# Synthesis and characterization of mannosylated pegylated polyethylenimine as a carrier for siRNA

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## ARTICLE INFO

## ABSTRACT

Regulation of gene expression using small interfering RNA (siRNA) is a promising strategy for research and treatment of numerous diseases. In this study, we develop and characterize a delivery system for siRNA composed of polyethylenimine (PEI), polyethylene glycol (PEG), and mannose (Man). Cationic PEI complexes and compacts siRNA, PEG forms a hydrophilic layer outside of the polyplex for steric stabilization, and mannose serves as a cell binding ligand for macrophages. The PEI-PEG-mannose delivery system was constructed in two different ways. In the first approach, mannose and PEG chains are directly conjugated to the PEI backbone. In the second approach, mannose is conjugated to one end of the PEG chain and the other end of the PEG chain is conjugated to the PEI backbone. The PEI-PEG-mannose delivery systems were synthesized with 3.45–13.3 PEG chains and 4.7–3.0 mannose molecules per PEI. The PEI-PEG-Man-siRNA polyplexes displayed a coarse surface in Scanning Electron Microscopy (SEM) images. Polyplex sizes were found to range from 169 to 357 nm. Gel retardation assays showed that the PEI-PEG-mannose polymers are able to efficiently complex with siRNA at low N/P ratios. Confocal microscope images showed that the PEI-PEG-Man-siRNA polyplexes could enter cells and localized in the lysosomes at 2 h post-incubation. Pegylation of the PEI reduced toxicity without any adverse reduction in knockdown efficiency relative to PEI alone. Mannosylation of the PEI-PEG could be carried out without any significant reduction in knockdown efficiency relative to PEI alone. Conjugating mannose to PEI via the PEG spacer generated superior toxicity and gene knockdown activity relative to conjugating mannose and PEG directly onto the PEI backbone.

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## 1. Introduction

Gene therapy using RNAi (RNA interference) has significant potential for treatment of a variety of diseases as well as a tool for biomedical research (Behlke, 2006). RNAi is a natural cellular mechanism by which a specific mRNA is targeted for degradation through inhibition of the synthesis of the encoded protein (Akhtar and Benter, 2007). Selectively silencing post-transcriptional mRNA by RNAi represents a promising new approach for the inhibition of gene expression *in vitro* and *in vivo* (Fire et al., 1998). SiRNA, 21–27 base pair double-stranded RNA, plays a vital role in initiating the RNAi mechanism (Caplen et al., 2001). However, siRNA is suscepti-ble to enzymatic degradation and has a relatively large molecular weight with a net negative charge that lowers its capacity to pen-etrate the cell membrane (Abe et al., 2009).

To overcome this challenge, both viral and non-viral vectors have been developed to make siRNA delivery more efficient (Aigner, 2007; Kim and Kim, 2009). Viral vectors such as adenoviruses and retroviruses are very effective delivery systems (Zhang and Godbey, 2006). However, concerns about their immunogenic nature and inadvertent gene expression changes following random integra-tion into the host genome still exist (Abbas et al., 2008; Martin and Caplen, 2007). Polyethylenimine (PEI) is a synthetic cationic polymer that has been widely used to deliver oligonucleotides, siRNA and plasmid DNA *in vitro* and *in vivo* (Abbas et al., 2008; Aigner et al., 2002; Boussif et al., 1995; Hobel and Aigner, 2010; Intra and Salem, 2008; Nimesh and Chandra, 2009; Pearce et al., 2008; Petersen et al., 2002; Zhang et al., 2008; Zintchenko et al., 2008). PEI electrostatically condenses nucleic acids and forms sta-ble nanoparticles or polyplexes (Intra and Salem, 2008). Branched PEI with higher molecular weights (<25 kDa) is more efficient

for *in vitro* transfection because it condenses nucleic acids more effectively than linear PEI (Godbey et al., 1999). Every third atom of PEI is a protonatable nitrogen atom, which enables the "proton sponge" effect over a wide range of pH. PEI will buffer the acidification within the endosome after endocytosis resulting in endosomal chloride accumulation. This results in osmotic swelling and rupture allowing for endosomal escape of the PEI/siRNA polyplexes (Boussif et al., 1995). Although cationic PEI has promising potential as a gene delivery vehicle, it is also associated with high toxicity relative to other non-viral vectors. (Fischer et al., 2003; Intra and Salem, 2008; Nimesh and Chandra, 2009; Swami et al., 2007). PEI can be modified to reduce toxicity and its free amine groups can be used to conjugate cell binding or targeting ligands (Beyerle et al., 2010; Biswal et al., 2010; Bonsted et al., 2008; Breunig et al., 2008; Diebold et al., 1999a; Kang et al., 2010; Kim et al., 2008; Merkel et al., 2009b; Moore et al., 2008; Ogris et al., 1999; Patnaik et al., 2010; Petersen et al., 2002; Zintchenko et al., 2008).
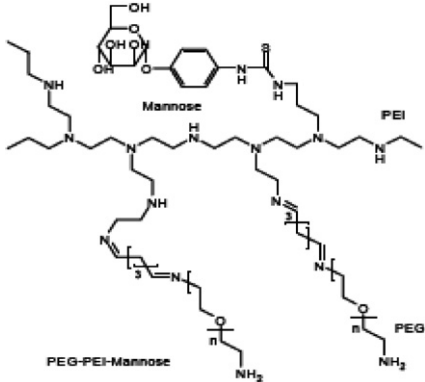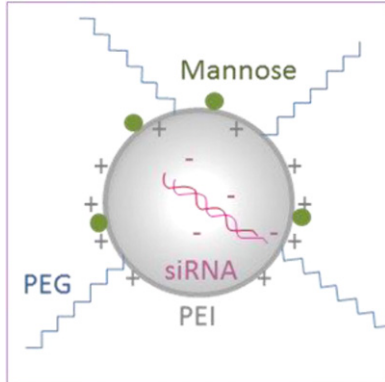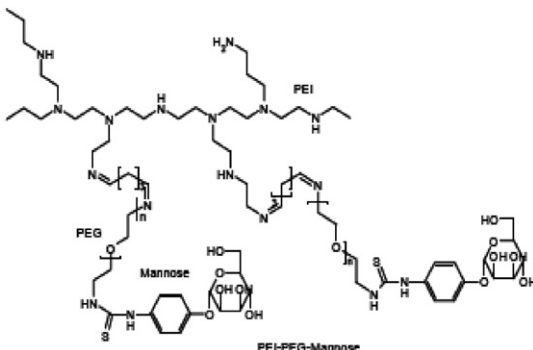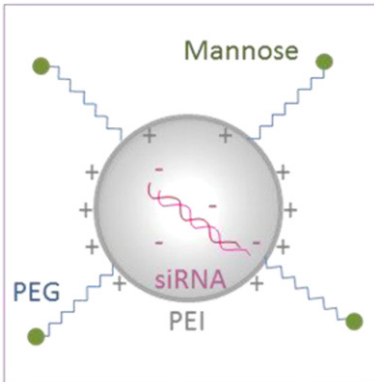
Various modifications have been applied to PEI to reduce toxicity and increase target specificity (Kawakami and Hashida, 2007). They include coupling to other macromolecules like polyethylene glycol (PEG), either alone (Katayose and Kataoka, 1997; Mao et al., 2006; Merkel et al., 2009a; Petersen et al., 2002; Sagara and Kim, 2002) or in combination with ligands for tissue specific targeting (Biswal et al., 2010; Hobel and Aigner, 2010; Kang et al., 2010; Kunath et al., 2003b; Ogris et al., 1999; Schiffelers et al., 2004; Sun et al., 2008). Conjugation of PEG to the polyplexes or nanoparticles creates a hydrophilic layer around the nanoparticles that provides steric stabilization thereby reducing aggregation of the particles. The PEG layer increases the circulatory half-life of the nanoparticles or polyplexes and reduces toxicity (Owens and Peppas, 2006; Peracchia et al., 1999). A surface PEG chain larger than 2 kDa is more efficient at steric stabilization and increasing the circulation half-life. This minimum MW is due to the inflexibility of shorter PEG chains (Gref et al., 1994; Leroux et al., 1995; Peracchia et al., 1997). PEG can be cleared by renal, fecal or hepato-biliary routes depending on their molecular weight or modification (Yamaoka et al., 1994, 1995). The majority of PEG 6 kDa is excreted in the urine 12 h after intravenous administration in humans. It is approved by the FDA for internal use due to its nontoxic and non-immunogenic characteristics (Fishburn, 2008; Pang and Fiume, 2001; Webster et al., 2007).

Specific ligands can bind to target cell surface receptors, which in turn can trigger receptor-mediated endocytosis (Park et al., 2008). Mannose is often used as a ligand that binds mannose receptors on cells to induce receptor-mediated endocytosis, which increases the delivery efficacy (Diebold et al., 1999a). Its receptor is expressed on the surface of antigen presenting cells (APCs; dendritic cell, macrophages) and liver endothelial cells (Diebold et al., 2002), etc. A number of studies have shown that mannosylated nanoparticles enter Raw264.7 cells via receptor-mediated endocytosis (Jiang et al., 2009; Kim et al., 2006; Park et al., 2008; Zhou et al., 2007).

In this study, we designed and synthesized a siRNA delivery system that incorporates PEI, PEG, and mannose. The mannosylated pegylated PEI delivery systems were designed in two different constructs. In our first approach, mannose and PEG chains were directly conjugated to the PEI backbone. In our second approach, mannose was conjugated to PEI via a PEG chain spacer (Table 1). The PEI-PEG-mannose constructs were then characterized for capacity to complex with siRNA, surface morphology and shape, gene and mRNA knockdown efficiency and toxicity.

**Table 1**

Schematical diagram showing chemical structures of two different mannosylated pegylated polyethylenimine constructs are depicted on the left column and schematical diagrams of the constructs after complexation with siRNA are shown on the right column. Mannose and PEG are directly conjugated to the PEI backbone in construct #1, whilst mannose is conjugated to the PEI via a PEG spacer in construct #2.

| Structure | Schematic diagram |
| --- | --- |
|  Construct #1 Mannose-PEI-PEG |  |
|  Construct #2 PEI-PEG-mannose |  |

## 2. Materials and methods

### 2.1. Materials

Branched PEI 25 kDa and α-D-mannopyranosylphenyl isothiocyanate (MPITC) were purchased from Sigma–Aldrich (St. Louis, MO). PEG 2 kDa with one amine terminus was purchased from Creative PEGWorks (Winston Salem, NC). DMSO, glacial acetic acid and EDTA were purchased from Fisher (Pittsburgh, PA). Bio-Gel P-2 Gel is from Bio-Rad (Hercules, CA). Maleic anhydride, toluene, glutaraldehyde 50%, methanol and sulfuric acid were purchased from Sigma–Aldrich (St. Louis, MO). All the siRNAs (DS scrambled negative control, RLuc-S1 DS positive control, Cy-3[TM], NC1, and HPRT) and pDNA (psiCHECK[TM]-2, Promega, Madison, WI) were kindly provided by Integrated DNA Technologies (Coralville, IA).

### 2.2. Synthesis

#### 2.2.1. PEI-PEG

500 mg PEG was dissolved in 10 ml toluene and heated up to 100 °C. Maleic anhydride solution (33.1 mg in 40 ml toluene) was added into the PEG solution in small increments. After the introduction of the maleic anhydride, the temperature was increased to 110 °C and then the mixture was refluxed for 12 h. Excess toluene was removed using rotary evaporation and the recovered product was dissolved in 2.5 ml deionized water which was then purified using a P-2 column. The elution was lyophilized and redissolved in 10 ml methanol. Then, 1 ml maleic anhydride-PEG was slowly added into 50% w/v glutaraldehyde solution containing 50 μl glutaraldehyde and 300 μl methanol. The reaction was stopped by 2 h and the mixture loaded into a P-2 column. The elution was immediately added to a 2 ml PEI solution (~150 mg PEI) for overnight reaction. PEI-PEG was recovered after dialysis (MWCO 10,000, Pierce Biotechnology Inc., Rockford, IL) for 3 days and lyophilized (Labconco FreeZone4.5, Kansas City, MO).

#### 2.2.2. Mannose-PEI-PEG

After the PEI was PEGylated, as described above, 80 μl of MPITC solution (dissolved at a concentration of 0.125 mg/μl in DMSO) was added to PEI-PEG for overnight mannosylation. The conjugate was recovered after dialysis and lyophilization.

#### 2.2.3. PEI-PEG-mannose

80 μl MPITC solution was added to 2 ml PEG solution (dissolved using NaHCO$_3$ buffer, 8.516 mg/ml, pH 8.4) containing 50 mg PEG for overnight reaction, followed by the addition of 50 μl of 50% w/v glutaraldehyde solution and 300 μl methanol. The reaction was stopped in 2 h using a P-2 column separation. The collection was added to PEI solution for overnight reaction. PEI-PEG-mannose was recovered after dialysis and lyophilization.

### 2.3. $^1$H NMR

Confirmation of the presence of mannose, PEI and PEG in the conjugate was achieved by dissolving the products in 1.0% w/v DCl/D$_2$O (Cambridge Isotope Lab, Andover, MA) at ~40 mg/ml and characterizing using proton nuclear magnetic resonance ($^1$H NMR, Bruker Avance 300).

### 2.4. Resorcinol assay

Resorcinol (Riedel-de Haen, Seelze, Germany) was dissolved at 6 mg/ml. D-(+)-mannose predried at 110 °C overnight was used as
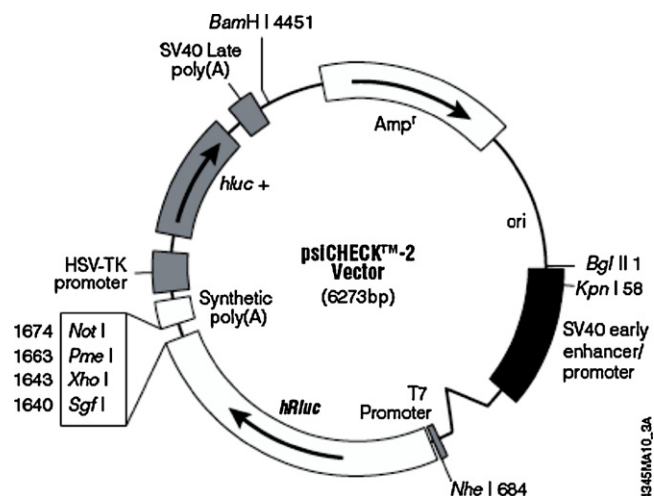


**Fig. 1.** PsiCHECK[TM]-2 vector map. It is a specifically designed 6.3 kb pDNA for RNAi experiments. It has two different reporter genes, firefly (hluc+) luciferase with HSV-TK promoter and *Renilla* (hRluc) luciferase with SV40 promoter, and a synthetic poly(A) sequence in between to reduce the potential for recombination events. This firefly reporter sequence has been specifically designed as an intraplasmid transfection normalization reporter, thus the *Renilla* luciferase signal can be normalized to the firefly luciferase signal.

a standard. Mannose was dissolved in 1% w/v HAc at a concentration ranging from 9.0854 to 908.54 μg/ml. PEI-PEG-mannose and mannose-PEI-PEG samples were dissolved using 1% w/v HAc at appropriate concentrations. Each test mixture, consisted of 20 μl mannose/sample solution, 20 μl resorcinol, 50 μl pristane (Acros, Fair Lawn, NJ), and 100 μl 75% w/v sulfuric acid, was subjected to vortex for 30 s, heating at 93 °C for 30 min and cooling down to room temperature for 30 min in a 96-well plate. The absorbance was recorded at 480 nm.

### 2.5. Cell culture

Raw264.7 cells (ATCC, Manassas, VA), a murine macrophage cell line that is known to express mannose receptors and that is typically hard to transfect, was selected for *in vitro* experiments because macrophages are a potential target for this delivery system. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Hyclone), penicillin-streptomycin (100 units penicillin; 100 ug streptomycin/ml, Gibco). The cells were maintained at 37 °C in a humidified, 5% carbon dioxide atmosphere.

### 2.6. Amplification and purification of pDNA

The psiCHECK[TM]-2 (Promega, Madison, WI) is a 6.3 kb pDNA designed to monitor a quantitative measurement of RNAi (Fig. 1). It has genes encoding for firefly (hluc+) luciferase and *Renilla* (hRluc) luciferase with each luciferase having a HSV-TK or SV40 promoter respectively. The firefly reporter gene has been constructed to serve as an intraplasmid standard so that the *Renilla* luciferase signal can be normalized to the firefly luciferase signal. The pDNA was transformed in *E. coli* DH5α (Invitrogen) and amplified in LB Broth media at 37 °C overnight on a plate shaker set at 250 rpm. The pDNA was extracted with Wizard® Plus Maxipreps DNA Purification System (Promega) followed by removal of bacterial endotoxin contamination with Endotoxin Removal Kit (MiraCLEAN®) according to the manufacturers' protocols. Purified pDNA was dissolved in Tris–EDTA buffer and its purity and concentration were determined by UV absorbance at 260 and 280 nm.
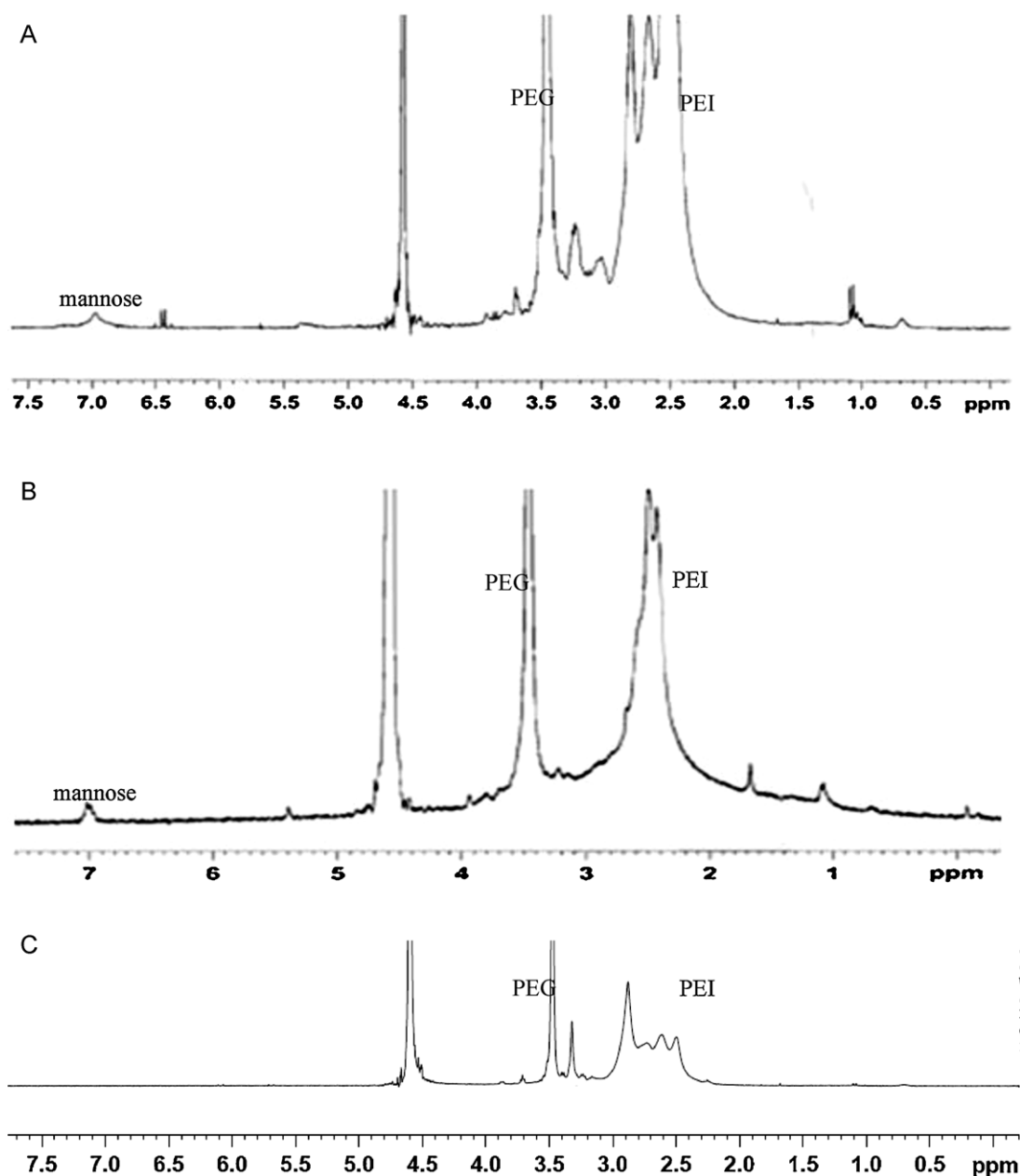
**Fig. 2.** $^1$H NMR spectras of mannose-PEI-PEG (A), PEI-PEG-mannose (B) and PEG-PEI (C). Mannose-PEI-PEG showed peaks of PEG (PEG-H) at 2.6 ppm, PEI (PEI-H) at 3.5 ppm and mannose (phenyl-H) at 7 ppm. PEI-PEG-mannose also showed each component at the same peaks.

## 2.7. Preparation of PEI-siRNA polyplexes

PEI/siRNA polyplexes were formed at desired N/P (nitrogen in cationic polymer per phosphate in nucleic acid) ratios with pre-determined amounts of siRNA and polymer solutions. A mass per phosphate of 325 Da for RNA and mass per charge of 43 for PEI were used to calculate N/P ratio. The polymer solution was dropped into siRNA solution, and then the mixture was vortexed for 20 s followed by 30 min incubation at room temperature.

## 2.8. Size distribution and surface morphology analysis

SiRNA/polymer polyplexes were formed as described above. Then the polyplex solutions were sprayed using All-Glass Nebu-lizer (PELCO, Redding, CA) onto silicon wafers. The silicon wafers were stained with 4% osmium tetroxide vapor under the hood

overnight and mounted on aluminum stubs using liquid colloid silver adhesives followed by overnight drying at room temper-ature. The specimens were observed using scanning electron microscopy (SEM, Hitachi S-4800). Polyplex size and zeta poten-tial measurements were conducted using the Zetasizer Nano ZS (Malvern, Southborough, MA). Briefly, the polyplexes were pre-pared as described above in nuclease-free deionized water at an N/P ratio of 7. The size measurements were performed at 25 °C at a 173° scattering angle. The mean hydrodynamic diameter was determined by cumulative analysis.

## 2.9. Gel retardation assay

SiRNA/PEI polyplexes were loaded in a 2% agarose gel with 0.5 μg/ml Ethidium Bromide and run at 60 V in TAE buffer for 45 min. The gels were visualized with a UV transilluminator

(Spectroline, Westbury, NY) and photographed by Panasonic DMC-FX30 digital camera.

### 2.10. Intracellular trafficking

RAW264.7 cells were plated in 0.01% (w/v) poly L-lysine coated 8-well chamber slides (Lab-Tak) at $1.2 \times 10^4$/well concentration and incubated overnight. For lysosomal staining, the cells were incubated with 75 nM Lysotracker® Green DND-26 (Molecular Probes®) containing opti-MEM media (Gibco) for 30 min then transfected with polymer/Cy3-labeled siRNA polyplexes in fresh opti-MEM. At pre-determined time points, the cells were washed with PBS and fixed with 4% paraformaldehyde and mounted using DAPI-containing Vectashield mounting medium for nuclear staining (VECTOR Laboratories, UK). Then the slides were covered with coverslips followed by 4 °C storage in the dark before visualization under the multiphoton/confocal microscope (Bio-Rad Radience 2100MP and Zeiss Confocal 710).

### 2.11. Assays to evaluate knockdown of gene and mRNA expression

#### 2.11.1. Dual Luciferase Assay

RAW264.7 cells were seeded on a T25 flask ($6.8 \times 10^6$ cells) on day 0 and incubated overnight. PsiCHECK™-2 was transfected using PEI in opti-MEM media on day 1 for 4 h, then trypsinized to be replated onto 24-well plates ($3 \times 10^5$ cells/well). Then RLuc-S1 DS positive control siRNA, which encodes a complementary sequence for *Renilla* luciferase gene in psiCHECK™-2 vector, was transfected using each different polymer in opti-MEM media on day 2 and the cells were incubated for 4 h followed by overnight incubation in complete media. On day 3, luciferase gene expression was analyzed using the Dual Luciferase Reporter System (Promega, Madison, WI) according to the product manual and using a Lumat LB 9507 (Berthold Technologies, Bad Wildbad, Germany). Plasmid DNA/PEI complexes or siRNA/polymer complexes were prepared as described above at precalculated N/P ratios. DS scrambled neg. siRNA, non-targeting sequences in the human, mouse, or rat transcriptome, served as a negative control. The *Renilla* luciferase gene expression was normalized to firefly luciferase as internal control expression and expressed as relative gene expression (100% to DS scrambled neg. transfected with PEI). The data was reported as mean ± standard deviation for triplicate samples using Microsoft Excel and Prism® software (GraphPad Software, Inc). Every experiment was repeated at least twice.

#### 2.11.2. Real-time PCR

Cells were seeded onto 48-well plate ($8 \times 10^4$ cells/well) for endogenous gene knockdown on day 0. Then NC1 (negative control) and HPRT siRNAs were transfected using various polymers in opti-MEM media on day 1 and incubated for 24 h. Total RNA was extracted (Promega, SV96 Total RNA Isolation System) on day 2 followed by cDNA synthesis and real-time PCR. 150 ng of total RNA was used for reverse transcription using Superscript II reverse transcriptase (Invitrogen, San Diego, CA). cDNA equivalent to 40 ng total RNA was analyzed by real-time PCR in triplicate using Immolase polymerase (Bioline, Randolph, MA) on AB7900HT (Applied Biosystems). The data was reported as mean ± standard deviation from triplicate RT-PCR reactions of each triplicate sample using Microsoft Excel and Prism® software (GraphPad Software, Inc). Every experiment was repeated at least twice.

### 2.12. Cytotoxicity assay

Cytotoxicity was determined using the MTS assay (CellTiter 96® AQ$_{ueous}$ One Solution Cell Proliferation Assay, Promega, Madison,

**Table 2**
PEI/PEG molar ratios and mannose content in PEI-PEG-mannose and mannose-PEI-PEG constructs. The PEI to PEG molar ratio was calculated based on the area of $^1$H NMR peaks. Resorcinol measurements were used to determine the content of mannose (μmol mannose per 1 mg polymer) in each construct.

| Formulation | PEI/PEG molar ratio | Mannose content (μmol/mg) |
|---|---|---|
| Mannose-PEI-PEG | 0.962 | 0.18679 |
| PEI-PEG-Mannose | 1.23 | 0.12122 |
| PEI-PEG | 3.704 | N/A |

WI) using the protocol provided by the manufacturer. In brief, the Raw264.7 cells were seeded in 96-well plates ($5 \times 10^5$ cells/well) and incubated overnight. The polymer containing DMEM media were prepared at different concentrations and added to cells for incubation. 20 μl MTS solution per 100 μl media was added to each well for further 1–3 h incubation at 37 °C in a humidified, 5% CO$_2$ atmosphere. The plate was then measured at 490 nm absorbance (SpectraMax Plus$^{384}$, Molecular Device) and the relative cell viability calculated using standard curves. Experiments were repeated in triplicate.

### 2.13. Statistical analysis

Group data are reported as mean ± SD. Differences between groups were analyzed by one-way ANOVA analysis with Tukey's post-test. Levels of significance were accepted at $P < 0.05$. Statistical analyses were performed using Prism software (Graphpad Software, Inc., San Diego, CA).

## 3. Results

### 3.1. PEI, PEG and mannose are detected in final product $^1$H NMR spectra

PEI-PEG-mannose and mannose-PEI-PEG showed peaks at δ 7.0 (MPITC phenyl-H), δ 3.5 (PEG-H), and δ 2.6 (PEI-H) indicating successful incorporation of PEI, PEG and mannose in the final product (Fig. 2). The signal at 7 ppm was relatively weak but indicated the existence of mannose (MPITC) which was further verified and quantified using the resorcinol assay. When comparing these spectra to the control spectra of PEI-PEG (Fig. 2C), the 3.5 ppm peak matched to PEG and the 2.6 ppm corresponded to the PEI hydrocarbon chain. The conjugation/substitution ratio was calculated based on the peak areas ratio between 3.5 ppm PEG and 2.6 ppm PEI (Table 2). The molar ratios of PEI to PEG were 3.704 in PEI-PEG, 0.962 in mannose-PEI-PEG and 1.23 in PEI-PEG-mannose. There was no significant difference between the $^1$H NMR spectra of mannose-PEG-PEI and PEG-PEI-mannose.

### 3.2. Mannose quantity is determined in final constructs using the resorcinol assay

The resorcinol assay is a commonly used method to determine the content of monosaccharides in polymeric structures (Diebold et al., 1999a). The mannose content in our products was determined using this technique (Table 2). PEI-PEG-mannose had 0.12 μmol/mg of mannose and mannose-PEI-PEG had 0.19 μmol/mg mannose.

### 3.3. Pegylated polyplexes are spherical and coarse in appearance

SEM images were used to observe surface morphology and shape of the polymer/siRNA polyplexes. The polymers/siRNA polyplexes without pegylation were spherically or semi-spherically shaped with porous but otherwise smooth surfaces (Fig. 3A). In
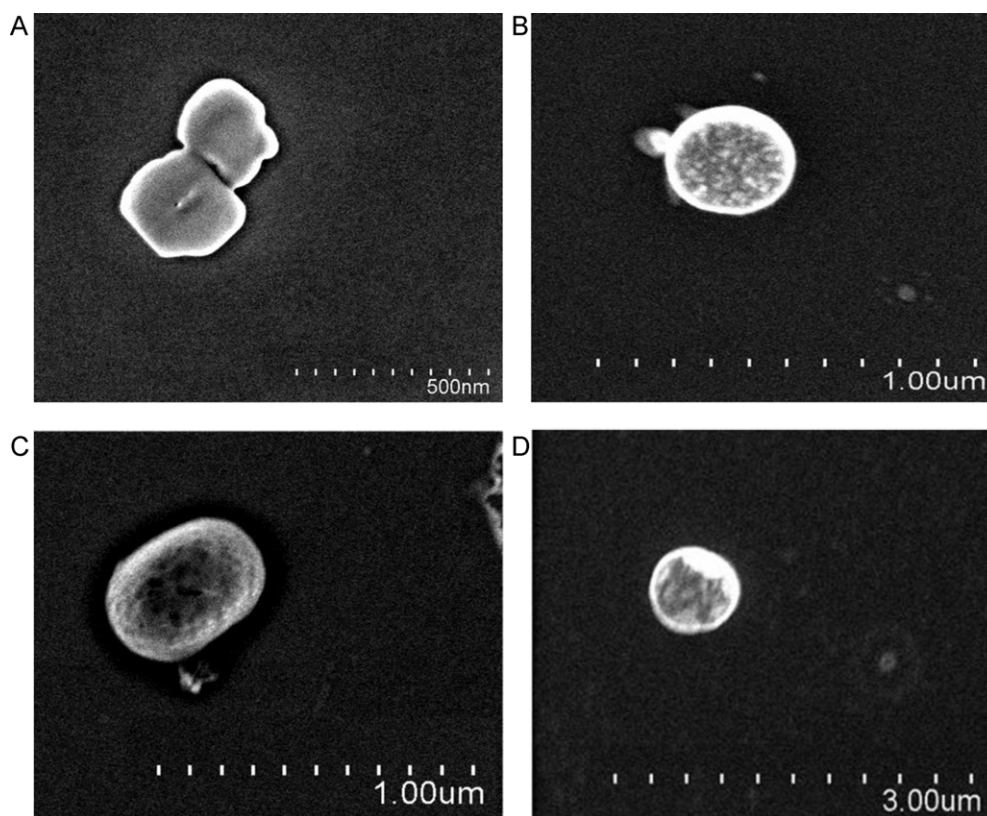
**Fig. 3.** SEM images of mannosylated pegylated PEI/siRNA polyplexes. (A) PEI/siRNA polyplexes, (B) PEI-PEG/siRNA polyplexes, (C) Mannose-PEI-PEG/siRNA polyplexes, (D) PEI-PEG-mannose/siRNA polyplexes. All the polyplexes were prepared with 1 μM siRNA at N/P ratio 5.

contrast, the PEGylated polyplexes had more coarse features on the surface (Fig. 3B–D). The particle sizes of various PEI polymers/siRNA polyplexes are shown in Table 3. PEI/siRNA polyplexes and PEI-PEG/siRNA polyplexes had 214.57 and 201.80 nm average diameters respectively. Man-PEI-PEG formed polyplexes with siRNA with an average size of 169.10 nm. PEI-PEG-mannose/siRNA polyplexes had an average size of 357.33 nm. The pegylated PEI/siRNA polyplexes and PEI-PEG-Man/siRNA polyplexes displayed zeta potentials (24.37 mV and 21.63 mV respectively) that were similar to unmodified PEI (21.94 mV). The zeta potential of polyplexes prepared using Man-PEI-PEG had a lower value of 10.46 mV.

### 3.4. PEI-PEG-mannose and mannose-PEI-PEG efficiently complex siRNA and retard siRNA migration in gel electrophoresis assays

For the mannosylated and pegylated PEI products we generated to be capable of delivering siRNA, they must be able to complex and condense the siRNA as efficiently as PEI alone. Cationic polymers form complexes with anionic siRNAs and compact them by ionic/electrostatic interactions. This complexation capacity was analyzed using a gel electrophoresis assay. The

**Table 3**
Hydrodynamic size and zeta potential of the polymer/siRNA polyplexes. All the polyplexes were prepared with 1 μM siRNA at N/P ratio 7. All data were represented as mean ± SD ($n = 3$).

| | Zeta potential (mV) | Size (nm) |
|---|---|---|
| PEI | 21.94 ± 0.50 | 214.57 ± 29.00 |
| PEI-PEG | 24.37 ± 1.63 | 201.80 ± 4.95 |
| Man-PEI-PEG | 10.46 ± 0.91 | 169.10 ± 9.54 |
| PEI-PEG-Man | 21.63 ± 6.31 | 357.33 ± 92.90 |

migration of siRNA on the agarose gel was retarded with the use of all the polymer constructs. All the constructs including PEI (Fig. 4A) and PEI-PEG (Fig. 4B) showed excellent complexation with siRNA even at low N/P ratios of 1 that prevented siRNA migration in the gel retardation assays. Both mannose-PEI-PEG and PEI-PEG-mannose showed complete exclusion of EtBr from N/P ratios 1–15 indicating a broad and strong complexation capacity with siRNA (Fig. 4C and D). Based on this result, the following studies were carried out using polyplexes prepared within this range of N/P ratios.

### 3.5. Mannosylated pegylated PEI/siRNA polyplexes are efficiently endocytosed by RAW264.7 cells

To track the cellular uptake and distribution of mannosylated pegylated PEI/siRNA complexes (N:P ratio of 7), siRNA and endosomes were labeled with red and green fluorophores, respectively. At 2 h post-transfection, most of the polymers/Cy3-siRNA complexes (shown in red) were successfully internalized in the RAW264.7 cells. PEI/siRNA and PEI-PEG/siRNA polyplexes were taken up by cells and localized in vesicular structures as seen by yellow fluorescence due to the green staining of lysosome and red siRNA signal in close proximity (Fig. 5A and B). Mannose-PEI-PEG/siRNA complexes were also found in the endosomes (Fig. 5C). PEI-PEG-mannose/siRNA polyplexes showed the most wide-spread endocytosis with relatively even distribution in a large group of cells (Fig. 5D and E). Several images including Fig. 5C and E showed polyplexes in the cytoplasm at the perinuclear region as observed by the separation of the green and red signal (nuclei stained with blue) suggesting release of the polyplexes from the endosomes.
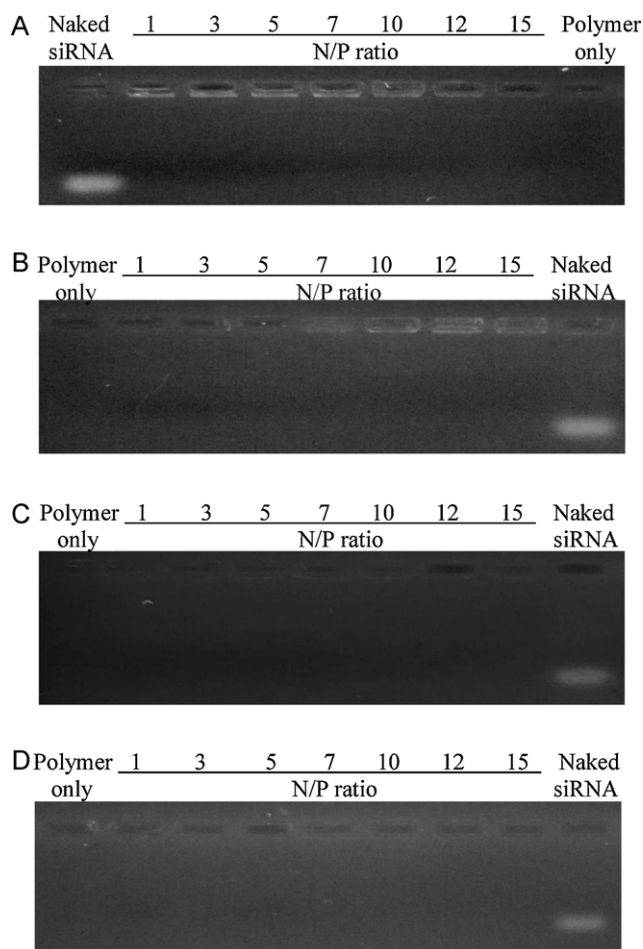
**Fig. 4.** Gel retardation assay results of various polyplex formulations. (A) PEI/siRNA polyplexes, (B) PEI-PEG/siRNA polyplexes, (C) Mannose-PEI-PEG/siRNA polyplexes, (D) PEI-PEG-mannose/siRNA polyplexes. All the polyplexes were prepared with 1 μM siRNA from N/P ratio 1–15.

## 3.6. Pegylation and mannosylation of PEI does not reduce knockdown efficiency relative to PEI alone

### 3.6.1. Dual luciferase assay

*In vitro* transfection was performed using the dual-luciferase reporter system to evaluate the siRNA delivery potential of mannosylated pegylated PEI delivery systems. Relative gene expression levels were analyzed after transfecting siRNA only targeting Renilla luciferase mRNA and leaving firefly gene expression as an internal control. All the polymers successfully delivered siRNA and reduced target gene expression (Fig. 6A). PEI/siRNA polyplexes inhibited *Renilla* gene expression to 33.6% at an N/P ratio of 3 and 18.8% at an N/P ratio 10. At an N/P ratio of 3, PEI-PEG showed the strongest knockdown efficiency of 88.7%, which is significantly higher than the other transfection groups including a commercial transfection reagent, siLentFect (51.8%). However, increasing the N/P ratio of PEI-PEG to 10 reduced the knockdown efficiency to 58.6%. There was no statistically significant difference in gene silencing efficiency of PEI-PEG between the N/P ratio of 3 and 10 ($P > 0.05$). Mannose-PEI-PEG/siRNA polyplexes generated 34.2% and 42.0% gene expression when used at N/P ratios 3 and 10, respectively. PEI-PEG-mannose decreased gene expression down to 19.9% at N/P ratio 3 and 22.9% at N/P ratio 10. No significant difference was found between the N/P ratios of 3 and 10 in both mannosylated pegylated PEI's ($P > 0.05$). Furthermore, the modified polymers showed no significant difference in gene silencing efficiency in

comparison to unmodified PEI ($P > 0.05$). Gene silencing efficiency in cells treated with polyplexes prepared at N/P ratios of 5 and 7 was not significantly different to cells treated with polyplexes prepared at N/P ratios of 3 and 10 (data not shown).

### 3.6.2. Real-time PCR

Endogenous gene knockdown was carried out using HPRT (Hypoxanthine-Guanine Phosphoribosyl Transferase) siRNA transfection. HPRT is a ubiquitously expressed enzyme that is commonly used as a positive control for endogenous gene knockdown experiments. PEI/siRNA polyplexes resulted in a 68.31% gene expression at N/P ratio 10 and PEI-PEG/siRNA resulted in a 65.80% remaining gene expression (Fig. 6B). The tricomponent polymers, mannose-PEI-PEG and PEI-PEG-mannose, further enhanced gene delivery showing 62.15% and 61.19%, respectively. Whilst pegylation and mannosylation marginally improved knockdown at the mRNA level, none of these differences were significant.

## 3.7. Pegylation of PEI reduces toxicity relative to PEI alone

Raw264.7 cells were treated with various groups of polyplexes to evaluate toxicity. At a working concentration of 0.0078 mg/ml, PEI showed the highest toxicity resulting in 37.5% cell viability (Fig. 7). PEI-PEG resulted in higher cell viability (79.1%) when compared to PEI alone. Mannose-PEI-PEG (68.9%) and PEI-PEG-mannose (53.9%) also resulted in higher cell viabilities than PEI alone. The modified PEI polymers demonstrated lower cytotoxicity relative to unmodified PEI. Cell viabilities were decreased by increasing concentrations of polymers for all the groups tested. However, the reduction in cytotoxicity when using modified polymers in comparison to unmodified PEI increased as the concentration of the polymers incubated with the cells increased (data not shown).

## 4. Discussion

In this study, we synthesized, characterized and tested a mannosylated pegylated PEI for potential delivery of siRNA. Pegylation has been previously shown to be of significant value in increasing the circulation time of nanoparticles and polyplexes (Brus et al., 2004; Owens and Peppas, 2006; Yamaoka et al., 1994). Mannose has previously been demonstrated to significantly increase binding of particles to cells that express the mannose receptor (Diebold et al., 1999a,b, 2002; Hashimoto et al., 2006; Jiang et al., 2009; Park et al., 2008; Zhou et al., 2007). To our knowledge, this is the first study to evaluate mannosylated pegylated PEI for siRNA delivery and it is the first study to characterize the effect of the location of the mannose ligand in mannosylated pegylated PEI constructs on knockdown efficiency.

PEG and mannose were either both directly conjugated onto the PEI backbone or mannose was conjugated to the PEI via a PEG spacer. $^1$H NMR spectra's confirmed that both constructs had PEI, PEG and mannose present and the peaks strongly corresponded to previously reported values (Handwerger and Diamond, 2007; Sagara and Kim, 2002). The signal for mannose at 7 ppm was weak due to the relatively low proportion of mannose in the overall construct composition. For this reason, we used the resorcinol assay to quantify the amount of mannose present in each construct.

The surface chain density of PEG is a critical factor in improving stealth shielding of nanoparticles and polyplexes. The PEI to PEG ratio of PEI-PEG was 3.704, which suggests that every 25 kDa PEI chain has 3.45 chains of 2 kDa PEG. PEI-PEG-mannose had a 1.23 PEI/PEG ratio indicating 10.16 PEG chains per PEI. The 0.962 PEI/PEG ratio of mannose-PEI-PEG suggests that there are 13.3 PEG chains for each PEI. PEG chains have a larger range of motion
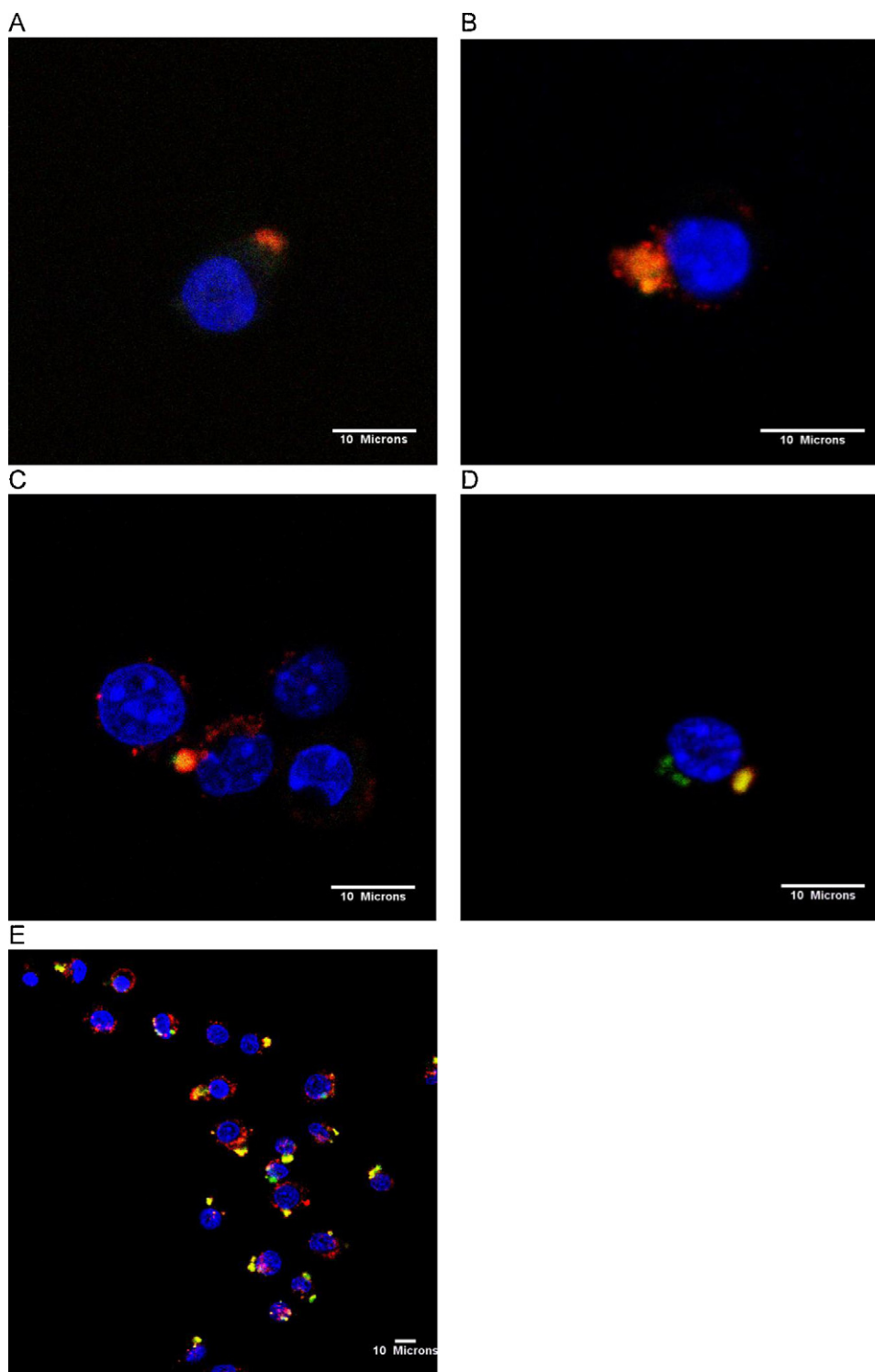
**Fig. 5.** Confocal microscopy images showing intracellular trafficking of polyplexes. Raw264.7 cells were stained with Lysotracker Green (green), incubated with polyplexes formed using Cy-3 labelled siRNA (red), and then mounted with DAPI containing mounting solution after fixation. Co-localization of polyplexes and lysosomes are shown as a yellow signal. (A) PEI/siRNA polyplexes, (B) PEI-PEG/siRNA polyplexes, (C) Mannose-PEI-PEG/siRNA polyplexes, (D) PEI-PEG-mannose/siRNA polyplexes, (E) PEI-PEG-mannose/siRNA polyplexes in lower magnification. Images were taken at 2 h after transfection and all the polyplexes were prepared with 1 μM siRNA at N/P ratio 7.

at low surface coverage, that can lead to gaps in the PEG protective layer (Storm et al., 1995). For PEG chains to fully cover the surface of PEI/siRNA polyplexes, six short PEGs (5 kDa) or one long PEG (20 kDa) are needed (Brus et al., 2004; Petersen et al., 2002). Therefore, 3.45–13.3 chains of 2 kDa PEG is expected to provide a satisfactory level of pegylation for steric stabilization. The 0.19 μmol/mg of mannose in mannose-PEI-PEG and 0.12 μmol/mg

in PEI-PEG-mannose represent an average modification of 4.7 and 3.0 molecules of mannose per PEI, respectively. This quantity of mannose is expected to be sufficient for selective binding of mannose receptors on cells.

Polymer/siRNA polyplexes exhibited sizes in the range of 169.10 and 357.33 nm. This particle size range is suitable for efficient endocytosis by RAW264.7 cells. Larger particles outside of this range
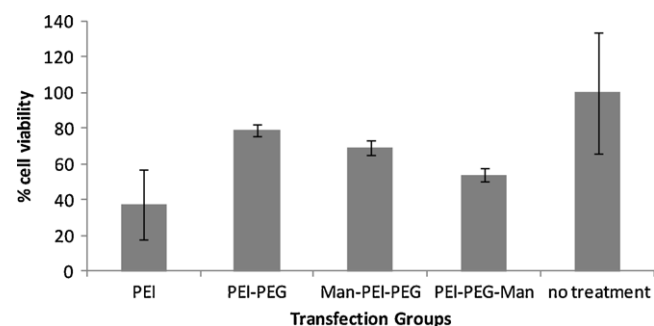
A

B
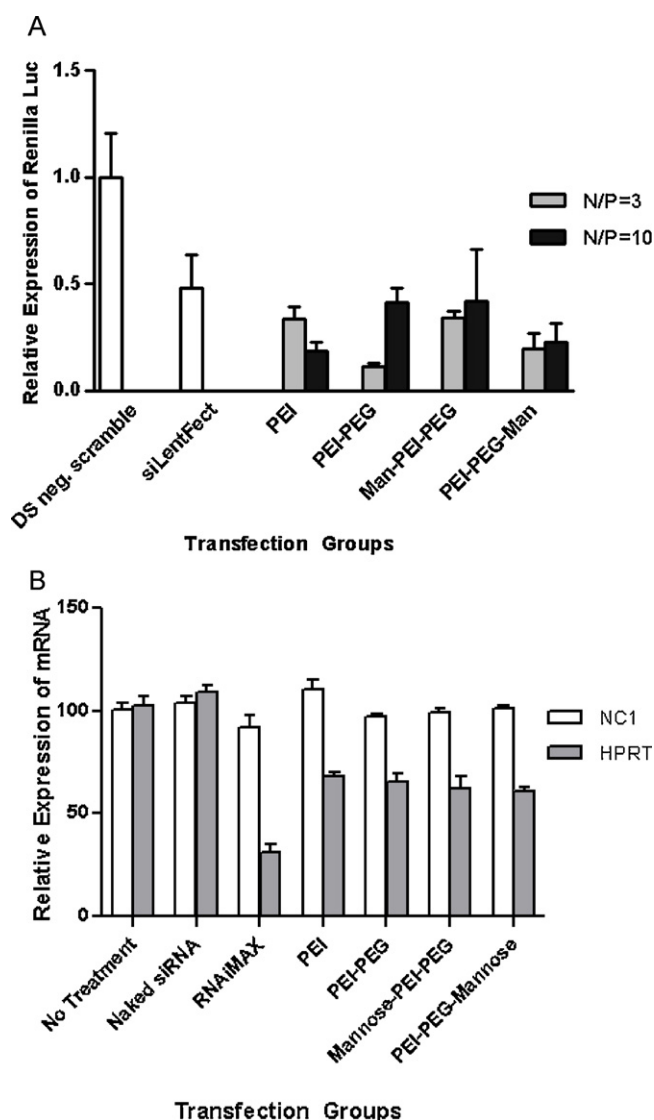
**Fig. 6.** Knockdown of luciferase expression and HPRT mRNA expression. (A) Relative gene expression of Renilla luciferase is presented with firefly luciferase used an internal control. From the left, DS scrambled neg with PEI, siLentFect/hRluc siRNA PEI/hRluc siRNA, PEI-PEG/hRluc siRNA, mannose-PEI-PEG/hRluc siRNA, and PEI-PEG-mannose/hRluc siRNA with N/P ratios at 3 and 10. (B) Relative expression of HPRT mRNA levels with NCI used as a control. From the left, no treatment, Naked siRNA, RNAiMax/HPRT siRNA, PEI/HPRT siRNA, PEI-PEG/HPRT siRNA, mannose-PEI-PEG/HPRT siRNA, PEI-PEG-mannose/HPRT siRNA.



**Fig. 7.** Cytotoxicity. Cytotoxicity of various polyplexes tested at the working concentration of 0.0078125 mg/ml. From the left; PEI, PEI-PEG, mannose-PEI-PEG, PEI-PEG-mannose, and no treatment group. The relative cell viability was calculated by normalizing to the non-treatment group.

observed by SEM are likely clusters or aggregates of these smaller particles. The zeta potentials of PEI-PEG-Man/siRNA polyplexes showed positive values that were approximately 21.63 mV and similar to unmodified PEI and pegylated PEI. This result suggested that PEI-PEG-Man could form stable polyplexes with siRNA. The zeta potential of Man-PEI-PEG/siRNA polyplexes was relatively low compared to PEI-PEG-Man/siRNA polyplexes. This could explain the lower cellular uptake of Man-PEI-PEG observed in our intracellular trafficking studies. The lower zeta potential could lead to weaker interactions with siRNA and the cell surface, which in turn could lead to decreased endocytosis of the polyplexes.

Branched PEI was selected as the backbone for our system because the complexation of branched PEI with siRNA has been reported to exceed that of linear PEI (Breunig et al., 2008). As a result, in gel retardation assays, no reduction in siRNA condensation properties was found with PEGylated PEIs. Overall, complete

binding of siRNA was achieved with all the various constructs at N/P ratios of 3 and higher.

We selected RAW264.7 cells for evaluation of polyplex uptake, trafficking and knockdown because the RAW264.7 cells are a murine macrophage cell line that are known to express mannose receptors and that are considered to be typically hard to transfect. Macrophages are also a potential target for our pegylated mannosylated PEI delivery system (Diebold et al., 2002).

In confocal images, the cells endosomes/lysosomes were stained with Lysotracker Green™. Co-localization of the green signals with red signals associated with the Cy-3 labelled siRNA indicated that polyplexes were being internalized by endocytosis, which is consistent with previous reports on uptake by PEI/nucleic acid polyplexes (Lecocq et al., 2000). In addition, separate red signals seen in some cells were most likely due to siRNA that had released from the endosomal compartments. Thirty minutes after incubation, polyplexes had been internalized by cells and localized in cytoplasm. From another cellular uptake study using green fluorescent-labeled (Oregon Green 488) polymers and red fluorescent-labeled (Cy3) siRNA, we observed the two signals were separated in the cytoplasm (data not shown) 2 h after transfection. This result indicated that siRNA complexes had been released from the lysosomal vesicles as well as from the polymers and were distributed in the cytosol. This release of siRNA is purported to be due to the proton sponge effect, which causes rupture of the endosomes because of the PEI's strong buffering capacity (Boussif et al., 1995). Intracellular trafficking plays an important role in the fate of siRNA polyplexes because their spatial distribution does not correspond to simple diffusion (Jen and Gewirtz, 2000). Perinuclear localization of siRNA, as seen in Fig. 5C and E, is required for successful gene silencing by interaction with RISC to induce RNAi. Interactions with RISC dictate siRNA localization even when siRNA is conjugated to cell-binding ligands such as the TAT peptide (Chiu et al., 2004). It suggests that the mannosylated pegylated PEI polymers developed in this study successfully protected siRNA during endocytosis and lysosomal escape in order to integrate siRNA into the RISC complex for correct RNAi processing.

Mannosylation and pegylation of PEI did not reduce gene knockdown efficiency relative to unmodified PEI showing no significant difference in relative gene expression. The advantages of pegylating polyplexes and incorporating cell binding ligands for *in vivo* applications have been well established (Beyerle et al., 2010; Ogris et al., 1999; Owens and Peppas, 2006; Sagara and Kim, 2002; Webster et al., 2007; Yamaoka et al., 1994, 1995). Furthermore, not only did pegylation have no adverse effect on knockdown efficiency, but it also reduced toxicity. This reduction in toxicity was slightly offset by the mannosylation but not significantly so. In addition to observing a reduction in cellular toxicity, we anticipate that pegylation of the polyplexes will also reduce toxicity at the systemic level by

reducing aggregation of the polyplexes and therefore the capillary embolism that has been associated with the use of unmodified PEI (Intra and Salem, 2008; Ogris et al., 1999).

One aspect of mannosylated pegylated PEI delivery systems that has not been explored before is the importance of the location in which mannose is bound to the construct. In our studies, PEI-PEG-mannose/siRNA polyplexes resulted in higher toxicity and higher knockdown efficiency than mannose-PEI-PEG. Furthermore, qualitatively we observe that PEI-PEG-mannose/siRNA polyplexes have more stable uptake by RAW264.7 cells than mannose-PEI-PEG/siRNA polyplexes. This observation is not clearly understood but could be explained by the structural difference between two constructs. The PEI-PEG-mannose construct has mannose moieties exposed at the tip of PEG chain whereas the mannose in mannose-PEI-PEG could be hindered by the PEG chains. Thus the mannose ligand–receptor interaction could be obstructed by the shielding effect of PEG chains. The use of a PEG chain can impair cell binding by shielding not only PEI but also the ligand (Kunath et al., 2003a). We carried out cellular uptake time-lapse studies at various time points including 0.5, 1, 2, 4, 8, and 24 h (data not shown). Throughout the course of experiment, we observed that PEGylated PEI had delayed endocytosis relative to PEI alone. The reason that PEG-PEI without mannosylaton at an N:P ratio of 3 still has better luciferase knockdown than either the mannose-PEI-PEG or PEI-PEG-mannose constructs could be attributed to its better cytotoxicity profile or the use of an optimized degree of pegylation for the PEI-PEG construct.

The endogenous knockdown generated by our modified PEIs was significantly less effective than RNAiMax at targeted mRNA reduction. In addition, in our hands, TransIT-TKO can routinely provide 80–90% knockdown, which is significantly more effective than the modified PEIs (data not shown).

The modified PEIs were more efficient at luciferase knockdown than that of another commercial transfection reagent, siLentFect, which was used following the manufacturers guidelines. Moreover, both mannose and PEG have well established attributes for *in vivo* delivery such as selected cell binding, reduced systemic toxicity and enhanced circulation. In particular, mannose ligands have shown significant potential for binding antigen presenting cells (APCs) such as mouse macrophages and dendritic cells. Furthermore, PEGylated PEI/siRNA complexes have been reported to have decreased random uptake into non-specific organs including liver and spleen compared to unmodified PEI.

Finally, the determination of the optimal location of cell binding ligands in delivery construct in this study is expected to have important implications in the design of several plasmid DNA, oligonucleotide and siRNA delivery systems currently in development that utilize alternative cell binding ligands and alternative cationic backbones such as chitosan.

## Acknowledgements

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