Published in final edited form as:

Biomaterials. 2009 February ; 30(4): 649–657. doi:10.1016/j.biomaterials.2008.10.003.

A ligand-mediated nanovector for targeted gene delivery and transfection in cancer cells

Omid Veisheh^a, Forrest Kievit^a, Jonathan Gunn^a, Buddy Ratner^b, and Miqin Zhang^{a,*}

^aDepartment of Materials Science & Engineering, University of Washington, Seattle, WA 98195, USA.

^bDepartment of Bioengineering, University of Washington, Seattle, WA 98195, USA.

Abstract

As conventional cancer therapies struggle with toxicity issues and irregular remedial efficacy, the preparation of novel gene therapy vectors could offer clinicians the tools for addressing the genetic errors of diseased tissue. The transfer of gene therapy to the clinic has proven difficult due to safety, target specificity, and transfection efficiency concerns. Polyethylenimine (PEI) nanoparticles have been identified as promising gene carriers that induce gene transfection with high efficiency. However, the inherent toxicity of the material and non-selective delivery are the major concerns in applying these particles clinically. Here, a non-viral nanovector has been developed by PEGylation of DNA-complexing PEI in nanoparticles functionalized with an Alexa Fluor 647 near infrared fluorophore, and the chlorotoxin (CTX) peptide which binds specifically to many forms of cancer. With this nanovector, the potential toxicity to healthy cells is minimized by both the reduction of the toxicity of PEI with the biocompatible copolymer and the targeted delivery of the nanovector to cancer cells, as evaluated by viability studies. The nanovector demonstrated high levels of targeting specificity and gene transfection efficiency with both C6 glioma and DAOY medulloblastoma tumor cells. Significantly, with the CTX as the targeting ligand, the nanovector may serve as a widely applicable gene delivery system for a broad array of cancer types.

Keywords

Nanoparticle; Cancer targeting; Chlorotoxin; Nanotechnology; Gene therapy

1. Introduction

Among the most devastating diseases, cancer annually accounts for approximately 10 million new cases worldwide[1], and 560,000 deaths in the United States [2]. Conventional therapies include surgical intervention, radiation treatment and chemotherapy, but these courses of treatment must finely balance the effectiveness of the therapy with the preservation of healthy tissue. Alternatively, the development of gene therapy against cancerous cells, a method of correcting the dysfunctional or deleted genes of damaged cells, could treat drug-resistant tissue, limit unwanted toxicity exposure to healthy cells, and present a platform for therapy against multiple forms of cancer [3–6].

Successful gene therapy has been demonstrated in controlled experimentation, but a successful transition into the clinic has stalled due to the lack of safe, efficient and controllable methods

[*]Corresponding author: Prof. Miqin Zhang, Department of Materials Science and Engineering, University of Washington, Seattle, WA 98195 (USA), Fax : (+ 1) 206-543-3100, E-mail: E-mail: mizhang@u.washington.edu.

of gene delivery to cancerous cells [7,8]. In response, two primary gene vehicle classes have been developed: recombinant viruses and non-viral vectors [6]. The former has been widely applied in gene-delivery studies, thanks to its evolutionarily rooted efficiency as a gene delivery vehicle [9,10]. However, safety concerns around random recombination immunogenicity, a lack of specific cell targeting, and limited nucleic acid loading capacity hinder the wide clinical application of recombinant viruses [7,11].

Alternatively, non-viral vectors including nanoparticles and liposomes can be engineered for reduced immunogenicity, infection or mutagenesis, and can transport large nucleic acid payloads [8,12–16]. These vectors typically employ cationic polymers such as chitosan, polylysine or polyethylenimine (PEI) complexed with anionic nucleic acids [8,12,17,18]. Among these charged polymers, PEI has demonstrated the highest gene delivery efficiency *in vitro* because of its large cationic charge that facilitates a high degree of DNA complexation and an effective ability to escape the cellular endosome upon internalization via the ‘proton sponge’ effect [18,19].

Unmodified PEI, though, is handicapped by an inherent toxicity and lack of cell specificity for selective transfection. This has been partially rectified by covalent modification of the cationic polymer with poly(ethylene glycol) (PEG) which has improved the biocompatibility of the vector [8,12,17]. Furthermore, cell-targeting by these PEI vectors has been accomplished, by ligand conjugation [20–25], with proteins such as antibodies for targeting HER2 expressing tumor cells [25], peptides such as RGD (arginine-glycine-aspartic acid) for integrin receptor targeting of endothelial cells of tumor metastases [21], and small organic molecules [24], including folic acid for folate receptor targeting in colon adenocarcinoma and oral epidermoid cancer cells [20].

While these previously developed systems have achieved enhanced transfection efficiencies through cell targeting, none have demonstrated broad cancer-type applicability, high-level specificity, or limited binding to non-tumor tissue. An alternative targeting peptide, chlorotoxin (CTX), has been proved to bind a broad class of cancer cells of the neuroectodermal origin in a membrane-bound matrix metalloproteinase-2 (MMP-2)-dependent manner [26]. This 4 kDa peptide has demonstrated high specificity and affinity for the vast majority of brain tumors (74 out of 79) [27], and recent research has further shown that CTX specifically binds to prostate cancer, sarcoma, and intestinal cancer [26]. These findings suggest that this peptide has broad implications as a cancer cell targeting ligand, demonstrating excellent targeting in previous *in vitro* and *in vivo* nanoparticle studies [28,29].

In this study, a non-viral vector system comprising a branched PEI polymer functionalized with grafted PEG, the Alexa Fluor 647 (AF) fluorochrome, and the CTX targeting peptide, is presented as a widely applicable nanovector delivery system (P-PEG-AF-CTX). The nanovector was designed to serve as an effective gene carrier with targeting and gene transfection capabilities specific to cancer cells. Physicochemical properties of the nanovector system were thoroughly characterized, and the DNA loading was optimized. The specificity and transfection of the targeting nanovector to cancer cells was studied with MMP-2 positive tumor cell lines C6 and DAOY, in comparison with non-targeting nanoparticle systems. The combination of PEG shielding to reduce toxicity of PEI and specificity to the target cells enabled by CTX may present an advancement towards the targeted gene therapy to cancer disease with reduced side effects.

2. Materials and methods

2.1. Materials

All materials were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. Chlorotoxin (Alomone Labs, Ltd. Jerusalem, Israel) is a peptide derived from venom of the Giant Israeli scorpion *Leiurus q. hebraeus*. It is a recombinantly produced peptide containing 36 amino acids, 8 cysteine residues, 4 disulfide cross-links. The sequence of Chlorotoxin is as follows: MCMPCFTTDHQMARCDDCCGGKGRGKCYGPQCLCR. The four disulfide bonds are between residues 2 and 19, 5 and 28, 16 to 33, and 20 to 35. Chlorotoxin is highly toxic to invertebrates, but non-toxic to mammals [30].

2.2. Plasmid DNA preparation

Enhanced green fluorescent protein (EGFP)-encoding DNA under control of the CMV promoter in the CS2 plasmid (CS2-GFP) was kindly donated by Dr. Jim Olson, and amplified in DH5 α E. coli (Invitrogen; Carlsbad, CA). Plasmid DNA was purified with the Plasmid Giga Kit (Quiagen; Valencia, CA) following the manufacturer's instructions. The final DNA stock was dissolved in TE buffer at 1 mg mL⁻¹.

2.3. Polymer conjugation

The P-PEG-AF-CTX construct was prepared using a multistep process. First, PEI (25 kDa) was pegylated through covalent attachment of PEG with the functional amino groups present on PEI using succinimidyl ester chemistry. Here, PEI was dissolved in water (100 mg mL⁻¹) and diluted in 100mM Na bicarbonate buffer and the solution was adjusted to pH 8.5. PEG succinimidyl ester (3.4 kDa; Nektar; San Carlos, CA), dissolved in water (100 mg mL⁻¹), was added to the buffered PEI solution at a PEG:PEI molar ratio of 4:1, and reacted for 2 hrs. The PEI-PEG (P-PEG) polymer solution was then reacted with the near infrared emitting fluorophore, Alexa Fluor 647 (AF) carboxylic acid succinimidyl ester (dissolved in dimethylformamide), at an AF:P-PEG ratio of 0.5:1 for 2 hrs at room temperature. Thereafter, the mixture was purified via gel permeation chromatography using Sephadex G-50 resin (GE Healthcare, Piscataway, NJ) equilibrated with 100mM Na bicarbonate buffer and adjusted to pH 8.5.

CTX attachment to the copolymer was performed using a three-step process. First, succinimidyl-6-(iodoacetyl)aminocaproate (SIAX; Molecular Biosciences; Boulder, CO), dissolved in 0.15 mL of dimethyl sulfoxide (DMSO), was added to a 1.5 mL suspension of P-PEG-AF in 100 mM Na bicarbonate (pH 8.5) at a molecular ratio of 7.5:1. The solution was protected from light and placed on a shaker for 2 hrs at room temperature. Free SIAX was removed using Sephadex G-50 resin equilibrated with a thiolation buffer (5 mM ethylenediamine tetraacetic acid in deoxygenated PBS; pH 8.0). Secondly, the CTX peptide (Alomone Labs Ltd.; Jerusalem, Israel) was modified with a sulfhydryl group via primary amine modification with Traut's reagent (Molecular Biosciences). Here, Traut's reagent (6 μ L at 10 mg mL⁻¹ in thiolation buffer) was added to 1.5 mg CTX (1 mg mL⁻¹ in thiolation buffer) and allowed to react for 1 hr at room temperature. Lastly, the peptide solution was mixed with the iodoacetate-functionalized polymer at a molecular ratio of 4:1 and allowed to react for 1 hr at room temperature. Excess CTX was purified from the conjugate by Sephadex G-50 chromatography equilibrated with 20 mM HEPES buffer adjusted to pH 7.4.

2.4. NMR spectroscopy

NMR spectra were obtained on a Bruker AVance 300 spectrometer operating at 300 MHz (¹H) and 298 K. The chemical shifts, δ_H (ppm), were assigned as follows: PEG (-O-CH₂-CH₂-) at 3.60–3.65, PEI (-NH-CH₂-CH₂-) at 2.50–3.10, and CTX at 2.60–3.10 and 3.65–3.80.

The molar ratio of PEG to PEI was determined using the integration values of corresponding PEG and PEI protons.

2.5. Formation and analysis of polymer-DNA complexes

CS2-GFP and the polymeric nanovectors were diluted in 20 mM HEPES buffer (pH 7.4) at concentrations equivalent to the w:w ratios tested. Equal volumes of CS2-GFP and polymer were mixed, vortexed, and incubated for >10 min at room temperature. Size and zeta potential analyses were performed using a DTS Zetasizer Nano (Malvern Instruments, Worcestershire, UK). DNA binding was determined using a gel retardation assay whereby vector-DNA complexes (500 ng DNA) were added to the wells of a 1% agarose gel. DNA was visualized by staining with ethidium bromide and imaged on a Gel Doc XR (Bio-Rad Laboratories, Hercules, CA). Separately, DNA binding to PEI and subsequent protection was quantified using an ethidium bromide exclusion assay [24].

2.6. Cell culture

C6 rat glioma, DAOY, and NIH3T3 mouse fibroblast cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic. Cultures were incubated at 37°C in a humidified incubator maintained at 5% CO₂.

2.7. Cell transfections

For all transfection experiments, cells were seeded 18 hrs prior to treatment. Vector-DNA complexes were prepared at a PEI:DNA ratio (w:w) of five. The complexes were then diluted with serum-containing cell culture medium and incubated with cells for 2 hrs. The medium was then removed and the cells were replenished with complete cell medium. The lipofectamine transfection (Invitrogen) was executed using the manufacturer's protocol.

2.8. Alamar blue cell viability analysis

Cells were grown to confluence on 12 well plates, and treated with DNA, nanovector complexes, or the lipofectamine reagent at 10 µg DNA mL⁻¹ in complete cell medium for 48 hrs. Cell counts of treated and untreated samples were compared to determine percent viability of treated samples (untreated cells assumed to represent 100% viability). After treatment, samples were washed with PBS three times, and incubated for 1 hr with 10% Alamar blue (Invitrogen) in phenol-free DMEM (supplemented with 10% FBS and 1% antibiotic-antimycotic). The percent reduction of Alamar blue was determined using the manufacturer's protocol.

2.9. Flow cytometry

Nanovector uptake and gene transfection efficiencies were quantified using flow cytometry. For both studies, cells were seeded on 12-well plates at 500,000 cells per well 18 hrs prior to transfection. Cells were treated with the nanovectors, as described, washed with PBS three times, trypsinized, and resuspended in 2% FBS in PBS. Flow cytometry analysis was performed on a BD LSRII (Beckton Dickinson, Franklin Lakes, NJ) using the 488 nm argon, and 633nm red diode lasers. Acquired data was analyzed using FlowJo software (Tree Star, Ashland, OR). A minimum of 10,000 cells was analyzed for each sample and three samples (wells) were evaluated for each treatment.

2.10. Microscopic analysis

C6 or NIH3T3 cells were plated on 24 mm glass cover slides (500,000 cells per slide) and grown for 18 hrs. Cells were then transfected as described, washed with PBS three times, and

fixed in a 4% formaldehyde/PBS solution (methanol free, Polysciences Inc., Warrington, PA) for 30 min. The fixative was then removed and cells washed with PBS three times. The membranes were then labeled with wheat germ agglutinin Alexa Fluor 555 (Invitrogen) according to the manufacturer's instructions. The slides were then mounted with DAPI-containing ProLong Gold antifade solution (Invitrogen) for cellular nuclei staining and fluorescence preservation. The slides were then examined by fluorescence and differential interference contrast (DIC) microscopy using a LSM 510 Meta confocal fluorescence microscope (Carl Zeiss Inc., Peabody, MA) equipped with 405 nm diode, 458 nm, 488 nm, 514 nm Argon/2, and 633 nm HeNe laser lines for excitation, and appropriate bandpass filters for collection of DAPI, FITC, TRITC, and NIR emission signals. In all fluorescence images the DAPI signal is depicted in blue, FITC signal in turquoise, and TRITC signal in green, and the NIR signal was translated into red pseudocolor.

3. Results and discussion

3.1. Synthesis of P-PEG-AF-CTX polymer

A multi-step reaction was performed for the synthesis of the branched P-PEG-AF-CTX nanovector construct, comprising PEG, CTX, and the near infrared emitting fluorophore Alexa Fluor 647 (AF), each covalently linked to a PEI backbone (P). Figures 1a and b show the chemical preparation scheme. As shown, a 25 kDa branched PEI was modified with PEG-N-hydroxy succinimidyl ester (3.4 kDa) via the exposed amino groups on the PEI to form pegylated PEI (P-PEG). The P-PEG was then fluorescently functionalized by reaction with AF-carboxylic acid, succinimidyl ester. This AF fluorophore provides reporter functionality during cell targeting analysis by flow cytometry and nanoparticle localization in microscopy analysis. Lastly, CTX was covalently bound to the PEI chain by a three-step reaction using the heterobifunctional linker molecules, iodoacetyl aminocaproate (SIAX) and Traut's reagent. Specifically, the SIAX molecule was reacted with the PEI chain to form a thiol-reactive iodoacetate moiety, while the Traut's reagent added a reactive thiol group to the CTX peptide. The thiolated peptide was then reacted with the iodoacetate functionalized PEI chain. CTX and PEG modification of PEI polymer was shown by ^1H NMR spectroscopy (Figure 2). In the P-PEG-CTX spectrum, the conjugate clearly shows the PEG peak (3.65–3.6 ppm) of the P-PEG-SIAX polymer as well as characteristic CTX peaks (2.60–3.10 and 3.65–3.80 ppm). Integrations of the PEG and PEI peaks yielded a PEG-to-PEI molar ratio of ~1.8:1.

In designing the nanovector, the 25 kDa branched form of PEI was chosen for its successful application in previous studies [18,31]. Its structure consists of primary (25%), secondary (50%), and tertiary (25%) amino groups. This unique chemical structure renders the material cationic and ideally suited for DNA complexation. Additionally, due to its amino group diversity, the polymer provides a significant buffering capacity over a wide pH range [18]. This enables a net positive charge at physiological pH, facilitating cell binding and endocytosis. Upon cellular uptake, the PEI polymeric nanovector undergoes endosomal escape via the 'proton sponge' effect [19], a unique ability retained by polymers with strong buffering capacities between pH 5.0 and 7.2. Here, it is believed that PEI may protect the nanovector by limiting the destructive enzymes within the endolysosome by raising the local pH, possibly affecting enzyme folding. This buffering also induces acidification of the endosome by proton absorption to the polymer, leading to vesicle rupture by increased lysosomal osmolarity and subsequent swelling. The contents of the endosome are then released into the cytosol, avoiding the destructive lysosomal processing pathway.

While essential for mediating efficient transfections, the PEI's cationic profile promotes non-specific cell interactions and contributes to its cytotoxicity and low blood circulation half-life *in vivo* [31]. To limit these negative attributes, PEG was grafted to the PEI backbone to shield its cationic charge. Pegylated PEI showed reduction in plasma protein adsorption and limited

sequestration by the reticuloendothelial system (RES), increasing the construct's blood half-life [8,32,33]. This approach has proven effective when the optimal length and grafting ratio of the PEG is used [31,32,34]. Thus, an optimal PEG length (3.4 kDa) polymer based on previous successes was employed in this study.

3.2. Nanovector Development

Electrostatic interactions between the negatively charged nucleic acids with the cationic P-PEG-AF-CTX construct induced formation of the P-PEG-AF-CTX/DNA nanovector, as illustrated in Figure 1c. The ideal polymer:DNA ratio, or DNA loading, for complete complex formation was found using a DNA retardation assay by agarose gel electrophoresis with PEI:DNA (w:w) ratios ranging from 0–4:1 (Figure 3a). As shown, a ratio of polymer:DNA ranging from 2:1 to 4:1 yielded good DNA complexation, and both CTX-modified (P-PEG-AF-CTX) and unmodified (P-PEG-AF-SIAX) copolymers showed a similar DNA binding capacity (Figure 3a). For quantitative analysis of DNA binding to the PEI polymer, an ethidium bromide exclusion assay was utilized [24]. As shown in figure 3b both versions of the polymers (CTX modified, and unmodified) demonstrated the ability to bind DNA at similar capacities and a polymer:DNA ratio of 5:1 yielded nearly complete DNA complexation. This experiment confirmed that incorporation of CTX in PEI nanoparticles does not alter the DNA loading capacity of the polymer.

Hydrodynamic size and zeta potential of non-viral vectors have been shown to dramatically influence their uptake by cells and their gene transfection efficiencies. To accurately compare the targeting efficiency of each of the tested constructs, the hydrodynamic size and zeta potential must be similar. The candidate P-PEG-AF-CTX/ DNA nanovectors and control P-PEG-AF-SIAX/DNA nanovectors, each with varying polymer:DNA ratios, showed similar hydrodynamic sizes and zeta potentials (Figure 4a). Specifically, nanovectors prepared with a polymer:DNA ratio of 5:1, which showed full DNA complexation, had zeta potential values of $+22.1 \pm 5.3$ (P-PEG-AF-CTX/ DNA) and $+17.6 \pm 7.5$ (P-PEG-AF-SIAX/DNA). The hydrodynamic sizes of these nanovectors were 134.8 (PDI: 0.273) and 128.0 (PDI: 0.217) for P-PEG-AF-CTX/DNA and P-PEG-AF-SIAX/DNA nanovectors, respectively. Figure 4b shows that the hydrodynamic size distributions of these nanovectors are similar except that a minimal amount of P-PEG-AF-CTX/DNA appears at a larger size. The cause for this phenomenon is unclear at present.

3.3. Receptor mediated internalization of nanovectors by cancer cells

The CTX peptide interacts specifically with the MMP-2-containing lipid raft complex, which is expressed only by cancer cells or upregulated on cancer cells [26,35]. Upon binding CTX, the lipid rafts, and the bound nanovectors, are endocytosed. Flow cytometry was used to determine the effectiveness of CTX as a targeting ligand for the nanovector system. Figure 5 shows that the CTX-modified nanovector (P-PEG-AF-CTX/ DNA) yielded higher uptake by C6 glioma cells than the non-functionalized nanovector (P-PEG-AF-SIAX/DNA) at doses of $0.1 \mu\text{g DNA mL}^{-1}$ or above, with the most dramatic uptake difference at a dose of $10 \mu\text{g DNA mL}^{-1}$ of DNA.

Additionally, uptake of both nanovector types by target cells (C6) was evaluated against the control cell line, NIH3T3, which not upregulated in MMP-2 expression. Here, C6 cells (MMP-2⁺) treated with the CTX-enabled nanovector showed significantly increased internalization levels when compared with C6 cells exposed to the non-functionalized vector (Figure 6). Conversely, the NIH3T3 cells showed little interaction with both nanovector types, indicating selective binding of the nanovectors presenting the CTX peptide. All cell lines were incubated with nanovectors at a dose of $10 \mu\text{g DNA mL}^{-1}$. At this DNA concentration (10

DNA $\mu\text{g mL}^{-1}$), there was an ~11 fold increase in uptake of CTX-modified nanovectors in C6 cells compared with control nanovectors.

3.4. Receptor mediated gene delivery

In addition to specific cell targeting, positive gene transfection by the CTX-enabled nanovector was also quantified by flow cytometry. A dose response study with C6 cells showed significantly higher transfection efficiencies of P-PEG-AF-CTX/DNA than P-PEG-AF-SIAX/DNA at 1 and 10 $\mu\text{g mL}^{-1}$ doses (Figure 7). At the highest DNA concentration tested, P-PEG-AF-CTX/DNA exhibited a gene transfection efficiency (55%) 5 fold more than P-PEG-AF-SIAX/DNA (11%).

3.5. Comparison of gene transfection efficiencies

A cross comparison of the transfection efficiencies of DNA (10 $\mu\text{g mL}^{-1}$) delivered to C6 and NIH3T3 cells (negative control cells) with the aid of PEI (P-AF/DNA), pegylated PEI (P-PEG-AF-SIAX/DNA), P-PEG-AF-CTX/DNA, the commercial transfection reagent, Lipofectamine, or as naked DNA was performed (Figure 8). Naked DNA introduced no transfection to either cell type, while the lipofectamine reagent showed preferential transfection in control NIH3T3 cells. P-AF/DNA induced the most efficient transfection rates in both target and control cell types, exhibiting no cell selectivity. PEG-modification of the PEI (P-PEG-AF-SIAX/DNA) decreased transfection efficiencies, while the addition of the CTX ligand to PEGylated-PEI (P-PEG-AF-CTX/DNA) significantly enhanced cell transfection for C6 cells only, demonstrating a high level of selective transfection of target cells by CTX-functionalized nanovectors. A 5 fold increase in transgene expression was observed in C6 cells (~55%) than in control NIH3T3 cells (< 10%) treated with P-PEG-AF-CTX/DNA. The non-targeting P-PEG-AF-SIAX/DNA nanovector showed similar transgene activity in either cell line.

This analysis also supports previous findings that showed unmodified PEI to be a highly effective gene carrier, but lacking cell selectivity [18,19]. PEGylation of PEI, in an attempt to reduce the toxicity of PEI, lowers gene transfection efficiencies of the vector significantly due to shielding of the polymer's cationic charge, limiting the nanovector's electrostatic affinity for cell surfaces [20,36]. Here, it was observed that with the addition of a targeting ligand (CTX), gene transfection of pegylated polycationic DNA carriers in target cells increased significantly while the gene transfection in control cells remained unchanged, an effect that can be attributed to enhanced interactivity of the nanovector with target cells due to the presence of the targeting ligand. Also significantly, the CTX-enabled nanovector demonstrated a 5× higher transfection efficiency compared with the nonspecific commercial reagent, lipofectamine. This indicates that the targeting nanovector could be applied *in vitro* for enhanced transfection of cells that are resilient to alternative transfection agents, and that cell specific nanovectors may provide an advantage in safely inducing transgene expression specifically in target cells.

Toxicities of the tested carrier agents towards both C6 and NIH3T3 cell lines were evaluated using an Alamar blue-based cell viability assay. Specifically, the cell lines were incubated with 10 $\mu\text{g mL}^{-1}$ of free DNA or a given nanovector complexed with DNA for 48 hours. Subsequently, cell viability was determined by dividing the numbers of treated cells remaining by the number of untreated cell populations (Figure 9). It was observed that unmodified PEI (i.e. P-AF/DNA) was highly toxic to both cell types, while PEGylation of PEI (i.e. P-PEG-AF-SIAX/DNA and P-PEG-AF-CTX/DNA) reduced the toxicity significantly in both tested cell types (4.2× higher viability than lipofectamine-treated C6 cells). Interestingly, lipofectamine showed selective toxicity towards C6 cells.

Visual confirmation of vector internalization, gene expression, and cell viability was established by confocal fluorescence and differential interference contrast (DIC) microscopy. Imaging of C6 (Figure 10a) and NIH3T3 (Figure 10b) cells further confirmed the results of the flow cytometry and Alamar blue-based cell viability studies. P-AF-/DNA-treated C6 cells showed high levels of vector internalization, gene expression, and cell toxicity as evidenced by altered morphology. Alternatively, cells treated with P-PEG-AF-SIAX/DNA showed minimal vector uptake with no detectable gene expression or changes in morphology. The targeting nanovector, P-PEG-AF-CTX/DNA, showed selective cell uptake and gene expression in C6 cells, with no evidence of cell toxicity.

To further verify the efficacy of the targeting nanovector to yield enhanced transgene expression in MMP-2⁺ cell lines, a second brain tumor cell line (DAOY) was tested for transgene expression levels against non-MMP-2 expressing NIH3T3 cells. For this analysis the three cell lines were either transfected with P-PEG-AF-CTX/DNA, or P-PEG-AF-SIAX/DNA nanovectors (5 µg DNA mL⁻¹), and analyzed for GFP transgene expression by measuring the mean FITC fluorescence intensity of each tested population (Figure 11). FITC intensity of untreated cells for each cell line is provided for reference. It was shown that the nanovectors targeting for MMP-2⁺ cells led to 6.1 and 3.9 fold increases in transgene expression for C6 and DAOY, respectively, compared to the non-targeting nanovectors. Conversely, the NIH3T3 cells displayed similarly low levels of transgene expression regardless of nanovector type.

4. Conclusions

We have demonstrated the functionality of a non-viral nanovector system that can be potentially used for the specific targeting and genetic therapy of an array of established cancer types. Normal DNA loading of the nanovector (P-PEG-AF-CTX/DNA) was demonstrated, and exhibited little change to the physical profile of an unmodified PEI-SIAX/DNA control. The specificity of the P-PEG-AF-CTX/DNA nanovector for MMP-2 positive tumor cells was demonstrated *in vitro*, yielding positive gene transfection over a range of exposure concentrations. Significantly, incorporation of CTX led to increased gene transfection efficiency that was otherwise substantially reduced by addition of PEG for reduced toxicity of PEI. There was little evidence of toxicity of the nanovector, providing initial evidence that the developed nanoparticle system could be a safe, efficient, and specific gene delivery. Interestingly, the nanovector demonstrated excellent transfection efficiency as compared with a commercially available reagent, indicating its potential use *in vitro* with cell lines typically resistant to transfection. Coupled with the large number of tumor types expressing MMP-2 complex, a CTX-enabled nanovector could provide a gene delivery platform demonstrating specific tumor targeting useful against many forms of cancer. This work could potentially lead to further use of this nanovector system *in vitro* and *in vivo* for bench-side and bedside applications as a selective, stealth, non-viral targeting agent.

Acknowledgments

This work is supported in part by NIH grants (R01CA9408, R01EB00643, and R01CA112350). O.V. would like to acknowledge support through an NCI/NSF IGERT fellowship, and J.W.G through an NIH training grant (T32GM0650). We would also like to acknowledge Dr. Jim Olson for providing the GFP plasmid DNA and Ni Mu for her laboratory assistance. Additionally, we would like to acknowledge the use of resources at the Department of Immunology's cell analysis facility and Keck Microscopy Imaging Facility at the University of Washington.

References

1. Stewart, BW.; Kleihues, P. World Cancer Report. Geneva: World Health Organization Press; 2003.

2. Ries, L.; Melbert, D.; Krapcho, M.; Stinchcomb, DG.; Howlader, N.; Horner, MJ., et al. NCI, editor. SEER Cancer Statistics Review, 1975–2005. Bethesda, MD: 2007. SEER web site http://seer.cancer.gov/csr/1975_2005/
3. Alton E, Ferrari S, Griesenbach U. Progress and prospects: Gene therapy clinical trials (part 2). *Gene Ther* 2007;14(22):1555–1563. [PubMed: 17984995]
4. Li SD, Huang L. Gene therapy progress and prospects: non-viral gene therapy by systemic delivery. *Gene Ther* 2006;13(18):1313–1319. [PubMed: 16953249]
5. McNeish IA, Bell SJ, Lemoine NR. Gene therapy progress and prospects: cancer gene therapy using tumour suppressor genes. *Gene Ther* 2004;11(6):497–503. [PubMed: 14762396]
6. Palmer DH, Young LS, Mautner V. Cancer gene-therapy: clinical trials. *Trends Biotechnol* 2006;24(2):76–82. [PubMed: 16380178]
7. Verma IM, Somia N. Gene therapy - promises, problems and prospects. *Nature* 1997;389(6648):239–242. [PubMed: 9305836]
8. Pack DW, Hoffman AS, Pun S, Stayton PS. Design and development of polymers for gene delivery. *Nat Rev Drug Discov* 2005;4(7):581–593. [PubMed: 16052241]
9. Edelstein ML, Abedi MR, Wixon J. Gene therapy clinical trials worldwide to 2007 - an update. *J Gene Med* 2007;9(10):833–842. [PubMed: 17721874]
10. Jang JH, Lim KI, Schaffer DV. Library selection and directed evolution approaches to engineering targeted viral vectors. *Biotechnol Bioeng* 2007;98(3):515–524. [PubMed: 17614321]
11. Kodama K, Katayama Y, Shoji Y, Nakashima H. The features and shortcomings for gene delivery of current non-viral carriers. *Curr Med Chem* 2006;13(18):2155–2161. [PubMed: 16918345]
12. Putnam D. Polymers for gene delivery across length scales. *Nature Materials* 2006;5(6):439–451.
13. Ohlfest JR, Freese AB, Largaespada DA. Nonviral vectors for cancer gene therapy: Prospects for integrating vectors and combination therapies. *Current Gene Therapy* 2005;5(6):629–641. [PubMed: 16457652]
14. Glover DJ, Lipps HJ, Jans DA. Towards safe, non-viral therapeutic gene expression in humans. *Nat Rev Genet* 2005;6(4):299–310. [PubMed: 15761468]
15. Ogris M, Wagner E. Targeting tumors with non-viral gene delivery systems. *Drug Discov Today* 2002;7(8):479–485. [PubMed: 11965397]
16. Wagner E. Targeting tumors with non-viral gene delivery systems. *J Gene Med* 2003;5(3):S7–S8.
17. Duncan R. Polymer conjugates as anticancer nanomedicines. *Nat Rev Cancer* 2006;6(9):688–701. [PubMed: 16900224]
18. Boussif O, Lezoualch F, Zanta MA, Mergny MD, Scherman D, Demeneix B, et al. A versatile vector for gene and oligonucleotide transfer into cells in culture and in-vivo - polyethylenimine. *Proc Natl Acad Sci U S A* 1995;92(16):7297–7301. [PubMed: 7638184]
19. Godbey WT, Wu KK, Mikos AG. Poly(ethylenimine) and its role in gene delivery. *J Control Release* 1999;60(2–3):149–160. [PubMed: 10425321]
20. Bennis JM, Mahato RI, Kim SW. Optimization of factors influencing the transfection efficiency of folate-PEG-folate-graft-polyethylenimine. *J Control Release* 2002;79(1–3):255–269. [PubMed: 11853936]
21. Kunath K, Merdan T, Hegener O, Haberlein H, Kissel T. Integrin targeting using RGD-PEI conjugates for in vitro gene transfer. *J Gene Med* 2003;5(7):588–599. [PubMed: 12825198]
22. Zanta MA, Boussif O, Adib A, Behr JP. In vitro gene delivery to hepatocytes with galactosylated polyethylenimine. *Bioconjugate Chem* 1997;8(6):839–844.
23. Zeng JM, Wang X, Wang S. Self-assembled ternary complexes of plasmid DNA, low molecular weight polyethylenimine and targeting peptide for nonviral gene delivery into neurons. *Biomaterials* 2007;28(7):1443–1451. [PubMed: 17156837]
24. Germershaus O, Merdan T, Bakowsky U, Behe M, Kissel T. Trastuzumab-polyethylenimine-polyethylene glycol conjugates for targeting Her2-expressing tumors. *Bioconjugate Chem* 2006;17(5):1190–1199.
25. Chiu SH, Ueno NT, Lee RJ. Tumor-targeted gene delivery via anti-HER2 antibody (trastuzumab, Herceptin (R)) conjugated polyethylenimine. *J Control Release* 2004;97(2):357–369. [PubMed: 15196762]

26. Veisheh M, Gabikian P, Bahrami SB, Veisheh O, Zhang M, Hackman RC, et al. Tumor paint: A Chlorotoxin : Cy5.5 bioconjugate for intraoperative visualization of cancer foci. *Cancer Research* 2007;67(14):6882–6888. [PubMed: 17638899]
27. Lyons SA, O'Neal J, Sontheimer H. Chlorotoxin, a scorpion-derived peptide, specifically binds to gliomas and tumors of neuroectodermal origin. *Glia* 2002;39(2):162–173. [PubMed: 12112367]
28. Sun C, Veisheh O, Gunn J, Fang C, Hansen S, Lee D, et al. In vivo MRI detection of gliomas by chlorotoxin-conjugated superparamagnetic nanoprobe. *Small* 2008;4(3):372–379. [PubMed: 18232053]
29. Veisheh O, Sun C, Gunn J, Kohler N, Gabikian P, Lee D, et al. Optical and MRI multifunctional nanoprobe for targeting gliomas. *Nano Lett* 2005;5(6):1003–1008. [PubMed: 15943433]
30. Shen S, Khazaeli M, Gillespie GY, Alvarez VL. Radiation dosimetry of I-131-chlorotoxin for targeted radiotherapy in glioma-bearing mice. *Journal of Neuro-Oncology* 2005;71(2):113–119. [PubMed: 15690125]
31. Eliyahu H, Barenholz Y, Domb AJ. Polymers for DNA delivery. *Molecules* 2005;10(1):34–64. [PubMed: 18007276]
32. Duncan, R.; Ringsdorf, H.; Satchi-Fainaro, R. *Polymer Therapeutics I: Polymers as Drugs, Conjugates and Gene Delivery Systems*. Berlin: Springer-Verlag Berlin; 2006. *Polymer therapeutics: Polymers as drugs, drug and protein conjugates and gene delivery systems: Past, present and future opportunities*. p. 1-8.
33. Peer D, Karp JM, Hong S, Farokhzad OC, Margalit R, Langer R. Nanocarriers as an emerging platform for cancer therapy. *Nat Nanotechnol* 2007;2(12):751–760. [PubMed: 18654426]
34. Hunter AC. Molecular hurdles in polyfectin design and mechanistic background to polycation induced cytotoxicity. *Adv Drug Deliv Rev* 2006;58(14):1523–1531. [PubMed: 17079050]
35. McFerrin MB, Sontheimer H. A role for ion channels in glioma cell invasion. *Neuron Glia Biol* 2006;2:39–49. [PubMed: 16520829]
36. Petersen H, Fechner PM, Fischer D, Kissel T. Synthesis, characterization, and biocompatibility of polyethylenimine-graft-poly(ethylene glycol) block copolymers. *Macromolecules* 2002;35(18):6867–6874.

Figure 1.

Nanovector preparation scheme. (a) PEGylation of PEI polymer and modification with Alexa Fluor 647 fluorophores (AF). (b) P-PEG-AF modification with SIAX, modification of chlorotoxin (CTX) with traute's reagent to produce free thiols on peptide and subsequent reaction of the thiol modified CTX peptide with reactive iodoacetate group of P-PEG-AF-SIX yielding P-PEG-AF-CTX. (c) The polymeric construct complexed with DNA to generate the targeting nanovector (P-PEG-AF-CTX/DNA).

Figure 2.

^1H NMR spectra showing characteristic peaks of the chlorotoxin peptide (CTX) at 2.60–3.10 and 3.65–3.80 ppm, SIAX-functionalized P-PEG polymer (P-PEG-SIAX) at 3.60–3.65 for the -O-CH₂-CH₂- repeating unit of PEG and at 2.50–3.10 for the -NH-CH₂-CH₂- repeating unit of PEI, and the CTX-polymer conjugate (P-PEG-CTX) which shows the characteristic peaks of both CTX and P-PEG.

Figure 3.

Agarose gel retardation assay of the polymeric nanovectors made with different polymer:DNA ratios (w:w). (a) Ethidium bromide-stained gel images of DNA-complexed P-PEG-AF-CTX and P-PEG-AF-SIAX nanovectors. (b) Ethidium bromide exclusion assay comparing percent complex formation as a function of polymer:DNA ratio tested for each nanovector.

Figure 4.

Zeta potential and hydrodynamic size analysis of nanovectors. (a) Zeta potentials of nanovectors prepared with varying polymer:DNA ratios (w:w). CTX-modified and unmodified nanovectors shared similar zeta potentials. (b) Hydrodynamic size, by intensity, of nanovectors formed at a polymer:DNA ratio of 5:1 (w:w).

Figure 5. Cell fluorescence intensity analysis by flow cytometry. C6 cells were exposed to either CTX-modified or unmodified nanovectors at varying DNA concentrations.

Figure 6.

Flow cytometry based analysis for relative uptake of nanovectors for targeted (C6) and non-targeted (NIH3T3) cells. C6 and NIH3T3 cells were exposed to either the targeting (P-PEG-AF-CTX/DNA) or non-targeting (P-PEG-AF-SIAX/DNA) nanovector at a DNA concentration of $10 \mu\text{g mL}^{-1}$.

Figure 7. Analysis of transgene expression of GFP by C6 cells. Transfection efficiency is measured by flow cytometry after treatment with either P-PEG-AF-SIAX/DNA or P-PEG-AF-CTX/DNA at varying DNA concentrations.

Figure 8.

Transfection efficiencies of C6 and NIH3T3 cells by DNA complexed with different polymeric vectors, as determined by flow cytometry. The commercially available Lipofectamine was tested as a reference transfection reagent.

Figure 9. Viability analyses of C6 and NIH3T3 cells after exposure to varying transfection vectors. An Alamar blue-based assay was used to determine cell viability.

Figure 10.

Confocal fluorescence and differential interference contrast (DIC) images of (a) C6 and (b) NIH3T3 cells treated with $10 \mu\text{g DNA mL}^{-1}$ without a delivery vector (DNA) or with vectors complexed with either PEI (P-AF/DNA), PEGylated PEI (P-PEG-AF-SIAX/DNA), or PEGylated and CTX-enabled PEI (P-PEG-AF-CTX/DNA). Cellular membranes are shown in green, nuclei in blue, polymeric vectors in red, and GFP expression in turquoise. Scale bars correspond to $40 \mu\text{m}$.

Figure 11.

Mean levels of transgene expression (mean FITC intensity) in C6, DAOY, and NIH3T3 cells treated with CTX modified (P-PEI-AF-CTX/DNA) or unmodified (P-PEG-AF-SIAX/DNA) nanovectors ($5 \mu\text{g DNA ml}^{-1}$). As reference the baseline fluorescence intensity of each cell type (Untreated) is also shown.