collected and centrifuged at 12 000 rpm. A volume of 20 μ L of each sample was placed in a 96-well plate, and luciferase activity was measured by a TECAN Safire II microplate reader (Tecan Systems, Inc.) after adding luciferase substrate (100 μ L). Total luminescence was measured for 1 s and the output was reported as relative light units (RLU). Protein concentration in the supernatant was determined using the BCA protein assay reagent (Pierce, Rockford, IL, USA). Cytotoxicity was obtained by incubating PC-12 cells with polyplexes at N/P of 2 for 1 day, followed by addition of MTS for measuring cell viability.

Dorsal root ganglion (DRG) isolation and uptake experiments

A pregnant C57Bl/6 mouse in the 16th day of gestation was anesthetized with an intraperitoneal injection of Beuthanasia (40 μ L per 25 g mouse). The abdomen was wiped with ethanol, the skin and peritoneum was cut to remove embryos which were placed in a Petri dish containing 1 $\times$ HBSS supplemented with 1% penicillin-streptomycin and 1% glucose. Skin and dorsal vertebral bones from the embryos were removed to the spinal cord, which was gently pulled away from the vertebrae and then removed from the vertebral cavity. The DRGs were removed from the cords or vertebral cavities if they did not come out together with the cord. DRGs were placed in cold 1 $\times$ HBSS with 0.1% trypsin ATV (antibiotic-trypsin-versene; GibcoBRL) and incubated for 5–10 min at 37 °C. After treatment with trypsin, the cells were centrifuged for 2 min at 1700 rpm and the media was removed replacing it with maintenance media containing DMEM/F12, 2% B27, 0.1% heparin, 1% glutamine and 20 ng/mL of NGF. Resuspended cells were plated on PLL-coated round glass coverslips placed in a 24-well plate (30 000 cells per coverslip). Media was supplemented with 20 ng/mL NGF every 3 days and half media changes were performed every week. Nanoparticles of bPEI, NT-PEI, and Tet1-PEI complexed with 1 μ g Cy3-pEGFP at N/P of 2 were formulated and diluted in DMEM/F12 medium. DRGs were treated with the nanoparticles for 1 h and washed with PBS twice. DRG cultures were fixed for 15 min with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100 for 5 min, and blocked with 10% donkey serum in 1 $\times$ PBS for 15 min. The cells were stained with a primary mouse antibody against NeuN for 3 h at RT, then secondary antibody AlexaFluor 488 anti-mouse (Molecular Probes) was applied for 2 h at RT.

Primary astrocyte culture and uptake experiments

A pregnant rat in the 13th day of gestation was anesthetized with an intraperitoneal injection of Beuthanasia. The abdomen was wiped with ethanol, and the skin and peritoneum was cut to remove embryos. Embryo brains were removed, placed in L-15 medium, and triturated. Medium was then replaced with fresh L-15 medium and cells centrifuged and plated in 24-well plates in DMEM/F12, 1% N2, 0.1% heparin, 1% glutamine and 20 ng/mL FGF. Cells were passaged after reaching 70% confluency and plated into 6-well plates and then 10 cm Petri dishes. After two passages, cells were placed in 12 mm laminin-coated glass coverslips

and differentiated into astrocytes in differentiation media (Neurobasal, 10% FBS, 1% pen-strep, 1% glutamine) for 14 days. Nanoparticles of bPEI, NT-PEI, and Tet1-PEI complexed with 1 μ g Cy3-pEGFP at N/P of 2 were formulated and diluted in DMEM/F12 medium. Cells were treated with the nanoparticles for 1 h and washed with PBS twice. Astrocytes were fixed for 15 min with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100 for 5 min, and blocked with 5% donkey serum in 1 $\times$ PBS for 30 min. The cells were stained with primary antibody anti-GFAP guinea pig (mature astrocyte marker) and anti-S100 β (immature astrocyte marker) rabbit for 3 h at RT. After three washes in PBS, secondary antibodies, AlexaFluor 488 antirabbit and 649 anti-guinea pig (Molecular Probes), were applied for 2 h at RT. DAPI was applied in the last wash with 1 $\times$ PBS.

Statistical analysis

Statically significant differences were analyzed by one-way analysis of variance (ANOVA). A *p* value less than 0.05 was considered statistically significant.

Results

Synthesis of NT-PEI and Tet1-PEI

NT-PEI was synthesized by pre-activating NT with bifunctional crosslinker SPDP and then conjugating the activated NT to PEI in the presence of Traut's reagent (Figure 1A). This approach has been demonstrated to give NT conjugates that retain receptor binding ability [20]. Reaction progress was monitored by measuring the absorbance of displaced pyridine-2-thione in solution. Tet1-PEI was synthesized by pre-activating PEI with SPDP followed by reaction with cysteine-terminated Tet1 (Figure 1B). Polymers were purified by gel filtration on a PD-10 column. The molar ratio of peptide conjugated to PEI was determined by assessing peptide concentration by UV absorbance and PEI concentration by formation of the cuprammonium complex upon copper(II) addition. The resulting peptide-PEI conjugates had peptide to PEI molar ratios of 8.2 and 0.6 for NT and Tet1, respectively.

Complex formation of NT-PEI and Tet1-PEI with plasmid

Cationic polymers like PEI, NT-PEI and Tet1-PEI form complexes (termed 'polyplexes') with polyanionic plasmids by self-assembly. Complex formation can be examined by agarose gel electrophoresis (Figure 2). Uncomplexed or loosely complexed plasmid migrates down the gel toward the positive terminal while complexed plasmid is retained in the loading well. Uncomplexed plasmid migration is shown as the 'P' lane in Figure 2. NT-PEI and Tet1-PEI form complexes with plasmid at N/P of 2 while complete plasmid complexation occurs with unmodified PEI at N/P of 1. This delayed complex formation observed with NT-PEI and Tet1-PEI is likely due to steric hindrance of the peptides attached on PEI. Conjugation of NT peptide converts the PEI primary amine into a charged amidine. Conjugation of Tet1 results in conversion of the PEI primary amine into an amide but the percentage of modification is very low (<1% of primary amines modified).

Particle sizes and surface charges of NT-PEI and Tet1-PEI polyplexes

The particle sizes of PEI, NT-PEI and Tet1-PEI complexed with plasmid at N/P of 2 were determined by dynamic light scattering (DLS) (Figure 3A) and their surface charges were measured by monitoring electrophoretic mobility in solution between electrodes (Figure 3B). PEI/DNA complexes formed polyplexes with diameters of 111.5 ± 5.6 nm. As expected, NT-PEI and Tet1-PEI polyplexes had larger particle sizes compared to bPEI polyplexes without any conjugated peptide (162.5 ± 28.3 and 139.6 ± 24.6 nm, respectively). NT-PEI polyplexes with higher peptide modification formed slightly larger particles than Tet1-PEI polyplexes which had lower peptide modification. All the polyplex formulations had similar particle

surface charges ranging from slightly negative to neutral. In order to assess the effect of ligand-mediated nanoparticle binding to cells in the absence of non-specific, charge-mediated association, complexes prepared at N/P of 2 were used in subsequent cell studies.

Specific binding to differentiated PC-12 cells^a

The binding of NT-PEI and Tet1-PEI nanoparticles with differentiated PC-12 neuron-like cells was investigated by flow cytometry (Figure 4). Nanoparticles were fluorescently labeled with YOYO-1, a DNA-intercalating flu-orophore. Experiments were conducted in quadruplicate and representative binding histograms of nanoparticle interaction with differentiated PC-12 cells are shown in fluorescence intensity vs. count plots (Figure 4A). The autofluorescence of untreated PC-12 cells is confined to the first decile (Figure 4A, top left panel). The fluorescence profile of cells exposed to PEI nanoparticles (N/P = 2) is similar to that of untreated cells, indicating minimal interaction between the nanoparticles and cells (Figure 4A, top right panel). In contrast, cells exposed to either NT-PEI nanoparticles (Figure 4A, bottom left panel) or Tet1-PEI nanoparticles (Figure 4A, bottom right panel) showed a subpopulation of cells with higher fluorescence resulting from nanoparticle binding. The extent of nanoparticle association with cells is quantified in Figure 4B. NT-PEI and Tet1-PEI nanoparticles displayed significantly higher affinity for differentiated PC-12 cells (12.7% and 16.3% cell binding, respectively) over unmodified PEI nanoparticles (0.6% cell binding). The overall binding levels of targeted nanoparticles have been qualitatively observed to correlate with the extent of cell differentiation to the neuron-like phenotype.

Imaging of nanoparticle interaction with differentiated neuron-like cells

The interaction of NT-PEI and Tet1-PEI nanoparticles (N/P = 2) containing Cy3-labeled plasmid with differentiated PC-12 cells was observed by confocal microscopy (Figure 5). Differentiated PC-12 cells were exposed to PEI, NT-PEI, and Tet1-PEI nanoparticles for 1 h. After removing unbound nanoparticles by washing, cells were fixed and counter-stained with DAPI to visualize cell nuclei. NT-PEI nanoparticles were associated with the plasma membranes of both soma and neurites, but few examples of internalized polyplexes were observed. In case of Tet1-PEI nanoparticles, nanoparticles were observed associated with cell membranes and internalized around the nucleus. Cells treated with bPEI nanoparticles had minimal associated fluorescence.

Binding of NT-PEI and Tet1-PEI nanoparticles to non-neuronal 3T3 fibroblasts

In order to verify the cell specificity of ligand-modified nanoparticles, cell-binding studies with the three nanoparticle formulations were repeated with NIH 3T3 fibroblast cells which do not express the neuronal neurotensin and tetanus toxin receptors (Figure 6). Representative fluorescence histograms of untreated, PEI-nanoparticle-treated, NT-PEI-nanoparticle-treated, and Tet1-PEI-nanoparticle-treated cells are shown in Figure 6A. In contrast to differentiated PC-12 cells, no significant increase in cell association was observed with the peptide-modified complexes although low levels of non-specific binding were observed with all formulations. The percentage of cells with nanoparticle association is shown in Figure 6B.

Competition assay with free ligands

Ligand-mediated binding to differentiated neuron-like cells was further verified by competition experiments with free peptide. Competition assays were conducted by first blocking the potential receptors of cells with free ligands at 4 °C for 30 min and then co-incubating a large excess (1000-fold) of free competitive ligands with targeted nanoparticles during the binding study (Figure 7). Binding was assessed by flow cytometry as described previously. NT-PEI and Tet1-PEI nanoparticles bound to ~30% of cells in the absence of competitive ligands but to significantly fewer (~5–7%) cells in the presence of competitive ligand.

Transfection of differentiated and undifferentiated PC-12 cells

The transfection efficiency of PEI, NT-PEI and Tet1-PEI polyplexes formulated at N/P of 2 with a luciferase reporter gene was assessed in undifferentiated PC-12 cells, PC-12 cells undergoing differentiation (treatment for 2 days with NGF), and differentiated PC-12 cells (treatment for 7 days with NGF) by luciferase assay. As expected, no significant difference in transgene expression between the three formulations was observed in undifferentiated PC-12 cells (data not shown). Exposure of PC-12 cells to NGF induces a neuron-like phenotype including neurite extension. Thy-1, which has been shown to bind to tetanus toxin C [25], is expressed within 24 h after NGF treatment [26,27]. The GT1b trisialoganglioside which was used to select for Tet1 peptide binding also has increased expression in PC-12 cells after NGF treatment; tetanus toxin C binding to PC-12 cells also increases with NGF treatment and correlates with GT1b expression [28]. The B2 bradykinin receptor, which has been shown to bind with low affinity to neurotensin, is expressed in NGF-treated cells [27,29]. Transgene expression from NT-PEI and Tet1-PEI complexes delivered to PC-12 cells undergoing differentiation was $6.5-(3.5 \times 10^5 \pm 2.9 \times 10^5 \text{ RLU/mg protein})$ and 7.0-fold ($3.7 \times 10^5 \pm 1.8 \times 10^5 \text{ RLU/mg protein}$) higher, respectively, than from untargeted PEI complexes ($5.2 \times 10^4 \pm 3.1 \times 10^4 \text{ RLU/mg protein}$) (Figure 8A). Large variance was observed in transfection studies and attributed to a combination of factors influencing gene delivery such as cell proliferation and receptor expression that is more heterogeneous in a mixed population of cells undergoing differentiation. However, the increase in gene transfection from Tet1-PEI complexes is statistically significant and reproducible in several separate experiments. Transfections of the three formulations conducted at N/P of 5 revealed no significant difference in transgene expression levels; this is hypothesized to reflect similar levels of nonspecific polyplex uptake due to electrostatic interactions of particles with membrane proteins. Fully differentiated PC-12 cells are non-mitotic; delivery of polyplexes to these cells resulted in no substantial transgene expression despite preferential binding by Tet1-PEI and NT-PEI complexes. The cellular toxicity of the PEI, NT-PEI and Tet1-PEI complexes was determined by MTS assay after transfection and compared to untreated cells (Figure 8B). The MTS assay assesses metabolic activity of live cells. No significant toxicity was observed for the formulations under these transfection conditions.

Polyplex delivery to primary DRG cells and astrocytes

The ability of targeted polyplexes to deliver genetic material to primary neurons was assessed by incubating fluorescently labeled PEI, NT-PEI and Tet1-PEI polyplexes with dorsal root ganglion (DRG) cells isolated from embryonic mice and visualizing binding and uptake by confocal microscopy. Neurons were stained with a NeuN antibody. Significant neuronal association and uptake was only observed with Tet1-PEI formulations (Figures 9A and 9B). Polyplex delivery to primary astrocytes revealed low levels of non-specific binding by all polyplex formulations (Figure 9C).

Discussion

Efficient *in vivo* delivery of genetic materials to neurons in the CNS has remained a major challenge because these cells are well insulated and protected from the surrounding environment in order to minimize unnecessary stimulus and damage [30]. Several strategies have been employed to overcome this challenge in the non-viral gene delivery field, one of which is to utilize materials that specifically recognize neurons. Neuron-specific toxin fragments, neurotransmitters and nerve growth factors have been employed as mediators of neuron recognition [13,14,16,31]. In this study, two materials, Tet1-PEI and NT-PEI, are evaluated for neuron-specific gene delivery. PEI was selected as the polycation carrier because it displays high *in vitro* delivery efficiency due to the presence of protonatable amino groups that are protonated at slightly acidic pH [32]. This polymer has been used for *in vivo* gene

delivery [33,34]. Tet1 is a novel 12-amino-acid peptide that competes with tetanus toxin for receptor binding [19] and neurotensin is a neuropeptide that is recognized by neurons in the CNS [35,36]. Tetanus toxin and neurotensin have both been applied in molecular conjugates for neuron targeting [15,18,37]. The neurotensin receptors are predominantly expressed by neurons in the CNS, with particularly high expression in the substantia nigra, hypothalamus, epithalamus, ventral thalamus, whereas tetanus toxin is capable of binding receptors of motor neurons at the neuromuscular junction, thus providing the possibility of minimally invasive delivery [38,39]. This work evaluates Tet1 as an attractive peptide-based alternative to tetanus toxin-based targeting.

Tet1-PEI and NT-PEI were synthesized by SPDP conjugation and complexed with plasmid DNA at N/P of 2 (Figure 1). The low charge ratio was selected to prevent formulation of highly cationic complexes that result in non-specific binding to anionic glycoproteins on cell surfaces; polyplex interaction with glycoproteins such as heparan sulfate has been shown to be a primary route of non-specific binding and internalization into cultured cells [40]. Complete DNA packaging at N/P of 2 was confirmed by gel electrophoresis (Figure 2). Tet1-PEI, NT-PEI, and PEI complexes with DNA formed nanoparticles of similar size (~100–150 nm, Figure 3A). Peptide-PEI nanoparticles were slightly larger, likely due to steric hindrance in complexation due to the conjugated peptides. The surface charge of nanoparticle formulations was determined by zeta potential measurement and found to be near neutral (Figure 3B). Therefore, charge-mediated interactions of nanoparticles and cells should be minimized with these formulations.

The specific recognition of differentiated PC-12 cells towards ligands on NT-PEI and Tet1-PEI nanoparticles was investigated by flow cytometry and confocal microscopy (Figure 4 and Figure 5). Targeted nanoparticles showed specific binding to cells within 1 h of co-incubation that was not apparent with unmodified PEI nanoparticles. Non-neuronal 3T3 fibroblasts did not demonstrate preferential binding toward NT-PEI and Tet1-PEI nanoparticles, indicating a specific interaction between NT and Tet1 ligands on nanoparticles and the receptors on the cells (Figure 6).

Receptor-mediated binding of NT-PEI and Tet1-PEI nanoparticles with differentiated PC-12 cells was confirmed by competition assay (Figure 7). Free peptide ligands, incubated in excess with the nanoparticles during the binding study, significantly blocked nanoparticle binding. Initial ligand competition studies were conducted by incubating nanoparticles with 1000 excess peptide in the presence of cells. However, minimal binding inhibition was observed. In the subsequent reported study, free peptide was first added to cells at 4 °C to block receptor binding sites before peptide and nanoparticles were added. Using this approach, specific binding inhibition was demonstrated. These results reveal the high binding avidity of targeted nanoparticles for neurons resulting from multivalent interactions of the nanoparticle with multiple receptors.

Gene transfection by PEI, NT-PEI and Tet1-PEI polyplexes was assessed in PC-12 cells during different stages of NGF-induced differentiation. Undifferentiated PC-12 cells do not express receptors for NT and tetanus toxin C; hence, no significant difference in gene expression was observed between the three formulations. Tetanus toxin C has been proposed to bind to GTb1 trisialoganglioside and also to Thy-1 [25,28]. To our knowledge, the expression of high-affinity neurotensin receptor has not been assessed in PC-12 cells; however, neurotensin has been shown to bind to B2 bradykinin receptors with low affinity in PC-12 cells [29]. Thy-1 mRNA levels in PC-12 cells are detectable by 24 h after NGF treatment, and peaks 4 days after NGF addition [26,27]. Tetanus toxin binding to PC-12 cells correlates with Thy-1 expression and increases significantly upon NGF differentiation [41]. GTb1 expression is also increased in PC-12 cells with NGF treatment [28]. Gunning and coworkers also showed that PC-12 cells

continue in log phase growth for 4 days after NGF differentiation before the growth curve plateaus [42]. Thus, transfection was also assessed in PC-12 cells after 2 days of NGF exposure. The rationale was that expression of neuron-specific receptors would be induced by this time, but continuing cell division would permit nuclear accumulation of internalized polyplexes and detection of transgene delivery. Low transgene expression was achieved by untargeted PEI polyplexes due to low cell binding. In addition to the near-neutral surface charge of the PEI polyplexes, NGF-treated PC-12 cells have increased amounts of N-sulfation in cell-associated heparan sulfate which has been hypothesized to contribute to reduced non-specific internalization of nanoparticles in differentiated PC-12 cells [43,44]. In contrast, NT-PEI and Tet1-PEI polyplexes retain their ability to transfect NGF-treated PC-12 cells under these conditions due to receptor-ligand interactions (Figure 8). Fully differentiated PC-12 cells are not transfected by any of the tested delivery formulations despite cell binding by NT-PEI and Tet1-PEI polyplexes. PC-12 cells treated for 7 days are in the plateau phase of the growth curve; in these cells, nuclear translocation becomes the limiting barrier to transfection. Thus, vehicles capable of efficient intracellular trafficking remain a major need in neuron gene delivery.

Tet1-mediated neuron targeting of nanoparticles was also demonstrated in primary neurons (Figures 9A and 9B) but not in primary astrocytes (Figure 9C). Tet1-PEI nanoparticles significantly bound to isolated DRGs whereas NT-PEI and PEI interacted minimally. Tet1 is recognized by receptors expressed by DRGs [19]. In contrast, NT-receptor expression was observed only in a subpopulation of DRGs with primary expression in small neurons and low expression in medium and large neurons [45]. Because the DRGs used in these studies were maintained under nerve growth factor selection and supplemented with glial cell-line derived neurotrophic factor (which promotes small neuron survival), low NTPEI nanoparticle binding to these DRGs was observed. The low levels of non-specific association of polyplex with astrocytes were similar to the binding trends observed with fibroblasts (Figure 6). No transgene expression was detected in DRGs, further emphasizing the need for delivery materials that can successfully maneuver to nuclei of non-dividing cells.

Successful neuron gene delivery requires, in addition to cell internalization, efficient retrograde transport in the axon, release of vehicles from the vesicular transport structures, and nuclear entry. Neurotensin-poly(L-lysine) conjugates require incorporation of the hemagglutinin HA2 membrane lytic peptide as well as a SV40 nuclear localization sequence in order to achieve high delivery efficiencies [46,47]. Here, PEI is proposed to mediate endosomal release through a proton sponge buffer effect [32]. The intracellular trafficking of the conjugates presented here and methods to improve nuclear delivery are currently being investigated.

In conclusion, Tet1-PEI and NT-PEI are promising neuron-specific gene carriers for *in vivo* gene delivery. Tet1-PEI is particularly attractive as a potential noninvasive delivery material for neuron targeting that can be administered to peripheral tissue for recognition by motor neurons. In future studies, Tet1-PEI will be used as adapted for *in vivo* studies. Tet1-PEI will be modified with a hydrophilic PEG group for nanoparticle stabilization. In addition, peptides that improve intracellular trafficking will be incorporated.

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Figure 1. Synthetic scheme of neurotensin-conjugated poly(ethylenimine) (NT-PEI) and Tet1-conjugated poly(ethylenimine) (Tet1-PEI)

Figure 2.

Agarose gel electrophoresis of branched PEI (bPEI), Tet1-PEI, and NT-PEI complexed with plasmid DNA at the various charge ratios; M: DNA 1 kb ladder marker, P: pEGFP-C1 plasmid

Figure 3. Particle sizes (A) and surface charges (B) of bPEI, NT-PEI and Tet1-PEI nanoparticles complexed with plasmid at N/P of 2, measured by dynamic light scattering

Figure 4. Binding of bPEI, NT-PEI and Tet1-PEI nanoparticles with differentiated PC-12 neuron-like cells. PC-12 cells were treated for 1 h with nanoparticles complexed with YOYO-1-labeled pEGFP at N/P of 2 and analyzed by flow cytometry. Each sample was measured in quadruplicate, and plotted as fluorescence intensity vs. count plot (A) and histogram of averaged cellular binding (B). $p < 0.001$ vs. PEI

Figure 5.

Confocal microscopy of bPEI, NT-PEI, and Tet1-PEI nanoparticles complexed at N/P of 2. Plasmid DNA was labeled with Cy3 and PC-12 cells were counterstained with DAPI after fixation. Differential interference contrast (DIC) imaging was merged with Cy3 and DAPI data to verify cellular binding of nanoparticles (bottom)

Figure 6. Binding of bPEI, NT-PEI and Tet1-PEI nanoparticles with non-neuronal NIH-3T3 fibroblast cells. The cells were treated for 1 h with nanoparticles complexed with YOYO-1-labeled pEGFP at N/P of 2 and analyzed by flow cytometry. Each sample was measured in quadruplicate, and plotted as fluorescence intensity vs. count plot (A) and histogram of averaged cellular binding (B)

Figure 7.

Competition assays with free ligand against the ligand-mediated binding of bPEI, NT-PEI and Tet1-PEI nanoparticles to differentiated PC-12 neuron-like cells. Free ligand in 1000-fold excess was incubated with the cells before and during treatment with nanoparticles. Cellular binding of nanoparticles was examined by flow cytometry

Figure 8.

(A) Luciferase activity of PC-12 cells transfected with bPEI, NT-PEI and Tet1-PEI nanoparticles complexed with luciferase plasmid at N/P of 2. PC-12 cells were differentiated for 2 days with NGF. * $p < 0.05$ vs. PEI. (B) Cell viability of PC-12 cells treated with bPEI, NT-PEI and Tet1-PEI nanoparticles

Figure 9.

(A) Binding of bPEI, NT-PEI and Tet1-PEI nanoparticles with dorsal root ganglion (DRG) cells. The cells were treated for 1 h with nanoparticles complexed with Cy3-labeled pEGFP (red) at N/P of 2 and imaged by confocal microscopy. Neurons were stained with NeuN (green). (B) Z-stack of DRG cells treated with Tet1-PEI. (C) Binding of bPEI, NT-PEI and Tet1-PEI nanoparticles with primary astrocytes. The cells were treated for 1 h with nanoparticles complexed with Cy3-labeled pEGFP (red) at N/P of 2 and imaged by confocal microscopy. Immature and mature astrocytes were stained with S100β (green) and GFAP (blue), respectively. Arrows indicate internalized polyplex