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Bimodal Targeting Using Sulfonated, Mannosylated PEI for Combined Gene Delivery and Photodynamic Therapy

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ABBREVIATIONS

TAM = Tumor associated macrophage; MMP = Matrix Metalloprotenase; PEI = Polyethyleneimine; m_x-PEI = PEI with x% mannosylation; s_x-PEI = PEI with x% sulfonation; Pyro = Pyropheophorbide-a; ADM = Allyl-D-(+)-Mannose; PDT = Photodynamic Therapy; PSGL-1 = P-selectin glycoprotein-1; DMSO = Dimethyl sulfoxide; DIPEA = N,N-Diisopropylethylamine; DCM = Dichloro methane; pDNA = plasmid DNA; CHO = Chinese hamster ovary; CHO-L = L-selectin overexpressing CHO cells; CHO-P = P-selectin overexpressing CHO cells; PBS = Phosphate buffer saline; FACS = fluorescence-activated cell sorting; EDTA = ethylenediaminetetraacetic acid; MTT = (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide); TGDP = Total gene delivery performance; eGFP = Enhanced green fluorescent protein.

ABSTRACT

Photodynamic therapy (PDT) and gene delivery have both been used to target both cancer cells and tumor-associated macrophages (TAMs). Given the complex nature of tumor tissue, there could be merit in combining these strategies simultaneously. In this study, we developed a bimodal targeting approach to both cancer cells and macrophages, employing materials conducive to both gene delivery and PDT. Polymers libraries were created that consisted of cationic polyethyleneimine (PEI) conjugated to the photosensitizer pyropheophorbide-α, with sulfonation (to target selectin-expressing cells) and mannosylation (to target TAMs). Polyplexes, consisting of these polymers electrostatically bound to DNA, were analyzed for transfection efficacy and cytotoxicity towards epithelial cells and macrophages to assess dual-targeting. This study provides preliminary proof of principle for using modified PEI for targeted gene delivery and PDT.

INTRODUCTION

Macrophages engulf foreign bodies and serve important roles in the immune system and are present in all human tissues. However, in the tumor microenvironment, tumor associated macrophages (TAMs) are implicated in tumor metastasis, angiogenesis and tumor initiation,

making them a target of interest.(1, 2) Macrophages can be classified as either M1 or M2 depending on their phenotype. M2 macrophages can provide a nutritional advantage to cancer cells and promote angiogenesis, whereas M1 macrophages are generally associated with innate immunity.(3) TAMs have served as a target for chemotherapeutic cargo delivery, imaging agents, and vaccines.(4) It has been demonstrated that the controlled reprogramming of M2 macrophages to an M1 phenotype can induce toxicity to the surrounding cancer cells.(5) TAMs secrete matrix metalloproteinases (MMP), which impact the tumor microenvironment.(4) For example, it has been shown that MMP-7 expressed by TAMs cleaves Fas ligand, causing the surrounding cells to become more resistant to cytotoxic drugs.(6) Thus, the ability to downregulate MMP-7 expression via nucleic acid delivery could improve the efficacy of chemotherapy. One mechanism that TAMs can be selectively targeted is via mannose receptors, an approach which has been explored in the recent years.(7)

Photodynamic therapy (PDT) is a clinically approved therapeutic procedure that uses light of specific wavelength and a photosensitizer to generate reactive oxygen species (ROSs), including singlet oxygen which cause cell death via apoptosis or necrosis.(8, 9) However, mainstream clinical applications of PDT for solid tumor treatment are limited by numerous factors including limited light penetration in tissues, and also limited tumor tissue specificity and consequential damage to adjacent healthy tissue. To improve specificity, extensive research efforts have examined PDT using activatable and targeted photosensitizers.(10, 11) Not only do TAMs play a role in PDT responses (12), but photosensitizers have been developed specifically to target TAMs, using receptors including the scavenger receptor and the mannose receptor (CD206).(13-15)

With the long term goal of improving tissue specificity of PDT, we previously conjugated the photosensitizer pyropheophorbide-α (pyro) to sulfonated polyethyleneimine (s-PEI), forming pyro-s-PEI.(16) This allowed for the selective PDT in cells overexpressing selectins, which are implicated in inflammation and cancer.(17, 18) As part of the inflammatory response under chronic inflammatory conditions, specific leukocyte sub-populations infiltrate into the site of inflammation through a cascade of events that is termed the 'multistep leukocyte adhesion cascade'. In the first step of this cascade leukocytes are captured and they roll on endothelial surfaces that express P- and E-selectins, and also interact with other already recruited white blood cells via L-selectin. P-selectin glycoprotein-1 (PSGL-1) is a well-known glycoprotein ligand for L- and P-selectin, with other sialofucosylated glycoproteins also acting as ligands of the selectins. Natural sulfated glycans are prominent entities that are well known to bind selectins under physiological conditions. These molecules typically contain either 6-O-sulfate or 3-O-sulfate groups attached to either N-acetylglycosamine (GlcNAc) or galactose (Gal), respectively. Prominent examples include GlyCAM-1(19) and sulfated L-selectin ligands that are expressed on the high endothelial cells of lymph nodes.(20) While these entities do not, strictly speaking, contain a sulfonate group (they are sulfates), both functional groups impart a local negative charge that enables selectin-ligation by engaging the lectin domain of the protein. Thus, they likely operate via the same mechanism. Such sulfated glycans bind L- and P-selectin with similar affinity are the core-2 sialyl Lewis-X entity that is part of PSGL-1.(21) In general, sulfonated macromolecules can bind L- and P-selectin.(16, 22, 23)

Previous studies have combined PDT with gene delivery.(24-26) PEI can be used gene delivery and with an abundance of amine groups can readily be modified with targeting moieties. Here, we explore the concept of using modified PEI conjugated to pyro for targeting TAMs and

selectin-expressing cells. PEI allows packing of DNA for cell internalization and pyro confers PDT capabilities. The polymer is modified with sulfate groups to target selectins and with mannose groups to target the mannose receptor expressed on TAMs. The strategy of targeting both TAMs and melanoma cells for PDT was recently reported using a viral method with cow-pea mosaic virus and dendrons was based on the selective expression of vimintin on the cells.(27)

MATERIALS AND METHODS

Materials. Branched PEI (10 kDa), 4-toluenesulfonyl chloride, D-(+)-mannose and other chemicals were obtained from Sigma. O-benzotriazol-1-yl-tetramethyluroniumhexaflurophosphate (HBTU) was obtained from VWR. Dialysis membranes (3.5 kDa pore size) were obtained from Spectra/ Por.

Synthesis. Sulfonated-PEI was synthesized as described previously.(28) Briefly, 0.5 mmol PEI was dissolved with vortexing in 50 mL of methanol. 8.5 mL (72 mmol) or 31 mL (266 mmol) of chlorosulfonic acid was added for the generation of 6% and 34% s-PEI respectively. The reaction was maintained at 60 °C for 30 min. A thick yellow paste was obtained after removing excess methanol and was dissolved in 5 mL water. The paste was vortexed until the product formed a homogenous solution and was added to methanol to form a precipitate. This was rinsed with methanol and the previous steps were thrice repeated in order to separate s-PEI. s-PEI was placed in a vacuum desiccator for 24 h to remove solvents.

Allyl-α-d-mannopyranoside (ADM; mannose) was synthesized by mixing 3 g (16.6 mM) of d-(+)-mannose with 8 mg (41.9 μM) toluenesulfonyl chloride in allyl alcohol (20 mL). The flask was maintained at 90 °C for 24 h under reflux and the product was concentrated at 35 °C using

vacuum distillation as previously described.(29) Mannosylated-PEI with varying mannose percentages (6% and 34%) was synthesized as follows. PEI was dissolved in dimethyl sulfoxide (DMSO) and allowed to react with varying amounts of ADM for 24 h at 90 °C to generate m-PEI. Unreacted mannose was removed via dialysis. Pyro was then conjugated to s-PEI and m-PEI as described below.

Pyro was synthesized as previously described.(30) 1 mL DMSO was added to PEI or s-PEI/ m-PEI and sonicated till the polymer completely dissolved. Pyro was added to DMSO containing polymer, followed by HBTU and diisopropylethylamine (DIPEA) addition and stirring for 24 h. Aqueous extraction was performed using 1:1 water and dichloromethane (DCM), and the aqueous phase was retained. Aqueous extraction was performed thrice by retaining the aqueous phase and adding equal volume of DCM to obtain pyro-labeled polymer. Small molecule impurities were removed from the aqueous layer was via dialysis against water for 4 h with three changes.

Gel shift assay. 500 ng of plasmid DNA (p-DNA) was mixed with indicated polymer in 25 mM NaOAc at pH 5.15 and the mixture was gently vortexed and incubated at room temperature for 15 min. Samples were loaded on to a 0.8% agarose gel, which was then subjected to electrophoresis and imaged with UV light and SYBR Safe DNA Gel Stain (Invitrogen). The lowest concentration at which pDNA could be completely complexed with the polymer was determined based on these results.

Polyplex characterization. Polyplexes were prepared by mixing polymer and pDNA in the desired ratio (1:50, 1:100 and 1:200) in 25 mM NaOAc buffer at a pH of 5.15. A few modified polymers, conjugated to mannose and sulfate, were not readily soluble so they were dissolved by

sonication. Polyplexes were diluted in phosphate buffer saline (PBS) and the zeta potential and size of the polyplexes was measured using Zetasizer nano-ZS90.

Cell viability assay and photodynamic therapy. Macrophages are known to overexpress mannose receptors and have been used to study mannose binding.(29, 31, 32) Selectin overexpressing cells, CHO-L and CHO-P, have been used to study sulfate binding.(16) CHO-L and CHO-P cell lines were generated by transient transfection of Chinese Hamster Ovary (CHO) cells with full length human L- and P-selectin. Individual clones were selected by serial dilution and antibody selection.(21) These cells contain 200-500 selectins/μm^2 as measured using Quantum Simply Cellular cytometry calibration beads (Bangs laboratories) and flow cytometry. Wild-type CHO cells, themselves, do not express either of the selectins. CHO and RAW 264.7 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS and 1% antibiotics. Cell densities for CHO and RAW 264.7 cells were $2x10^4$ per well and $1x10^4$ per well, respectively, and the cells were allowed to adhere for 24 h. In vitro experiments were carried out in 96-well plates. For PDT experiments, media was replaced with polymer solution (pyro concentration: 500 nM) diluted in PBS. Polymer treated wells were irradiated with laser to determine targeting and PDT efficacy. A fluence of 10 J/cm^2 (power density: 29.84 mW/cm^2) was achieved using a LED based 665 nm custom built laser box capable of irradiating the area of the box with uniform power as previously described (16). 24 hours later, cell death induced by PDT was measured using XTT assay. XTT (2, 3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide) and PMS (N-methyl dibenzopyrazine methyl sulfate) were diluted in PBS to achieve a final concentration of 50 µg/mL and 30 µg/mL, respectively. Absorbance values were read at 450 and 630 nm after incubating cells with 100µl of PBS containing XTT and PMS for 3 h. Cell viability was calculated after subtracting the values at 630 nm from 450

nm readings. The ratio of treated cells to untreated cells after subtracting XTT background was used to determine the cell viability. All cell experiments were performed in triplicate and the error bars represent standard deviation.

Cell transfection studies. Cell seeding densities for RAW264.7 and CHO cells in 96-well plates were 30,000 and 20,000 per well, respectively. After 24 hours, media was removed and 30 µL polymer:pDNA polyplexes were added to each well containing 100 µL fresh media. Media containing polyplex was replaced with fresh DMEM after 4 h and, 48 h later, gene delivery efficiency was determined with the help of a flow cytometer. Luciferase expression level was determined using Bright Glo Assay (Promega) and the total values were normalized to the total protein in each well. Total protein in each well was determined using a luminometer, and was calculated with the help of Micro BCA Protein Assay Kit (Pierce).

Transfection efficiency of the polyplexes was determined by calculating the number of transfected cells using flow cytometry. 48 h post-transfection, media was removed and ice cold PBS was used to rinse the wells in order to remove trace media before detaching the cells. Cells were detached for fluorescence-activated cell sorting (FACS) analysis using either 0.05% trypsin/ EDTA or cell scraper. Equal amount of DMEM containing serum was added in order to deactivate trypsin. Cells were then resuspended in PBS after centrifuging at 250 g for 5 min. Flow cytometer was used to determine the percentage of transfected cells.

MTT assay. Cells were seeded in a 96-well plate and allowed to adhere for 24 h. Cells were transfected at the aforementioned concentrations and similar procedure for incubation were followed. Media was replaced with 10% v/v 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) solution (5 mg/mL) diluted in PBS after 24 h. 100 µL of MTT solution was added to each well and incubated for 3 h. MTT solution was replaced with

DMSO to dissolve formazan crystals. After agitating gently for 1 h, the plate was read at 570 nm using a microplate reader and 630 nm readings were used as background.

Measurements. NMR measurements were made with INOVA-500 in deuterated-DMSO at 500 MHz. A Zetasizer Nano ZS90 was used to determine the size and zeta-potential of the polyplexes and a Synergy 4 Multi-mode Microplate Reader was used to measure the luminescence in the well-plates. A Calibur flow cytometer was used for flow cytometry measurements.

RESULTS AND DISCUSSION

Synthesis and characterization of polyplexes

Sulfate or mannose was conjugated to PEI via reaction with the amine groups of PEI as shown in figure 1. The polymer itself is represented by a sphere in Figure 1 and a representative segment is shown. Allyl-α-d-mannopyranoside was incubated with PEI overnight, allowing amines to react with the monosaccharide. Sulfonated-PEI was produced by the addition of chlorosulfonic acid to PEI dissolved in methanol, as previously described (16). Both s-PEI and m-PEI were subjected to further purification steps in order to remove the impurities. Conjugation of sulfate and mannose to PEI was confirmed by NMR (Figure S5). Pyro was conjugated to s-PEI and m-PEI by dissolving polymer along with pyro in DMSO in the presence of HBTU and DIPEA. Free carboxylic group on pyro reacts with primary amines on the surface of modified polymers to generate pyro-labeled sulfated- or mannosylated-polymers. Post conjugation, pyro-polymer conjugates were extracted using DCM-water extraction and then dialyzed overnight to remove impurities. The number of pyro units was found to be between 2 and 4 per PEI polymer based on pyro absorbance.

To evaluate the formation of polymer-pDNA polyplexes, gel electrophoresis was performed at different ratios (1:1 to 200:1) of polymer to pDNA (Figure S2-S4). Based on gel electrophoresis, the minimum polymer concentration required for successful polyplex formation was determined (table S1 and figures S2, S3, and S4) and the ratios 1:50, 1:100, and 1:200 were used to study transfection efficiencies of the library of polymers. Polyplex size and zeta potential were also measured (Figure S1). The average size of the polyplexes was found to be spread between 100 and 350 nm. Pristine PEI polymers showed a mean diameter of 150 nm. However, modified polymers showed variable size based on the conjugation ligand. With increasing sulfonation, the size of the polyplex increased for s-PEI polymers (Fig S1A). At higher polymer concentrations, these polyplexes tend to clump together due to increased electrostatic interaction, which may explain the size increase. Although, pyro-labeled m_6-PEI was an exception with mean size ranging between 300 and 400 nm indicating they might have partially aggregated. Zeta potential measurements showed that the positive charge on the polymer was only slightly reduced with increasing sulfonation (Fig. S1B). However, the decrease in surface charge rendered s-PEI polymers less toxic to both CHO (Figure 2) and RAW264.7 cells (Figure 5). This might be explained by the fact that cationic groups on PEI are toxic to the cells, (33) and thus modified PEI polymers with fewer primary amines show higher cell viability. Conjugation of mannose to PEI did not have a marked influence on surface charge. Although no such trend was observed with m-PEI polymers, unexpectedly, mannosyation seemed to increase the zeta potential of the modified polymers. Moreover, conjugation of mannose to s-PEI polymers showed higher positive charge overall compared to PEI or s-PEI polymers alone. The reason for the increase in charge is not clear, purity of polymers or the interaction between DNA and polymers might be the cause.

Total gene delivery performance

Transfection magnitude or transfection efficiency alone cannot predict the performance of the vector accurately. Therefore, to assess the gene delivery capabilities, the total gene delivery performance (TGDP) has been used.(29, 34) Polymer modification in general had a positive influence on the TGDP efficacy of the polyplexes. For example, at 1:50 polymer-pDNA ratio, transfection efficiency of s_{34}-PEI was 40% higher than PEI. Transfection magnitude, a measure of total luminescence following luciferase transfection, presented similar trends, indicating successful binding and uptake of polymers by selectin expressing cells. Although non-pyro-labeled polymers showed similar luminescence values for CHO and CHO-L cells (expressing L-selectin), CHO-P cells (expressing P-selectin) showed 4-5 fold higher transfection magnitudes for sulfonated-polymers compared to unmodified PEI. Pyro-labeled polymers also exhibited similar trends for eGFP transfection. Luciferase transfection increased with the sulfonation of PEI. Transfection magnitude of sulfonated polyplexes increased by 4-5 folds for CHO-P and CHO-L cells compared to CHO cells, indicating sulfonated polymers have better transfection capabilities for selectin-expressing cells. Surprisingly, CHO cells exhibited an inverse relation between polymer-pDNA ratio and transfection magnitude for pyro-labeled polymers. For example, transfection magnitude for pyro-PEI at 50:1, 100:1 and 200:1 were 200, 100 and 75 RLU per µg of protein respectively. On the other hand, CHO-P and CHO-L cells displayed an increase in transfection magnitude with increase in the concentration of the polymer.

In general, transfection efficiency and transfection magnitude showed pronounced effects in CHO-P cells. In comparison, the transfection magnitude of CHO-L cells was lower than CHO-P cells, suggesting that CHO-P cells might be binding sulfonated-polymers with higher specificity. Although PEI has performed as an excellent transfection agent, it has been linked to higher

toxicity and decreased cell proliferation.(33) Altering the surface amine groups of PEI with anionic sulfate groups exhibited an increase in the cell viability suggesting modified PEI to be less toxic compared to pristine PEI. Pyro conjugation to s-PEI enhanced the cell viability further, which might be due to the decrease in amines on the surface of PEI/ s-PEI owing to the amide bond formation between the carboxylic group of pyro and amine group of PEI.

Conjugation of mannose (6%) to PEI or s-PEI decreased the transfection efficiency in CHO cells slightly but trends were similar to non-mannosylated polymers (figure 3). CHO cells showed 50-60% transfection efficiency before mannose conjugation and 40-50% with m-PEI polymer. CHO-L and CHO-P also displayed a decrease in transfection efficiency by 10-15% but were generally higher than CHO cells showing sulfates to be still interacting with selectins after mannose conjugation. However, luminescence assay results showed a drastic decrease in transfection magnitude of CHO-P and CHO-L cells resulting in similar transfection magnitudes for all three cell lines. m_6-s_{34}-PEI showed slightly higher transfection magnitude on CHO-L and CHO-P cells indicating availability of excess sulfate moieties might overcome the hindrance due to mannose modification. Mannosylation did not seem to affect pyro-labeled sulfonated-polymers since they displayed similar transfection trends with eGFP and luciferase assay. Mannosylation of PEI or s-PEI increased the cell viability at higher polymer-pDNA ratio but it did not seem to have any effect on the cell viability at lower polyplex ratios.

Conjugation of higher percentage mannose (34%) to s-PEI polymers decreased the transfection efficiency of the polymers on CHO cell lines (Figure 4). For example, if we compare the performance of m_{34}-s_6-PEI versus s_6-PEI on CHO and CHO-L cells, transfection efficiency of CHO and CHO-L cells for m_{34}-s_6-PEI remained unchanged at approximately 40%, whereas s_6-PEI was nearly 25% higher for CHO-L cells at 1:50 polyplex ratio. Transfection magnitudes of

s_6-PEI and m_{34}-s_6-PEI showed similar trends in both CHO and CHO-L cells suggesting transfection magnitudes to be unaffected by mannose modification. However, a comparison of the same polymers on CHO and CHO-P cells suggested the transfection magnitude to be drastically lowered by mannose conjugation. CHO-P cells showed a 4-folds higher transfection magnitude with s_6-PEI when compared with m_{34}-s_6-PEI, pointing towards lower binding and therefore lower transfection. Although the modified polymers showed lower efficiency, pyro-labeled polymers displayed identical trends with or without mannose modification. Moreover, pyro-s_6-PEI and pyro-m_{34}-s_6-PEI showed similar transfection magnitudes on CHO-P and CHO-L cells. In general, pyro-conjugated polymers showed higher transfection efficiency with luciferase assay compared to their non-pyro counterparts.

To verify if mannosylation would have any influence on transfection efficiency in macrophages, we tested the gene delivery efficiency of mannosylated polymer libraries on RAW264.7 cells. With increase in mannosylation, TGDP of the polymers improved, indicating that binding and internalization was via mannose receptors on the surface of macrophages (Figure 5). Transfection efficiency of mannosylated PEI (m-PEI) and s-PEI also showed a marked improvement. Influence of mannosylation on transfection efficiency was verified by comparing m_6-PEI with m_{34}-PEI. Increase in mannosylation from 6% to 34% resulted in 5% improved transfection efficiency of m_{34}-PEI. In comparison to PEI, at lower polymer-pDNA ratio, m_6-PEI and m_{34}-PEI showed two and three fold increase in transfection efficiency respectively. At 200:1 ratio, m_{34}-PEI showed the highest EGFP transfection, but at the same time it was also toxic with just 70% cell viability. Performance of pyro labeled m-PEI polymers decreased slightly with increasing sulfonation at lower polymer-pDNA ratio. At 1:200 ratio pyro-m_{34}-s_6-PEI performed the best with 40% transfection efficiency.

The luminescence assay showed a slightly different trend for m-PEI polymers. Transfection magnitude increased with increasing mannosylation percentage and the trend was clearly visible at 1:200 polymer-pDNA ratio. However, the cell viability of m-PEI polymers decreased with increasing polymer-pDNA ratio. At 1:50 ratio, cell viability of PEI polymers was nearly 100%, upon increasing the concentration of the polymer in the polyplex, cell viability decreased by 20-30% indicating unmodified PEI to be toxic at higher concentrations. Mannosylation alone did not have much influence on cell viability however m_x-s_x-PEI polymers proved to be less toxic to RAW264.7 cells even at higher concentrations. Pyro conjugation to m_x-s_x-PEI polymer reduced their toxicity further. m_{34}-PEI, which performed the best with respect to transfection efficiency, had a TGDP value of 38. However, m_{34}-PEI was toxic at higher polymer:pDNA ratios. Considering all the metrics (cell viability, transfection magnitude, and efficiency) pyro-m_{34}-s_6-PEI showed optimal performance on macrophages with a TGDP value of approximately 30.

In the absence of mannosylation, gene delivery efficiency of s-PEI polymers to macrophages was low compared to their performance with CHO-P or CHO-L cells. Although s-PEI polymers were less toxic to macrophages in comparison to PEI, eGFP or luminescence assays did not yield high transfection efficiency on macrophages, even at higher polymer-pDNA ratios, leading to poor TGDP (Figure 5). Previous studies have shown mannosylated polymers to undergo receptor mediated endocytosis which accumulate in endosomes.(35, 36) Therefore, higher TGDP of m-PEI polymers can be explained on the basis of improved targeting via mannose receptor. On the other hand, s-PEI polymers displayed better PDT efficacy after short incubation but longer incubation time did not translate to improved gene delivery. Clatherin mediated endocytosis usually leads to a less efficient gene delivery owing to the degradation of polyplexes in lysosomes.(37) Presence of lectins on the surface of cells might be leading to improved binding

and uptake of sulfated polymers.(38) However, s-PEI polymers might be undergoing lysosomal degradation after being internalized by macrophages leading to lower TGDP.

These results suggest that, in addition to dual-targeting capabilities, incorporating sulfate and mannose on to a single polymer leads to higher transfection efficiency with improved cell viability. However, while using dual targeting systems, studies involving a library of polymers might be able to identify optimal system for gene delivery.

Photodynamic therapy

PDT with pyro-conjugated sulfonated and mannosylated polymers was assessed in vitro with CHO, CHO-P, CHO-L, and RAW 264.7 cells. After a short 4 minute incubation of pyro-labeled polymers with cells, they were irradiated with 665 nm light and cell viability was assessed. Sulfonated-PEI polymers exhibited better targeting capabilities and hence more efficient PDT with CHO-P and CHO-L cells compared to CHO cells (Figure 6). s-PEI polymers showed marked reduction in cell viability for CHO-P, CHO-L, and RAW264.7 cells whereas decrease in cell viability of cells incubated with pyro-PEI polymer was insignificant. Decreased viability observed in CHO-P and CHO-L for pyro-m6-PEI can be explained based on the selectin binding to sulfates on the surface of modified polymers. Macrophages might be binding to lectins present on the surface of the leading to improved PDT efficacy.(38) In parallel, we established the PDT efficacy of pyro-labeled mannosylated-PEI polymers on macrophages. Since macrophages are known to express mannose receptors on their surface, we incubated mannose and sulfonated polymer-pyro conjugates with RAW264.7 cells. Mannosylated-PEI has been shown to effectively target mannose receptors.(39) Incubation of pyro-m_6-PEI with RAW264.7 cells followed by laser irradiation demonstrated higher cytotoxicity to macrophages,

indicating successful targeting which corroborated with the previous studies. On the other hand, CHO cells remained unaffected when treated with pyro-m_6-PEI suggesting carbohydrate targeting to be specific to RAW264.7 cells. These results collectively indicate that bimodal targeting can be achieved *in vitro*.

Many studies have involved the use of either gene delivery or PDT as a standalone treatment for therapy, synergistic use of PDT and gene delivery for therapy can enhance the efficacy of the carriers. Some studies have shown the delivery of siRNA along with a photosensitizer enhances the therapeutic efficacy for cancer therapy *in vitro*.(24) Other studies have demonstrated the use of photosensitizer in light triggered release for gene delivery.(40) Thus, DNA packed polymers conjugated to photosensitizers can serve multiple purposes.

CONCLUSION

A library of PEI polymers modified with varying amounts of mannose and sulfate moieties was developed for targeted PDT and gene delivery. Conjugating a photosensitizer to sulfonated and mannosylated polymers furnished them with PDT capabilities. Pyro-conjugated sulfate and carbohydrate modified PEI polymers were able to induce cytotoxicity in selectin and mannose receptor expressing cell lines thus exhibiting targeting and therapeutic capabilities. Modified polymers enhanced the gene transfection efficiency and reduced cell toxicity compared to unmodified polymer. Although higher percentages of sulfation and mannosylation resulted in higher efficacy, trends were not clearly visible in a few polymers. Nevertheless, results from these studies show that sulfonated and mannosylated polymers can serve as simultaneous targets for epithelial cells and TAMs and also enable dual therapeutic modalities (PDT and gene delivery).

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SUPPORTING INFORMATION

Additional Supporting Information is available in the online version of this article or from the author:

Figure S1. Polyplex characterization summary. Polyplexes were prepared at three polymer:pDNA weight ratios and characterized using dynamic light scattering for A) particle diameter (nm) and B) zeta potential (mV).

Figure S2. Gel shift assay of first generation polymers. Lane 1 is unbound polymer, whereas the other lanes contain polymers. Specifically, L2 – 1:1, L3 – 5:1, L4 – 10:1, L5 – 25:1, L6 – 50:1, L7 – 100:1, L8 – 200:1.

Figure S3. Gel shift assay of second generation polymers. Lane 1 is unbound polymer, whereas the other lanes contain polymers. Specifically, L2 – 1:1, L3 – 5:1, L4 – 10:1, L5 – 25:1, L6 – 50:1, L7 – 100:1, L8 – 200:1.

Figure S4. Gel shift assay of third generation polymers. Lane 1 is unbound polymer, whereas the other lanes contain polymers. Specifically, L2 – 1:1, L3 – 5:1, L4 – 10:1, L5 – 25:1, L6 – 50:1, L7 – 100:1, L8 – 200:1.

Figure S5. 1H NMR spectra of all the polymers.

Table S1. Polymer:pDNA minimum binding ratio determined from gel electrophoresis.

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FIGURE CAPTIONS

Figure 1. Generation of sulfonated and mannosylated PEI. Step 1: Sulfonation of PEI. Step 2: Pyro conjugation of s-PEI. Step 3: Synthesis of Allyl-α-d-mannopyranoside and mannosylation of PEI/ s-PEI. Step 4: Pyro conjugation of mannosylated PEI/ s-PEI. The polymer is shown as a sphere, the sulfonate groups in blue and mannose in red.

Figure 2. Effect of sulfonation (6% and 34%) of PEI on total gene delivery performance (TGDP). A) CHO B) CHO-L (CHO cells expressing L-selectin) C) CHO-P (CHO cells expressing P-selectin) transfected with polyplexes prepared at ratio of 50:1,100:1 and 200:1 of polymer-pDNA. TGDP is the product of APC cell viability after transfection, transfection efficiency (percent EGFP positive cells) and magnitude (luciferase). s_x-PEI and pyro-s_x-PEI indicate sulfonated-PEI and pyro conjugated sulfonated-PEI respectively.

Figure 3. Effect of mannose conjugation (6%) of PEI on total gene delivery performance (TGDP). A) CHO. B) CHO-L. C) CHO-P transfected with polyplexes prepared at ratio of 50:1,100:1 and 200:1 of polymer-pDNA. TGDP is the product of APC cell viability after transfection, transfection efficiency (percent EGFP positive cells) and magnitude (luciferase).

m_x-PEI and pyro-m_x-PEI indicate mannosylated-PEI and pyro conjugated mannosylated-PEI respectively.

Figure 4. Effect of mannose modification (34%) of PEI on total gene delivery performance (TGDP). A) CHO. B) CHO-L. C) CHO-P transfected with polyplexes prepared at ratio of 50:1,100:1 and 200:1 of polymer-pDNA. TGDP is the product of APC cell viability after transfection, transfection efficiency (percent EGFP positive cells) and magnitude (luciferase).

Figure 5. Effect of sulfate and mannose modification of PEI on total gene delivery performance (TGDP) of RAW264.7 cells. RAW264.7 cells were transfected with polyplexes prepared at three polymer-pDNA weight ratios of A) sulfonation modified (pyro-) PEI, B) 6% mannosylation modified s_x-(pyro-) PEI, and C) 34% mannosylation modified s_x-(pyro-)PEI. Subsequent TGDP was calculated as the product of APC cell viability after transfection, transfection efficiency (percent EGFP positive cells) and magnitude (luciferase).

Figure 6. Photodynamic Therapy. PDT cytotoxicity caused by pyro–labeled polymers in CHO cells expressing P- or L-selectin and macrophages. Indicated polymers were incubated with cells in a 96-well plate and then wells were irradiated with a fluence rate of 10 J/cm^2. Cell viability was assessed 24 h later using the XTT assay. Mean ± std. dev. for n = 4.