pH-Responsive Sulfonamide/PEI System for Tumor Specific Gene Delivery: An in Vitro Study

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The feasibility of pH-sensitive polymeric nanoparticles that effectively target the acidic extracellular matrix of tumors is demonstrated. Plasmid DNA was complexed with polyethyleneimine (PEI) and further with a pH-sensitive diblock copolymer, poly(methacryloyl sulfadimethoxine) (PSD)-*block*-PEG (PSD-*b*-PEG), to obtain naonparticles. The shielding/deshielding of nanoparticles was tested along with cell viability and transfection efficiency at physiological and tumor pH. The nanoparticles composed of DNA/PEI/PSD-*b*-PEG were 300 nm in size and showed low cytotoxicity and transfection at pH 7.4 due to shielding of PEI by PSD-*b*-PEG. The PSD-*b*-PEG bound to PEI/DNA complex decreased the interaction of PEI positive charges with cells and reduced the cytotoxicity by 60%. At pH 6.6, the nanoparticles demonstrated high cytotoxicity and transfection, indicating PSD-*b*-PEG detachment from the nanoparticles and permit PEI to interact with cells. PSD-*b*-PEG is able to discern the small difference in pH between normal and tumor tissues and hence has remarkable potential in drug targeting to tumor areas.

1. Introduction

Since the introduction of *nitrogen mustard* during World War II as the first chemotherapeutic agent, a variety of chemical and biological agents have been developed to combat cancer.[1] Even after half a century, we are still struggling with problems associated with delivery of anticancer moieties, be it chemotherapeutic agents or biological agents. The chemical system is fraught with difficulties such as drug specificity and side effects; biological systems such as biological therapy, gene therapy, etc. have also not proven to be effective alternatives for cancer therapy.[2]

With advances in our understanding of cancer and its physiology, we have been able to improve treatment to a certain extent. It is now known that nanosized particles accumulate at tumor sites due to what is termed as enhanced permeability and retention effect.[3,4] This passive targeting mechanism coupled with targeting agents such as antibodies and ligands which interact with antigens and receptors, respectively, expressed preferentially among tumor cells have significantly improved tumor targeting.[5] More recently, targeting systems have been developed that exploit the use of external stimuli and the difference in physiology between normal cells and cancer cells.[6] Thermo-responsive micelles, which change from soluble form to aggregates by increasing temperature, have shown enhanced release of chemotherapy agents at tumor sites when locally heated.[7] The relatively high negative charges on the membranes of tumor cells, compared to normal cells, have been exploited by some drug carriers. These carriers have membrane-disrupting lytic peptides conjugated to their backbone, which selectively destroy cancer cells.[8,9]

Gene therapy is a promising biological tool for the treatment of cancer. The therapy treats the cause and not the symptoms of the disease.[10] It involves incorporation of therapeutic nucleotides inside the nucleus, where the nucleotide either repairs or blocks a damaged gene. There are many obstacles to effectively deliver the therapeutic gene into the cancer cells. The body would recognize the gene as a foreign object and rapidly clear it. At the cancerous region in vivo, the transfection is poor and only about 1% of what is present outside the cells is actually taken up.[11] Over the years, researchers have tried to solve these problems by complexing the gene with transfection promoting agents that also protect the gene during systemic circulation. The cationic polymers such as polyethyleneimine (PEI) and poly(L-lysine) have become popular for nonviral gene delivery.[10,12,13]

To get high transfection efficiency, excess polycation is usually complexed to the gene which results in a particle with a net positive charge. Neutral particles are preferred as our body has electrolytes and enzymes which recognize the charges on foreign particles and eliminate them.[14] One problem with complexing genes with PEI polymer is high cytotoxicity: the more the PEI content in the complex, the higher the cytotoxicity. The high content of PEI, however, increases the transfection to a certain extent, so typically a compromise is made between toxicity and transfection efficiency for the gene delivery.[15,16] Development of nanoparticles which shield the DNA/PEI complex during systemic circulation and expose the complex only at the tumor sites would eliminate the main drawback of nonviral gene delivery system and make it a viable option.

An interesting aspect in the physiology of tumors is the relatively lower pH of their extra cellular matrix (ECM) compared to the normal body pH. This acidic pH profile arises due to their high metabolic rate which leads to production of excess lactic acid and hydrolysis of ATP under hypoxic conditions.[17] Consistent results over the last few decades both by electrical and chemical probes have shown that the mean pH of tumor ECM is pH 7.06 with a range of 5.7−7.8.[18] This acidic pH is now regarded as a phenotype of the cancer cells

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necessary for their growth and invasiveness.[19] This small but significant difference in pH between tumors and normal tissues has instigated many investigators to try and develop pH sensitive materials.[20−22]

Most of the carriers developed so far do not have the high sensitivity that is required to respond to such small differences in pH. This is because the carriers were designed around the pH sensitive carboxylic group whose pK_a is not near physiological range.[23] In the past few years, our group has developed ultra pH-sensitive polymers which show sharp transition ranges around physiological pH.[24−27] The different systems developed, which are mainly based on sulfonamide pendent groups, are as follows: pH-sensitive polymeric micelles, nanoparticles, and self-assembled hydrogels that respond to minute changes in pH near physiological conditions.[28−30]

Here we discuss nanoparticle complexes formed by electrostatic interactions between plasmid DNA, PEI, and poly(methacyloyl sulfadimethoxine). Physicochemical properties of the nanoparticles were investigated in terms of size, pH-sensitivity, and zeta-potential. Cell cytotoxicity and transfection efficiency on a cancer cell line were also analyzed.

2. Materials and Methods

2.1. Materials. Sulfadimethoxine [4-amino-*N*-(2,6-dimethoxy-4-pyrimidynyl)benzenesulfonamide] (SD), fluorescein isothiocyanate (FITC), *N*-hydroxysuccinimide (HOSu), dicyclohexyl carbodiimide (DCC), and branched PEI (M_w = 25 000 Da) were purchased from Aldrich Chemical Co. (Milwaukee, WI) and used without further purification. α-Hydroxy-ω-carboxymethyl poly(ethylene oxide) (PEG monoacid) was synthesized and purified by Zalipsky's method.[31] Methacryloyl chloride (Aldrich) was distilled under reduced pressure (10 mmHg) at 30 °C, and dimethyl sulfoxide (DMSO, Aldrich) was purified by vacuum distillation at 75 °C at 12 mmHg. 2,2′-Azobisisobutyronitrile (AIBN) (Aldrich) was recrystallized in methanol twice. All other chemicals were of reagent grades and used without purification.

2.2. Synthesis of PSD-b-PEG. The synthesis of poly(methacyloyl sulfadimethoxine) (PSD) is described in our previous report.[29] Briefly, methacryloyl SD was prepared in 20 mL of acetone/water (1:1) and sodium hydroxide (0.01 N). SD was first dissolved in the acetone/water mixture in the presence of NaOH, and then methacryloyl chloride (10 mmol) was added dropwise into the mixture with simultaneous stirring at 0 °C. The precipitated product was filtered and washed with distilled water three times. A white powder was obtained after drying in vacuum at room temperature for 3 days.

Semitelechelic SD polymer was prepared by free radical solution polymerization of methacryloyl SD in the presence of 2-aminoethanethiol (0.15 mmol) as a chain transfer agent in DMSO. AIBN (0.2 mol % for monomer) was added as an initiator. Methacryloyl SD, 2-aminoethanethiol and AIBN were dissolved in 80 mL of DMSO. The mixture was degassed by freeze−thaw cycles. The round-bottom flask containing the reaction mixture was sealed under reduced pressure and immersed in an oil bath and kept at 60 °C for 20 h. After polymerization, the content was poured into deionized (DI) water to precipitate out the product. The polymer was collected by filtration. Unreacted monomers were eliminated by dissolving dried polymer in 10-fold excess methyl alcohol. Reactions were confirmed and monitored with IR and ^{1}H NMR spectra. The molecular weight was determined to be 3 KDa (PDI = 1.16) using MALDI-TOF mass spectroscopy. Thus, PSD is an oligomer rather than a polymer.

For the coupling of PSD with PEG monoacid (M_w = 2000 Da), PEG was activated in 30 mL of tetrahydrofuran (THF) by HOSu with a molar ratio of PEG:HOSu:DCC = 1:2:2. The PEG was dissolved in 30 mL of THF with HOSu and stirred, then SDM was added (mole ratio 1:1 with PEG), and the reaction continued for 1 h. DCC was then added, and the mixture was allowed to react for 12 h. The contents were poured into 300 mL of *n*-hexane for precipitation of the conjugate. Monomers and other impurities were removed by dissolving the polymer in DMSO and dialyzing (MWCO 3 KDa) against water for 3 days. The conjugation was confirmed by mass spectrometry.

2.3. Nanoparticle Assembly. DNA expressing Luciferase was first complexed with various amounts of PEI to form DNA/PEI complexes, resulting in different charge ratios (the ratio of the number of ionizable groups in each polymer, N/P for polycation/gene complex). Gel electrophoresis, particle size, and zeta-potential measurements were performed to obtain the optimum charge ratio. After fixing the N/P ratio at 4, the excess positive charge on the DNA/PEI complex was used to complex with the negatively charged PSD-*b*-PEG. Complexes of PEI/PSD-*b*-PEG were made with a charge ratio of 1.

2.4. pH-Sensitivity Studies of PSD and PSD-b-PEG using Light Transmittance. PSD, PSD-*b*-PEG, and PEI (control) were separately dissolved in 15 mL of 0.1 N NaOH solution at a pH of 12 (50 μmol/L). The solutions' pH was brought down to the required level using various quantities of 0.05 N HCl. The optical transmittance of the solutions at varying pH was measured at 500 nm using Varian Cary 1E UV/vis spectrometer. The transmittances at varying pH were expressed as percentage relative to the transmittance at pH 8.0.

2.5. Zeta Potential and Particle Size Measurements. Zeta potential and particle size were measured using laser light scattering technique on a Malvern Zetasizer 3000HS (Malvern Instruments, Worcestershire, U.K.) equipped with He−Ne laser (633 nm). The complexes were made by mixing various components at individual pH which had a 2 mM NaCl concentration. PEI (0.01 mg/mL) was complexed with PSD or PSD-*b*-PEG to make neutral particles. For DNA/PEI/PSD-*b*-PEG experiments, the complexes were formed at pH 9.0 at a PEI concentration of 0.1 mg/mL and the resulting particles were diluted in PBS buffer at various pH to get a final concentration of 0.01 mg/mL of PEI. For ionic strength experiments, the samples were assembled using DI water and diluted in various ionic strength PBS buffers.

2.6. AFM Imaging. AFM was employed to observe the polymeric complex morphology and particle size. Fresh mica surfaces were cleaved from 5 mm disks and soaked in magnesium acetate solution overnight. The mica was then cleaned with DI water and sonicated for 30 min. To remove all organic impurities, the dried mica surface was plasma-cleaned under oxygen. Dilute samples (0.01 mg/mL) were pipetted onto the mica surface for 2 min and washed gently to remove all buffer salts. Finally, the surface was dried under nitrogenous atmosphere. The samples were then analyzed with an AFM microscope (Pico Plus, Molecular Imaging INC., AZ) under MAC mode. The images were captured and edited with WSxM 4.0.[32]

2.7. Cell Viability Assay. The A2780 human ovarian carcinoma cell line was cultured in RPMI 1640 media (Sigma) supplemented with 10% fetal bovine serum (FBS: Gibco) and 10 mg/L insulin and kept at 37 °C in a humidified atmosphere of 5% CO_2.

Cells were seeded in 12 well plates at a density of 2.5×10^5 cells per well and incubated for 24 h. The medium with serum was replaced with media without serum, half the wells with medium at pH 6.6 and the other half with medium at pH 7.4, for 1 h before addition of various complexes. Serum free incubation was done to prevent any non specific interaction between the serum protein and polyplexes. Various complexes (0.5 μg DNA; N/P=4) were added and the cells were incubated for 4 h. The two pHs were used to discern the difference in attachment/detachment of the pH sensitive sulfonamide polymer (PSD-*b*-PEG). The serum free media were replaced with serum containing medium at pH 7.4 and incubated for 24 h. This incubation was done to bring the test cells into stable and comparable environment. A total of 100 μL of MTT solution was then added to each well and the well plates were incubated at 37 °C for 4 h. The medium was then removed, 500 μL DMSO was added and again incubated at 37 °C for 10 min. Percentage viability was calculated by transferring 100 μL of this solution into a 96-well plate reader and detecting absorbance at 570 nm, using a plate reader (Bio Rad USA).

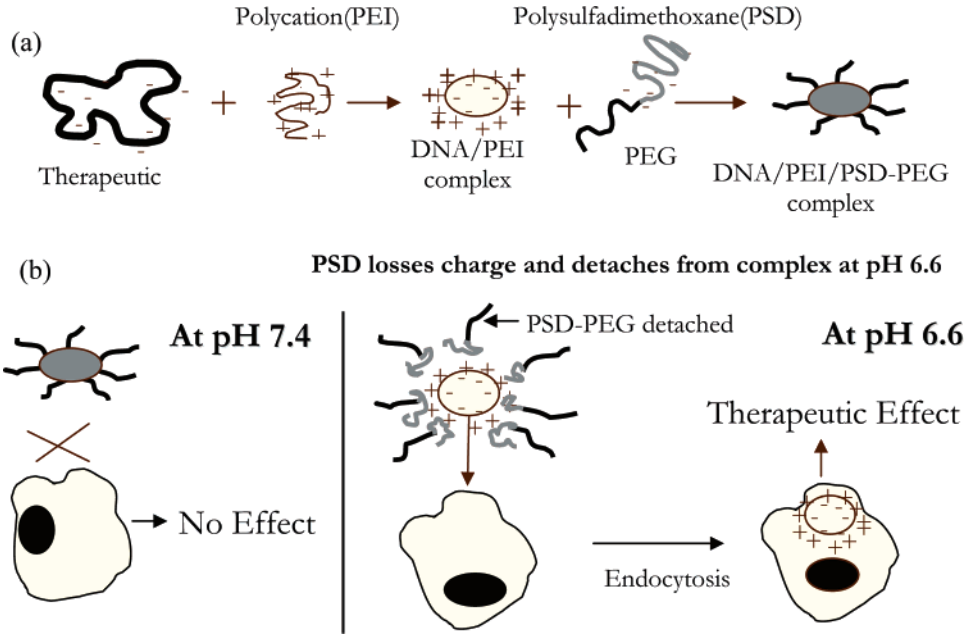

Figure 1. Targeting based on difference in pH: (a) shows formation of the nanoparticle complex through charge−charge interaction between DNA, cationic polymer (PEI) and PSD-*b*-PEG; (b) shows the complex shielded at physiological pH and deshielded at cancer pH.

2.8. Luciferase Assay. Cells were seeded in 6 well plates at a density of 5×10^5 cells per well and incubated for 24 h. The procedure for addition of complexes at pHs 6.6 and 7.4 was similar to cell viability assay. After incubation for 24 h, the medium was removed, and the cells were washed twice with PBS buffer solution. Lysis buffer (200 μL) was added to each well, and the plates were stored in −20 °C for 12 h. These cells were then analyzed for Luciferase activity using standard protocol and were normalized for protein content using BCA protein assay kit (Pierce, U.S.A.).

The Luciferase activity was determined using Luciferase assay system kit (Promega, U.S.A.) on a Plate Lumino Luminescence Analyzer.

2.9. Statistical Analysis. All of the experiments were repeated at least three times with a minimum sample size of three. Student's *t*-test was used, using STATA statistical software (StataCorp LP, Texas), to test for statistical differences between samples. Comparisons having '*p*' values ≤0.05 were considered significant.

3. Results and Discussion

The polymeric nanoparticle formed through electrostatic attraction is designed in such a way that the final particle is neutral. The neutral particle has the least interaction with the body and would be more stable compared to anionic and cationic particles, during systemic circulation. The central idea of this design is that when the particles experience a decrease in pH as they extravasate into tumor tissue due to enhanced permeability and retention effect, the sulfonamide groups would lose their charge and get detached from the carrier complex. The detachment of sulfonamide from the complex exposes pCMV-Luc DNA condensed with cell transfecting PEI, to act on cancer cells. The schematic is shown is Figure 1.

3.1. pH-Sensitivity of PSD and PSD-b-PEG. The pH response of PSD and PSD-*b*-PEG were examined by making the polymer solutions at various pH and measuring their turbidity. Figure 2a shows the pH-dependent turbidity profiles of these two polymers along with PEI (control). It is clearly seen that both PSD and PSD-*b*-PEG show very sharp pH transitions. PSD shows a transition, from being in solution phase to forming aggregates, between pH 6.6 and 6.4, whereas PSD-*b*-PEG shows a transition between pH 6.9 and 6.8. The transition point of PSD-*b*-PEG has shifted a little higher from PSD. The reason is not clear at the moment but is probably due to the conjugation of PEG which influences the local environment and PSD solubility. Other factors may include increased overall MW and removal of the primary amino group from PSD.

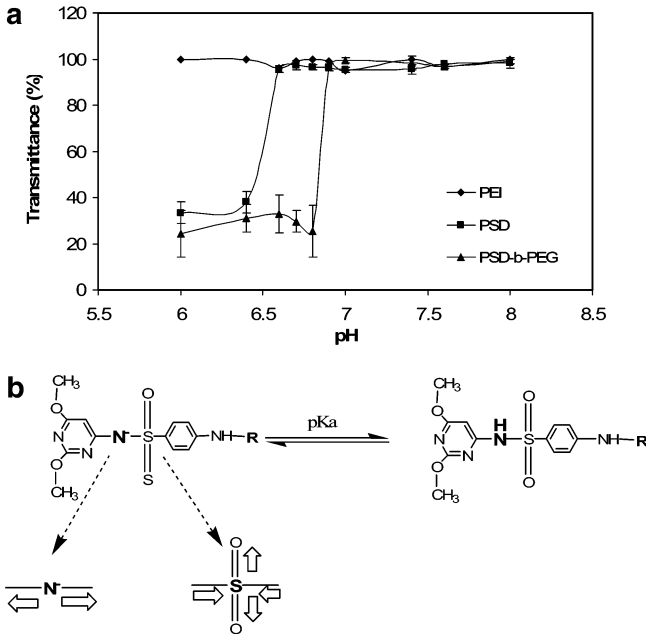

Figure 2. (a) pH response of various polymers. PSD shows sharp transition between pH 6.6 and pH 6.4. At the same time PSD-*b*-PEG has a transition at between 6.9 and 6.8. ($p < 0.05$ for both PSD polymers near their transition pHs). (b) Chemistry of sulfonamide pH transition.

The carboxylic acid based polymers show transitions in about one pH unit which is very broad, and that transition is much below the physiological and tumor pH range, whereas the PSD-*b*-PEG polymer shows transition within 0.2 pH units between the physiological and tumor pH. These sulfonamide polymers, compared to polymers based on carboxylic groups, have a much sharper transition.[33,34] The sulfonamide group is readily ionizable

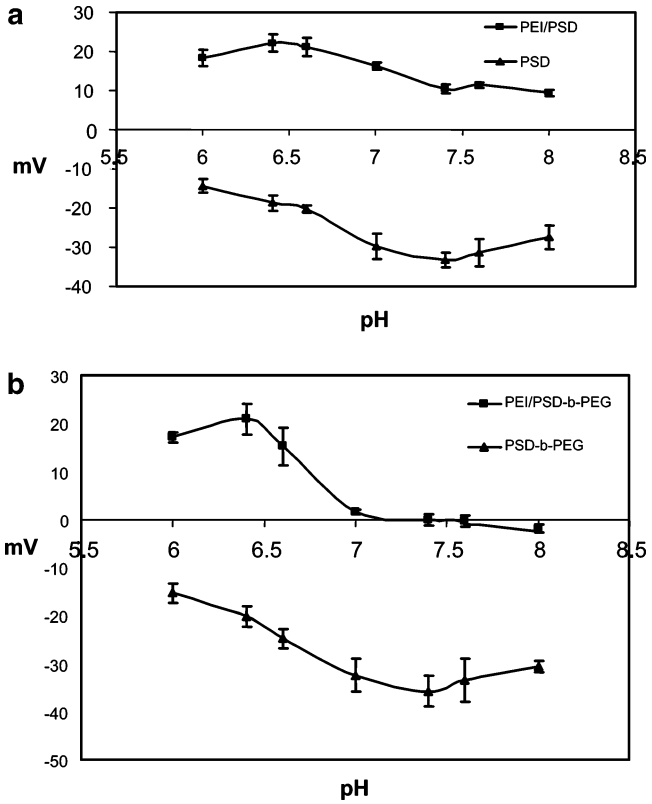

Figure 3. Shielding effect of PSD and PSD-*b*-PEG on PEI. (a) Zeta potential of PSD decrease with decrease in pH. When complexed with PEI there is good shielding until pH 7 after which we see a higher zeta potential indicating detachment. (b) Similar trend is shown by PSD-*b*-PEG.

at high pH because the strong electronegativity of the oxygen atoms of the sulfonyl group draws electrons from the sulfur atom, which in turn pulls the electrons from the nitrogen atom (Figure 2b). This results in nitrogen pulling electrons from the N−H bond and thus releasing the proton. The pK_a of the sulfonamide compound is governed by the dimethoxypyrimidine group, which is a electron withdrawing group. Here the polymer used has a low degree of polymerization (DP ≈ 7) and very narrow polydispersity (PDI = 1.1), confirmed by MALDI-TOF analaysis, due to which all of the chains of the polymer experience similar forces. This may be one of the main reasons for the polymer to show such sharp transition. It is noted here that this crucial transition of the PSD-*b*-PEG is ideally suited for targeting to tumor cells.

3.2. Shielding of Cationic PEI by PSD and PSD-b-PEG. The shielding effect of the pH-sensitive PSD and PSD-*b*-PEG, on cationic polymer PEI, was measured at different pH using the zeta potential. A negative or neutral zeta potential means the positively charged PEI is completely complexed and is being shielded (Figure 3).

PSD shows negative zeta potential of about −30 mV between pH 8.0 and 7.0 and the potential gradually increases at lower pH, indicating that the degree of ionization of PSD decreases below pH 7.0. Shield effect of PSD on PEI is good from pH 8.0 to 7.0, as indicated by the low positive zeta potential, after which there is an increase in zeta potential. This is most likely due to the positively charged PEI being separated from PSD which is only partially ionized. The polymer PSD-*b*-PEG also shows a similar trend like that of PSD, but it seems like it has a better shielding property with PEI than PSD by itself because the polymer is able to keep the zeta potential at near neutral levels till pH 7.0 compared to PSD which shows complex

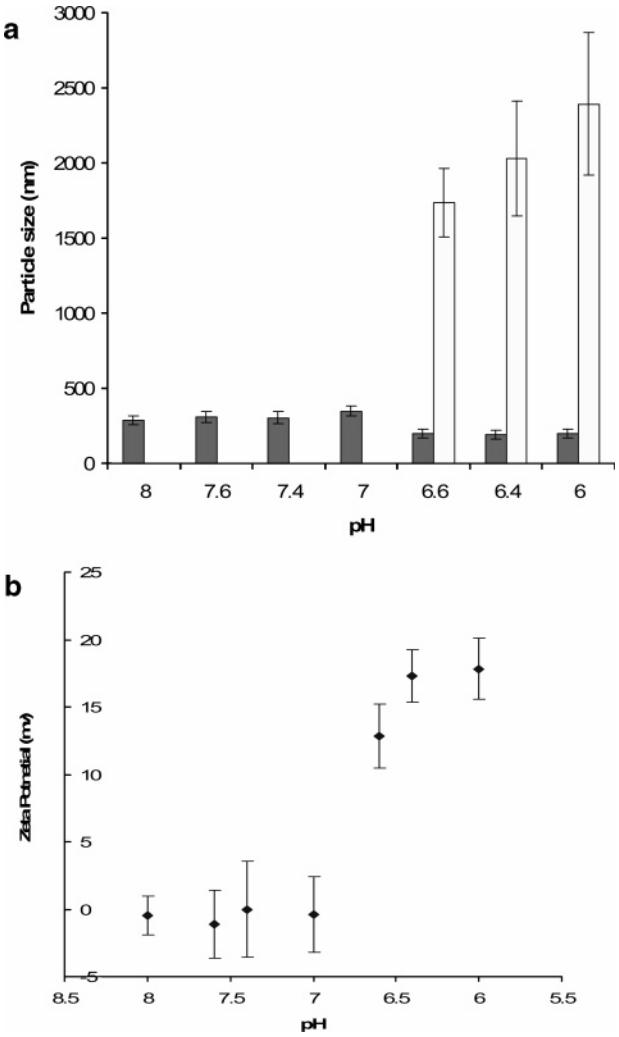

Figure 4. Particle size and zeta potential of DNA/PEI/ PSD-*b*-PEG complexes at various pHs. (a) Particle size of the complex from pH 8.0 to pH 6.0. From pH 8.0 to 7.0 unimodal particle distribution was observed, indicating PSD-*b*-PEG complexed with DNA/PEI, 6.6 to 6.0 showed bimodal particle distribution, the solid bars could be DNA/PEI complex and the open bars may be the aggregates of neutral PSD-*b*-PEG. (b) Zeta potential study indicates similar trend, the particles show near zero zeta potential between pH 8.0 and 7.0 that indicates complete complexation of PSD-*b*-PEG; positive potential from pH 6.6 to 6.0 indicates decomplexation.

potential with PEI at 10 mV. The better shielding may be due to the presence of PEG in PSD-*b*-PEG, which has been proven to shield charges in a complex.[35] The zeta potential of PSD-*b*-PEG polymer by itself is similar to that of PSD alone. Figures 2 and 3 substantiate the claim that PSD-*b*-PEG is highly pH-sensitive near the tumor pH range and should be able to release a positively charged carrier near an acidic tumor environment.

3.3. Particle Size and Zeta Potential Studies of DNA/PEI/ PSD-b-PEG as a Function of pH and Ionic Concentration. After determining the optimum N/P ratio for the plasmid (DNA)/PEI complex with agarose gel electrophoresis, zeta potential and particle size (data not shown), we selected the charge ratio of PEI/DNA as 4 (N/P = 4). This ratio was used for all particle size, zeta potential, AFM, and in vitro studies. The particle size of the polyplex DNA/PEI/PSD-*b*-PEG is about 300 nm from pH 8.0 to 7.0 and population distribution is unimodal (Figure 4a). The normal size of DNA/PEI complexes are about 200 nm.[36] The addition of negatively charged PSD-*b*-PEG to the polyplex seems to have increased the final size. The charge ratio of N/P = 4 for DNA/PEI has net positive charge on the outside

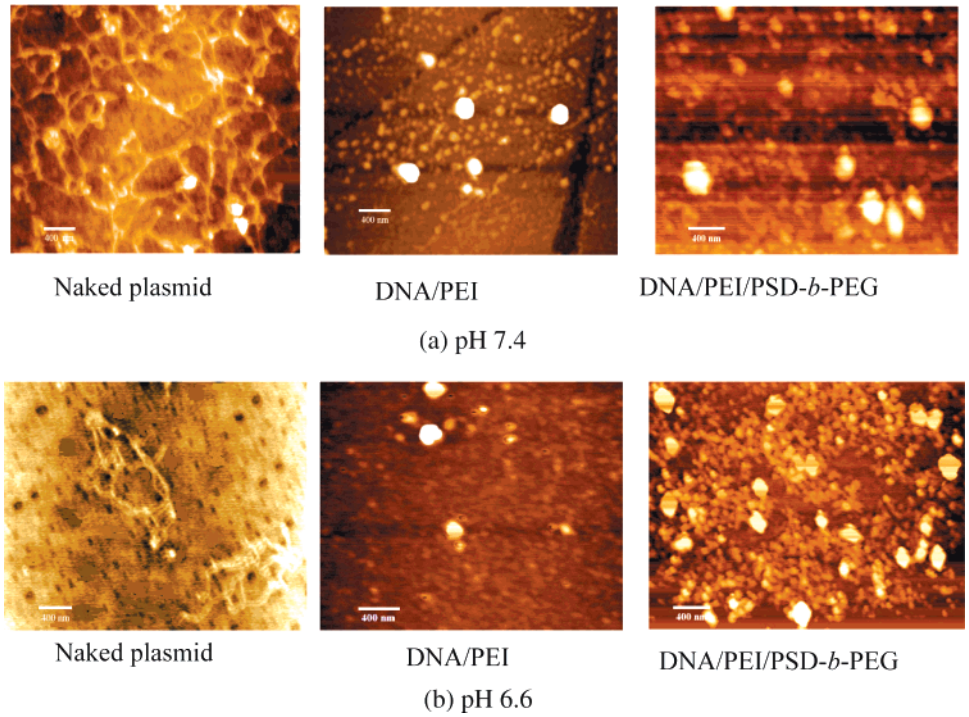

Figure 5. AFM images of complexes at different pHs. (a) complexes at pH 7.4 are shown. Plasmid in circular disks of about 1000 nm can be clearly seen. Addition of PEI condenses the plasmid to form particles of about 200 nm. Addition of PSD-*b*-PEG expands the complex to about 300 nm, indicating interaction of PEI with both DNA and PSD-*b*-PEG. (b) Similar trend is observed at pH 6.6 except with DNA/PEI/PSD-*b*-PEG complex. Here the addition of PSD-*b*-PEG does not affect the size, indicating low or no interaction. (Scale bar: 200 nm)

of the complex and this would result in repulsion within the particle. PSD-*b*-PEG, on complexation with PEI reduces the electrostatic interaction between PEI and DNA resulting in slight destabilization of the particle. This destabilization manifests as an increase in particle size. Between pHs 6.6 and 6.0, a bimodal distribution of particles is observed. This strongly indicates that pH sensitive PSD-*b*-PEG may have detached from DNA/PEI polyplex. One population is on an average 200 nm, which may correspond to the PEI/DNA polyplex and the other population is in micro-size range and may be that of aggregated PSD-*b*-PEG. The aggregated particle size increases with decrease in pH, this may be because more PSD-*b*-PEG is detached at pH 6.0 compared to pH 6.6. The zeta potential experiments yielded similar results (Figure 4b), wherein the potential remained near zero between pH 8.0 and 7.0 and increased sharply to about 15 mV at pH 6.6 and below. These results agree with those of Wolff et al. where they report that introduction of polyanions into a DNA/polycation complex increases the size.[37] Their complexes were of the ratio 1:2:1 and the polyanion had a high charge density due to which the polyanion destabilized the initial complex. The present complexes were formed at 1:4:3 ratio and the polyanion used was of comparatively low molecular weight (PEI 25 kDa; PSD 2 kDa).

Figure 5 shows AFM images of DNA, DNA/PEI complex (N/P=4) and DNA/PEI/ PSD-*b*-PEG (N/P = 4; the remaining three positive charges of PEI complexed with PSD-*b*-PEG) at two pH 7.4 and 6.6. Addition of PEI to plasmid condenses it from being irregular shaped 1000 nm rings to a condensed particle of about 200 nm. At pH 7.4 with the addition of PSD-*b*-PEG this particle expands to about 300 nm.

A similar trend is seen in AFM analysis at pH 6.6. Only here, addition of PSD-*b*-PEG does not increase the particle size of the complex. This indicates that at pH 6.6, sulfonamide has lost its charge and is aggregated, due to which it is not able to complex with the charged PEI. Some clusters are also seen on the AFM image at pH 6.6. These clusters may actually be the aggregates formed by the PSD-*b*-PEG. The potential problems in future in vivo tests associated with PSD-*b*-PEG aggregation could be reduced by a diluted environment and employing quickly degradable PSD after decomplexation.

The particle sizes of the DNA/PEI/PSD-*b*-PEG complexes were measured as a function of ionic concentration to determine if the particles would be stable at physiological pH (Figure 6a). It was observed that the particle size increased steadily with ionic concentration. The ionic concentration of 5 mM NaCl yielded the smallest particle size (275 nm) and ionic concentration of 250 mM NaCl yielded the largest particle (550 nm) and particle size at physiological ionic concentration (150 mM) was 425 nm. The particles were stable over a period of 3 days at 37 °C as observed in Figure 6b and did not show any disintegration or aggregation. A similar trend is observed for DNA/PEI complex as well. All of the in vitro studies were done in RPMI medium having an ionic concentration of 150 mM. The particle size of 425 nm at physiological ionic concentration may not be very effective in utilizing the EPR effect[38] and would need more time to extravasate into tumor sites but the pH-sensitive shielding of the carrier should be able to protect the gene during this prolonged circulation.

3.4. pH Dependent Cell Viability. After establishing that the PSD-*b*-PEG polymer has good pH sensitivity, the shielding effect was tested in vitro by performing MTT assay on the A2780 ovarian adenocarcinoma cancer cell line. Figure 7 shows the results of the viability of this cell line in various test conditions. The cells were incubated in RPMI medium at pH 7.4 or pH 6.6 with various complexes for 4 h and then replaced with normal medium (pH 7.4) having serum and incubated for 24 h before performing the viability tests. The lowering of pH to 6.6 for 4 h did not affect the cell viability of these cells, as there is no significant difference between cells at pH 7.4 and pH 6.6 where there has been no addition. Addition of just the plasmid to cells does not reduce the viability at both pHs because the negatively charged DNA does not interact with the

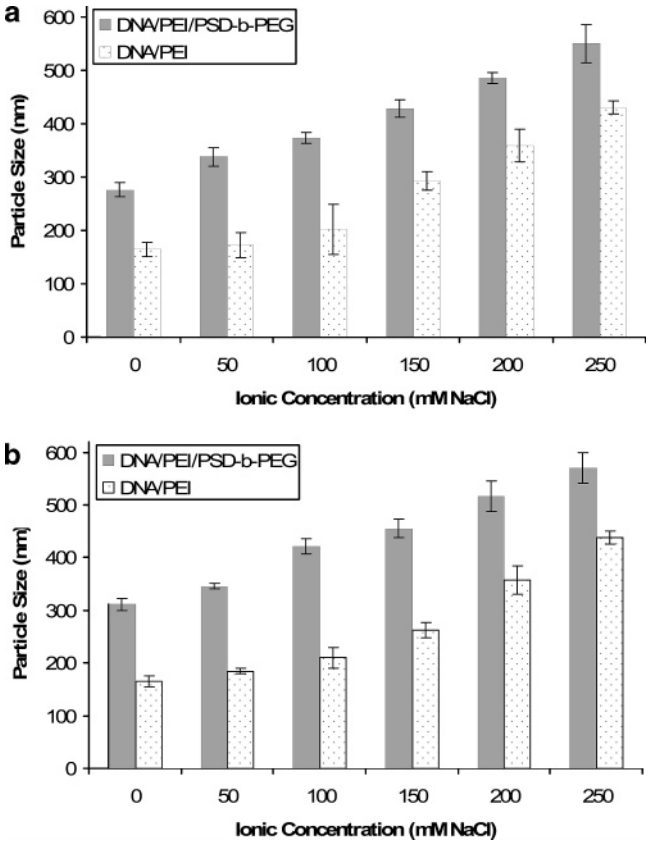

Figure 6. Stability of DNA/PEI and DNA/PEI/ PSD-*b*-PEG complex as a function of ionic strength. (a) Particle size variation with ionic concentration at N/P ratio 4 for DNA/PEI/PSD-*b*-PEG and DNA/PEI complexes. (b) Size of the same complexes after 3 days of incubation at 37 °C.

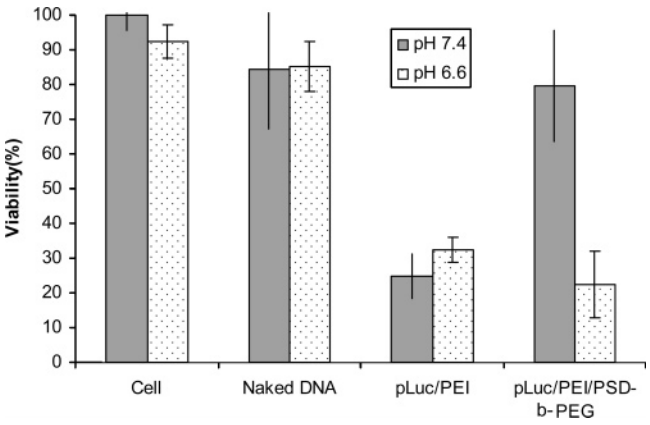

Figure 7. Cell viability of adenocarcinoma cells A2780. (a) Addition of plasmid to cells does not reduce the viability at both pHs. When DNA/PEI complex is added the viability is reduced to about 30% at both pHs. Complexation with PSD-PEG is able to sustain the viability at 80% at pH 7.4 but not at pH 6.6 where the viability is similar to DNA/PEI.

negatively charged plasma membrane of cells. When the DNA/PEI complex is added the viability is reduced to about 30% at both pHs. The charge ratio of N/P = 4 has excess of PEI which promotes transfection but also increases cytotoxicity of cells.[39] The cytotoxicity is produced due to the interaction of PEI with the cell membrane followed by disruption of the lipid bilayer.[40]

The PSD-*b*-PEG polymer at pH 7.4 is able to sustain the viability at 80% compared to 30% for DNA/PEI complex (statistically significant, $p < 0.05$). This means the sulfonamide of the polymer is complexed with PEI and the PEG component

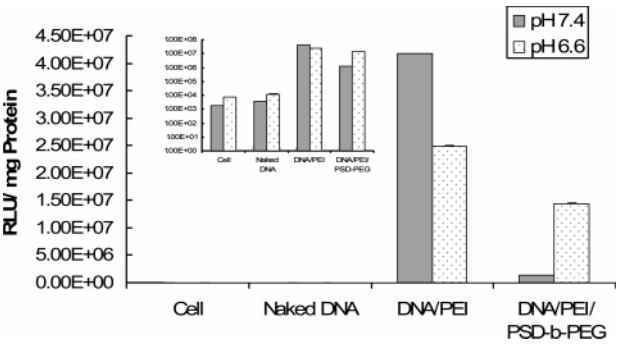

Figure 8. Luciferase gene transfection of various complexes on adenocarcenoma cells A2780; The control cells and plasmid DNA shows negligible transfection at pH 7.4 and 6.6. The DNA/PEI complex shows high transfection at both pH. The DNA/PEI/ PSD-*b*-PEG complex shows low transfection at pH 7.4 indicating PEI shielding, at the same time pH 6.6 shows high transfection, indicating low shielding. Inset figure: Same graph in logarithmic scale.

is preventing PEI to interact with the cell membrane. In the meantime, the PSD-*b*-PEG polymer is not able to increase viability at pH 6.6. At pH 6.6, due to formation of hydrophobic aggregates, the sulfonamide is not able to complex with PEI and the exposed PEI is able to interact with the cell membrane thus increasing the cytotoxicity of the cells.

3.5. Gene Internalization by the Nanoparticles. An analysis of the extent of internalization of the nanoparticles helps understand the behavior the polymers and their complexes at the two pHs. The plasmid DNA is pCMV−Luc that encodes the Luciferase gene, which is a common reporter for plasmid integration into the nucleus.[41] The internalization of these nanoparticles was followed by performing the Luciferase assay (Figure 8) on the A2780 cell line.

Cells without any addition (controls) and plasmid DNA addition show negligible transfection at both pHs. The DNA/PEI complex shows high transfection at both pHs because of the PEI present in the complex. The DNA/PEI/PSD-*b*-PEG complex shows low transfection at pH 7.4 compared to pH 6.6 (statistically significant, $p < 0.05$) indicating PEI shielding. The electrostatic charge−charge interaction between PEI and PSD-*b*-PEG is preventing the PEI to interact with the cells to enhance transfection. It is observed that for the same nano particles, pH 6.6 shows high transfection indicating de-shielding of PEI due to unionization and subsequent hydrophobic aggregation of PSD-*b*-PEG. There is a 10 fold decrease in transfection at pH 7.4 compared to pH 6.6 which indicates good shielding and deshielding. The cell viability and gene internalization studies indicate the same phenomenon of shielding at 7.4 and de-shielding at pH 6.6. These data together with the particle size, zeta potential and transmission data clearly establish that the pH sensitive polymer PSD-*b*-PEG is able to recognize the small difference in pH and may be suitable for targeting acidic tumors.

The difference between transfection efficiencies of DNA/PEI and DNA/PEI/PSD-*b*-PEG at pH 6.6 is small but suggests that perhaps not all of PSD-*b*-PEG detaches from the complex at this pH. We are trying to quantitate the binding and detachment efficiency and thereby use the information to optimize the polymer.

This polymeric carrier may not be useful for tumors that do not have a pH below 7.0. The systemic administration of glucose has been shown to effectively decrease the pH of tumor areas and this technique can be used along with the polymeric carrier.[21] This artificial decrease can benefit in two ways: (1) tumors that are not very acidic can be made acidic which would enable the use of the pH sensitive polymeric carrier and (2) the

tumors that are acidic can be made more acidic thus making the pH sensitive polymer more effective.

4. Conclusion

In the present study, we have shown that the small difference in pH may be exploited by use of a pH sensitive material. Our ultra pH sensitive polymer, which shows a sharp pH profile, was able to shield positively charged complexes at physiological pH of 7.4. The pH sensitive polymer was able to detach from the complex when the pH environment decreased to pH 6.6. This detachment helped to expose the active therapeutic, which was shown by an increase in transfection of the reporter gene at pH 6.6. Designs similar to the one described here can be employed to mask highly toxic moieties and/or can be combined with any electrostatically charged carrier system to bestow specificity.

A main disadvantage of our polymer is that it is nondegradable and the aggregates formed may be toxic. It is also noted that this system may not be effective in tumors which have pH very close to physiological range. A polymer that would shield during systemic circulation but after detachment from the complex would quickly degrade is under development. We are currently investigating the in vivo efficacy of this carrier in preferentially activating genes at the tumor site.

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