**Conjugation with Betaine: A Facile and Effective Approach to Significant Improvement of Gene Delivery Properties of PEI**

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Supporting Information

**ABSTRACT:** Herein, we developed a new gene delivery vector by grafting a betaine monomer (N,N-dimethyl-(acrylamidopropyl)ammonium propane sulfonate, DMAAPS) onto 25 KDa polyethylenimine (PEI 25K) via the Michael addition reaction. The graft ratio for betaine on PEI polymer could be readily controlled, and in this study three PEI-betaine conjugates PEI-DMAAPS23%, PEI-DMAAPS55%, and PEI-DMAAPS95% were prepared with their graft ratios of 23, 55, and 95%, respectively. The PEI-betaine conjugates exhibited much lower protein adsorption and cytotoxicities compared with PEI 25K, and they also showed little or no hemolytic effect. Moreover, the PEI-betaine conjugates display satisfactory DNA condensation capability; and in the absence and presence of serum, PEI-DMAAPS23%/pEGFP and PEI-DMAAPS55%/pEGFP complexes exhibited remarkable gene transfection efficiencies determined by flow cytometry, which are in general several times higher than that of PEI 25K. With these favorable properties, the PEI-betaine conjugates hold great potential for use as efficient gene delivery vectors. This study suggests that the betaine monomer may serve as a biocompatible modifying agent and this facile strategy may provide a facile and effective way for constructing some other biocompatible materials.

**INTRODUCTION**

Polyethylenimine (PEI), an off-the-shelf material, has been used as a gene-delivery vector since 1995 and become one of the most promising and widely studied gene carriers1−7 due in large part to efficient escape from the endocytic pathway through the proton-sponge mechanism.8,9 When PEIs are used as the gene carriers, the high cationic charge densities in the polymeric chains result in tight DNA condensation, leading to highly disruptive properties that frequently cause high cytotoxicities,10−12 the strong binding with negatively charged proteins which disrupts uptake in vitro; however, high cationic charge densities also bring about some severe problems, for example, the membrane-disruptive properties that frequently cause high cytotoxicities,13−15 the strong binding with negatively charged proteins which deteriorate serum stability of the polyplexes, and so on.9−11 Hence, PEI has been chemically modified in different ways so that additional functions or enhanced performances were achieved for a better gene delivery, and the modification methods include the incorporation of short hydrophobic substituents to yield more effective carriers,12−14 PEGylation or hydroxylolation to improve the serum stability during circulation,15−17 linking to polysaccharides to enhance biocompatibility,18,19 cross-linking of low molecular weight PEI to reduce cytotoxicities,20,21 and attachment of some functional molecules to target specific cells or tissues.22−24

Among the PEI modification approaches, PEGylation is a commonly applied conjugation method that affords the polypexes with stealth behavior, because PEG takes on a ballooning-type conformation that increases solution viscosity and acts as a water barrier that prevents proteins from approaching the surface.25 Furthermore, PEG reduces the tendency of particles to aggregate by steric stabilization, thereby producing formulations with increased stability during storage and application.26,27 Currently, PEG remains the most used polymer and the gold standard in biomedical applications. On the other hand, the search for potential alternatives with comparable or even better properties is attracting attentions due to some limitations of PEG such as compromised bioactivity in some applications.28 In recent years, zwitterionic polymers, such as phospholipid-based, polymer-like poly(2-methacryloyloxyethyl phosphorylcholine) (PMPC)29−36 and betaine-based, polymer-like poly(carboxybetaine) (PCB) and poly(sulfobetaine) (PSB),37,38 have been applied to a broad range of biomedical and engineering materials. Due to the electrostatically induced hydration, surfaces modified by (via conjugation or coating) zwitterionic polymers are highly resistant to nonspecific protein adsorption, bacterial adhesion, and biofilm formation. For examples, the pioneering studies performed by Ishihara et al.
have shown that the MPC polymers exhibit excellent biocompatibility based on inhibition of protein adsorption and activation on the surface, and the MPC-based polymers can be applied for surface coating of many medical devices including implantable artificial organs.\(^49\) On the other hand, Jiang and co-workers\(^39\)–\(^41\) have ingeniously developed a series of betaine-based zwitterionic polymers that are more resistant to nonspecific adsorption of proteins and can provide stability, prolonged circulation, reduced degradation, and increased water solubility when covalently attached to a protein. As for prolonged circulation, reduced degradation, and increased overall gene delivery properties of PEI (25 kDa) by coating a zwitterion polymer with PEI; thereby, conjugating the latter enhanced gene delivery properties along with good biocompatibility. The conjugation of zwitterion monomer with PEI is technically much simpler and more efficient than the reaction of zwitterion polymer with PEI; thereby, conjugating zwitterion monomer with PEI might become a technically simple and highly efficient method for constructing biocompatible and high-performance gene delivery vector.

With this idea in mind, in this study, we demonstrate an alternative and facile strategy to significantly improve the overall gene delivery properties of PEI (25 kDa) by coating a large amount of betaine monomers (not its polymer) to PEI through a Michael addition reaction. This betaine-monomer-modified PEI exhibits extraordinary gene delivery efficacy, low cytotoxicity and protein absorption, excellent anticoagulant property, and excellent serum tolerance; in addition, for this betaine-betaine-modified PEI, the overall performance concerning gene transfection is outstanding among various PEI derivatives. More importantly, this simple and safe approach is highly reproducible, which is essential for the practical applications of manufactured biomaterials.

**EXPERIMENTAL SECTION**

**Materials.** N-[3-(Dimethylamino)propyl] acrylamide (DМААA, 98%) and 1,3-propanesultone were purchased from TCI Shanghai. The Dulbecco’s modified Eagle’s medium (DMEM), trypsin-ethylenediaminetetraacetic acid (Trypsin-EDTA), penicillin-streptomycin, and fetal bovine serum (FBS) were obtained from Invitrogen. A 25 kDa branched polyethylenimine (PEI, 25 kDa, primary amine content ~25% in mole), ethidium bromide (EB), and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma. The plasmid DNA [EGFP-N1 (4.7Kb) encoding enhanced fluorescent protein] was purchased from Clontech (Palo Alto, CA) and stored at ~20 °C until the transfection experiments. All other reagents were of analytical grade and used as received. Van Kampen and Zijlstar solution was purchased from Shanghai Rongbai Biotechnological Technology Co. Ltd.

**Synthesis of N,N-Dimethyl(acrylamidopropyl)ammonium Propane Sulfonate (DMAAPS).** DMAAPS was synthesized by the ring-opening reaction of 1,3-propanesultone with N,N-dimethylamino-propylacrylamide (DMAАA) in anhydrous acetone at ambient temperature.\(^32\) DMAАA (4.0 g, 25.6 mmol) and acetone (15 mL) were charged into a 50 mL dried round-bottom flask. 1,3-Propane-sultone (3.0 g, 24.6 mmol) solution in 15 mL of anhydrous acetone was added dropwise into the flask in 0.5 h and stirring was proceeded for 16 h at the same temperature. The resulting white precipitate was filtered, washed with acetone, and dried under vacuum, yielding a white powder, 92% (6.29 g, 22.6 mmol). \(^1^H^\) NMR (δ ppm, in D2O, Figure S1): 2.07 (m, 2H), 2.22 (m, 2H), 2.90 (t, J = 8 Hz, 2H), 3.12 (m, 6H), 3.40 (m, 4H), 3.49 (t, J = 8 Hz, 2H), 5.80 (d, J = 12.0 Hz, 1H), 6.19–6.52 (m, 2H). In mass spectrum (Figure S2), mass peaks at m/z 278.9 corresponds to [DMAАA + H].

**Synthesis of DMAAPS-Modified PEI (PEI-DMAAPS).** In a typical run, to a reaction flask containing 25 kDa PEI (450 mg, 0.018 mmol) in 12 mL of methanol under a nitrogen atmosphere, DMAАA (436 mg, 1.6 mmol for the graft ratio of 55%) was added. The reaction mixture was stirred at room temperature for 3 days and then dialyzed (MW cutoff: 3500) for 48 h against distilled water. The PEI-DMAАA conjugate was collected after freeze-drying; the final yields 56% (476 mg, 0.01 mmol).

**Characterization.** \(^1^H^\) NMR spectra of the samples was recorded on a Bruker Avance 400 MHz NMR spectrometer using D2O as the solvents at 25 °C. The absorbance measurements were carried out using a Hitachi U-3010 UV–vis spectrophotometer. The particle size was determined by DLS using a Malvern Nano-ZS90 Zetasizer. The cell imaging was performed on an Olympus IX71 inverted fluorescence microscope equipped with a DP72 color CCD (excitation: 460–490 nm). The gene transfection efficiency was evaluated by scoring the percentage of cells expressing EGFP using a Beckman Coulter Epics XL flow cytometer with EXPO32 ADC software (Beckman Coulter, U.S.A.).

**Cell Culture.** Two cell lines (HeLa and HepG2) were cultured in DMEM supplemented with 10% FBS (penicillin/streptomycin 100 U/mL). All cells were incubated at 37 °C in a humidified 5% CO2 atmosphere.

**Buffering Capacity.** The buffering capacity of PEI-DMAАA samples were determined by acid–base titration assay. Briefly, 0.2 mg/mL of each sample solution was prepared in 30 mL of 150 mM NaCl solution. The pH was adjusted to 10 by using a 0.1 M NaOH solution. The polymer solution was then titrated with 0.1 M HCl at room temperature. The pH values for all solutions were measured using a microprocessor pH meter (Sartorius PB-10).

**Hemolysis Test.** The hemolysis test was adapted from Standard Practice for Assessment of Hemolytic Properties of Materials (ASTM designation: F 756-08). The total blood was freshly collected from three rabbits and mixed. Determination of total blood hemoglobin was accomplished by using cyanmethemoglobin reagent (Van Kampen and Zijlstar Solution) and measuring the absorbance of free hemoglobin in solution at 540 nm. The total blood hemoglobin concentration was used to adjust the hemoglobin content of the blood sample to 10 ± 1 mg/mL by adding blood and an appropriate amount of 1× PBS solution. Rabbit blood, 1 mL, was incubated with 50 μL polymer or complex solution in PBS at 37 °C for 3 h under gentle shaking. After 3 h, samples were centrifuged at 750 g for 15 min. Supernatant was collected, added 1:1 to equivalent volume of cyanmethemoglobin reagent and incubated for 5 min before measurement. Absorbance measurements at 540 nm were then recorded using a Hitachi 3010 spectrophotometer. Triton X-100 and PBS were used as positive and negative controls, respectively. Total hemoglobin released from whole blood diluted in the cyanmethemoglobin reagent was determined as 100% hemoglobin release. Hemolysis was expressed as the percentage of hemoglobin released to total content. Tests were done in triplicate.

**Protein BSA Adsorption Assay.** A total of 1 mL of polymer solution (1 mg/mL) and 1 mL of BSA solution (2 mg/mL) were mixed and shaken at 37 °C for 0.5 h. The mixtures were centrifuged and the supernatants were collected carefully for obtaining the characteristic UV absorbance at 280 nm. The BSA concentrations in supernatants were determined using a calibration curve obtained from...
BSA solutions of known concentrations. The protein adsorption values \( A \) are defined as

\[
A = \frac{C_V - C_{V_i}}{m}
\]

where \( C_i \) and \( C_f \) are the initial BSA concentration (2 mg/mL) and the BSA concentration in the supernatant determined by adsorption experiments, respectively; \( V_i \) is the initial volume of the BSA solution (1 mL); \( V_f \) is the final volume of the BSA after adsorption measurement (2 mL); and \( m \) is the weight of the polymer (1 mg) added into the solution.

**Cytotoxicity Assay.** The cytotoxicities of the polymer vectors were examined by MTT assay and 25 kDa PEI (PEI 25K) was used as the positive control. For the assays, HeLa and HepG2 were seeded into a 96-well plate at a density of 1 \( \times \) 10\(^5\) cells per well, respectively. After 24 h of incubation at 37 °C, cells were washed with prewarmed PBS buffer. Then, the cells were treated with PEI-DMAAPS conjugates or PEI 25K dissolved in media. After 48 h of incubation, the plates were washed with PBS buffer and incubated for another 4 h with RPMI 1640 medium containing 0.5 mg/mL MTT. After discarding the culture medium, 150 μL of DMSO was added to dissolve the precipitates and the absorbance was recorded with a Thermo MK3 ELISA reader at 570 nm and the statistical mean and standard deviation were used to estimate the cell viability. Untreated cells served as control with 100% viability, and wells having MTT reagent only (without cells) were used as blank to calibrate the spectrophotometer to zero absorbance.

**Preparation of Polymer/pDNA Complexes.** All polymer/pDNA complexes were freshly prepared before use. Complexes at different N/P ratios were prepared by slowly adding appropriate volume of polymer solution (1.0 mg/mL in 150 mM NaCl solution) into 2 μL of plasmid EGFP-N1 DNA (1.0 μg/μL in pure water) with gentle vortexing and incubated for 30 min at room temperature to ensure effective formation of polymer/DNA complexes. The N/P ratio can be calculated using the following formula:

\[
N/P \text{ ratio} = M / (Q \times 3)
\]

where \( M \) represents the nitrogen atoms of PEI in the carriers (nmol) and \( Q \) equals the quantity of DNA (μg).

**Agarose Gel Electrophoresis.** The efficiency of DNA condensation was assessed by agarose gel electrophoresis. The solutions of complexes with varied N/P ratios were prepared and their final volumes were made up to 20 μL with 6× loading buffer containing xylene cyanol dye. The stability of the complexes was analyzed on 0.8% agarose gel in Tris-acetate-EDTA (TAE) buffer solution. Electrophoresis was carried out at 100 V for 60 min in a Biorad electrophoresis system (Biorad Laboratories, Hercules, CA, U.S.A.). The DNA bands on the gels were visualized and photographed upon ethidium bromide staining using a Gel Doc XR+ Imaging System (Biorad Laboratories, Hercules, CA).

**Particle Size and Zeta Potentials Measurements.** The prepared complex solutions were diluted in 10 mM NaCl or 150 mM NaCl aqueous solution. The mean hydrodynamic diameter and polydispersity of the complexes were determined by photon correlation spectroscopy via dynamic light scattering measurement using a Malvern NanoZS90 Zetasizer (Malvern Instruments, UK). Measurements were performed at a temperature of 25 °C and at a detection angle of 90° and with a He–Ne 633 nm laser as the light source. The instrument was routinely calibrated using standard reference latex particles (Malvern Instruments, U.K.). The particle size of each sample was expressed as the mean hydrodynamic particle diameter ± standard deviation of three measurements. Surface charge of polymer/DNA complexes was also measured at 25 °C with the same instrument after calibration with a polystyrene dispersion (Malvern Instruments, UK) of known zeta potential. Given values were the average of at least five runs. A PSS0012 Marvern Instruments’ DTS Application Software running on Windows environment was used for the data analysis.

**In Vitro Transfection.** HeLa and HepG2 cells were seeded in 24-well plates at 5 × 10\(^4\) cells/well and were incubated for 12 h. pEGFP was complexed with PEI-DMAAPS samples (of varied DMAAPS graft ratios) at N/P 15, 30, and 60, or with 25 kDa PEI (the control) at N/P 10. The medium was removed from each well and the cells were washed once with 1× PBS (200 μL). Thereafter, the medium in each well was replaced with serum-free media or fetal bovine serum media. The complexes of PEI-DMAAPS/pDNA at different N/P ratios (with 2 μg plasmid DNA in each sample) were incubated for 6 h. The transfection medium was aspirated, replaced with 600 μL growth medium (DMEM with 10% FBS), and the cells were further incubated for 48 h. The transfected cells were washed once with PBS and detached with 0.25% trypsin. Transfection efficiency was evaluated by scoring the percentage of cells expressing pEGFP with the flow cytometer. Moreover, the transfected cells were imaged using the fluorescence microscope.
RESULTS AND DISCUSSION

Synthesis of PEI-DMAAPS Conjugates. First, a vinyl-containing betaine compound \(N,N\)-dimethyl-(acrylamidopropyl)ammonium propane sulfonate (DMAAPS, namely the betaine monomer) was synthesized and characterized, as shown in Scheme 1 and Figure S1, and three PEI-DMAAPS conjugates with varied graft ratios were then obtained through Michael addition reaction between DMAAPS and PEI 25K in methanol. The unconjugated DMAAPS were readily removed by dialysis. The mole percentages of DMAAPS relative to the amino groups of PEI in the feed were set at 30, 60, and 100%, respectively. \(^1\)H NMR spectra for the three samples were given in Figure 1. Based on the \(^1\)H NMR spectra,

![Figure 1.](image-url)

Figure 1. \(^1\)H NMR spectra (in D\(_2\)O) of PEI-DMAAPS conjugates with varied graft ratios.

the graft ratio (mole percentage of DMAAPS relative to the total amino groups in PEI 25K before the Michael addition reaction) for the resulting conjugates were estimated to be 23, 55, and 95%, respectively, by calculating the ratio between the integral area of the peaks for DMAAPS moieties and that of PEI (Figures S2–S4). The three samples are hereinafter labeled as PEI-DMAAPS\(_{23\%}\), PEI-DMAAPS\(_{55\%}\), and PEI-DMAAPS\(_{95\%}\). In this study, it was found that the Michael addition readily took place and the graft ratio could be easily controlled, this ensures repetitiveness of the facile approach.

Protein BSA Adsorption of PEI-DMAAPS Conjugates. Proteins adsorbed onto the surface of biomaterials can trigger thrombosis and blood coagulation, leading to life-threatening situations or causing functional failure when biomaterials are used in artificial organs, blood vessels, and other medical devices in contact with blood. In system circulation, serum proteins could bind to polymer/pDNA complexes in a nonspecific manner, nonspecific protein adsorption is the first interaction event that occurs at the interface between polymers and human blood. We determined the BSA adsorption by the polymer (Figure 2) and the polymer/pDNA complex (Figure S5).

As shown in Figure 2, the BSA adsorption of PEI 25K is much higher than PEI-DMAAPS derivatives. The absorption was induced mainly by electrostatic interactions between negatively charged BSA and the positively charged polymer, leading to protein BSA adsorption. The protein adsorption values for the three PEI-DMAAPS conjugates were at similar level, about half of that for PEI 25K. The results also indicate that the reduction of protein adsorption on the PEI-DMAAPS conjugates was associated with the betaine monomer (DMAAPS) moieties on the PEI. Similarly, as shown in Figure S5, the complexes formed by PEI-DMAAPS derivatives and the pDNA exhibit much lower protein adsorption than that formed by PEI 25K and pDNA. Previously, the betaine polymers have been shown to exhibit ultrahigh resistance to nonspecific protein adsorption,\(^5\) while this result demonstrates that the small molecular betaine, after being coated on PEI surfaces, also displays resistance to protein adsorption.

Hemolysis Test for PEI-DMAAPS. The red blood cell (RBC) hemolysis assay was performed to evaluate the blood compatibility of PEI-DMAAPS conjugates. The compatibility of any gene delivery vector with blood indicates its suitability for introduction into the systemic circulation. The hemolysis tests were performed following ASTM F756-08. The materials are usually considered with little to no hemolytic reaction when the hemolysis rate was lower than 5%.\(^5\) Figure 3 shows the hemolysis test results of the polymers (pristine PEI 25K and the betaine monomer-modified PEIs) and the polymer/pDNA complexes at two different concentrations. As shown in Figure 3A, the PEI-DMAAPS polymers exhibit much lower hemolysis degrees than PEI 25K. At the polymer concentration of 300 and 500 \(\mu\)g/mL, PEI 25K displays a hemolysis rate of 6 and 17%, respectively, in contrast, all the PEI-DMAAPS polymers exhibit hemolysis rates less than 3% at both concentrations. On the other hand, the hemolysis tests were also conducted for different polymer/pDNA complexes, and the PEI-DMAAPS/ pDNA complexes also display much lower hemolysis rates than that of PEI 25K/pDNA complex, as shown in Figure 3B.

The high hemolytic damage of PEI could be owing to the high charge density, which cause excessively strong interaction with membrane proteins and phospholipids and thus disturbs membrane structure and function.\(^1\) After surface modification of PEI with DMAAPS, excessive amino groups were substituted by DMAAPS, and these zwitterionic molecules on the polymer surfaces can help to avoid the disruption of blood cell membranes.

In Vitro Cytotoxicity of PEI-Betaine Conjugates. Before carrying out the transfection experiments, it is important to evaluate the cell viability properties of the modified PEI gene vector. The cytotoxicity of PEI-DMAAPS conjugates and PEI 25K in the concentration range from 10 to 150 mg/L were evaluated in HeLa and HepG2 cell lines by MTT assays, as

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Figure 2. Protein adsorption for PEI-DMAAPS conjugates and PEI 25 K. ***P < 0.001 vs PEI 25K. Two-tailed Student’s t-test (n = 3, error bar represents SD).
shown in Figure 4. As anticipated, the highest cytotoxicity was seen for PEI 25K, significant toxicity was observed when its concentration was higher than 25 mg/L. In this case, the cell viability for PEI was found to be lower than 20% for HeLa cell line and 40% for HepG2 cell line as the concentration of PEI is above 20 mg/L. On the other hand, the PEI-DMAAPS conjugates exhibit much lower and graft-degree-dependent cytotoxicity, and the betaine monomer DMAAPS exhibits no cytotoxicity, as shown in Figure 4. The cytotoxicity of the conjugates decreases with increasing graft degree. This result agrees well with the accepted opinion that the cell biocompatibility has a strong association with the charge density on polycation surface. It is believed that the positive charges facilitate endocytosis through negatively charged cell surface, but such interactions also cause significant cell death due to the aggregation of these polymers on the cell surface impairing the important membrane function like protein kinase activity.54,55 Moreover, we also determined the cytotoxicities for the complexes formed by pDNA and PEI-betaine conjugates or PEI 25K, as shown in Figure S6, which indicates that the complexes formed by using PEI-betaine conjugates exhibit lower cytotoxicity.

The conjugation of the betaine molecules on the PEI chains can substantially alleviate the cytotoxicity, and we suppose the betaine moieties have 2-fold role in relation to the cytotoxicity. First, the conjugation of betaine can reduced the cation density on the polymer surface, thereby lessening the damage to cell membrane. Second, the betaine monomers, when uniformly distributed on the polymer surfaces, are equivalent to zwitterionic polymers and highly resistant to nonspecific protein adsorption, and this may contribute to the enhanced biocompatibility and higher cell viability of the vector materials.

Buffering Capacity of PEI-DMAAPS Conjugates. Buffering capacity of the polymer is one of the important properties that enables it to buffer the endosomes and helps in the rupture and subsequent release of the genetic material into the cytosol. Acid–base titration experiments were carried out for PEI 25K and PEI-DMAAPS conjugates to evaluate the buffering capacity of the new polymer. As shown in Figure S7, PEI-DMAAPS conjugates exhibit lower buffering capacities than PEI 25K, and with the increasing number of betaine moieties on the polymer, the buffering capacity decreases. For the three PEI-betaine conjugates, with increasing degree of graft, the number of amines decreases and fewer amines are available for charge neutralization. Interestingly, although the efficient buffer capacity was important for endosomal release of pDNA into the cytoplasm, previously published results also indicated proper reduction of PEI buffering capacity sometimes may be good for in vitro gene delivery activity.56

Complex Formation of PEI-DMAAPS with pEGFP. To examine the stability of the PEI-DMAAPS/pEGFP complexes, the formation of the complexes was examined by their electrophoretic mobility on an agarose gel. As shown in Figure S5, the three conjugates can efficiently condense pDNA and fully retard the movement of pDNA along the gels. Moreover, the

Figure 3. Hemolysis test results for the polymers (A) and polymer/pDNA complexes at N/P = 60 (B) at the polymer concentration of 300 or 500 μg/mL. The tests were performed following ASTM F756-08. ***P < 0.01, ****P < 0.001 vs PEI 25K. Two-tailed Student’s t-test (n = 3, error bar represents SD).

Figure 4. Cell viability profile for PEI-DMAAPS conjugates, PEI 25K and betaine monomer (DMAAPS) in HeLa (A) and HepG2 (B) cells. Percent viability of cells is expressed relative to control cells. ***P < 0.01, ****P < 0.001 vs PEI 25K. Two-tailed Student’s t-test (n = 3, error bar represents SD).
three complexes exhibit similar electrophoretic migration patterns as a function of the N/P ratio, which refers to the number of nitrogen atoms in PEI of the carrier per DNA phosphate. For the conjugates, the complete retardation of DNA was achieved at N/P ratios of 1.5, while for PEI 25K, the ratio is 2. This result suggests that these betaine-modified PEI polymers have satisfactory capacity to form stable complexes with pEGFP. As can be seen from Scheme 1, the coupling of each betaine monomer onto the PEI chain turns one primary amino group into a tertiary amino group and introduces a cationic quaternary amine and a negative sulfonic group. The cationic quaternary amine group can also complex with DNA, hence, PEI-DMAAPS conjugates still retain a better DNA condensation capability, though the incorporation of betaine molecules forms a barrier exerted by sulfo-anions on the PEI’s surface. Moreover, for gene delivery, tertiary amino groups are highly desirable because they have pK_a values in the physiological range and can be engineered into delivery vehicles to buffer endosomes. In this experiment, we added xylene cyanol into the gel as a color marker to monitor the process of agarose gel electrophoresis, and we found the addition of the dye did not cause obvious interference for the result, as shown in Figure S8.

Size and Zeta Potential Measurements. The size and zeta potential of the polymer/pDNA complexes in 10 mM NaCl solution was determined by DLS method using a Malvern Nano-ZS90 Zetasizer. The averaged sizes and zeta potentials as a function of N/P ratio are displayed in Figure 6, while the size distribution curves and the size polydispersity indices (PDI) are displayed in Figure S9. The mean diameters of the complexes determined in 10 mM NaCl solution were found to be in a range of 220–500 nm, and for the complexes formed by pDNA and PEI-betaine conjugates at higher N/P ratios (15, 30, and 60), the diameters are in the range of 260 to 380 nm, suggesting that these complexes may be used for gene delivery. In addition, as can be seen from Figure S9, the calculated PDI for the complexes determined in 10 mM NaCl solution are generally rather high, ranging from 0.30 to 0.65. In general, the complexes with lower N/P ratios (N/P = 3 and 7.5) exhibit relatively low PDI.

For surface charge measurement in 10 mM NaCl water solutions, PEI 25K/pDNA complexes exhibit a slightly positive zeta potential (+4 mV) at N/P = 3, and with increasing N/P ratio, zeta potentials slowly increase and reach plateaus about 60 mV at N/P ratios between 15 and 60. The high zeta potential for PEI-DMAAPS/pDNA complexes at N/P = 3 is not quite clear at present; we suppose, as the strong electrolyte cation, the quaternary ammonium in the betaine moieties may contribute more greatly to the positive zeta potential than the amines (weak electrolyte cation) in PEI chains. The high positive zeta potentials for the PEI-DMAAPS/pDNA complexes are beneficial to their attachment to anionic cell surface and endocytosis, thus, improving the gene transfection.
In Vitro Transfection. The in vitro transfection of PEI-DMAAPS conjugates and PEI 25K were assessed in HeLa and HepG2 cells using pEGFP as a reporter gene. Polymer/pDNA complexes prepared at N/P ratios of 15:1, 30:1, and 60:1 were selected for transfection studies; PEI 25K/pEGFP at its optimal N/P ratio of 10:1 was chosen as the positive control. The transfection experiments were performed using different media, that is, in the presence or absence of 10.0% serum. The transfection efficiency of PEI-DMAAPS conjugates were compared with that of PEI 25K.

The transfection efficiency of pEGFP in HeLa and HepG2 was quantified using flow cytometry in terms of cell population expressing EGFP. Figure 7A shows the percentage of the HeLa cells expressing EGFP at different N/P ratios using PEI-betaine conjugates and PEI 25K. Interestingly, the complexes based on PEI-DMAAPS\(_{23\%}\) and PEI-DMAAPS\(_{55\%}\), give remarkable levels of transfection; their transfection efficiencies are substantially higher than that of PEI 25K. Specifically, for the N/P ratios we tested, both PEI-DMAAPS\(_{23\%}/p\)EGFP and PEI-DMAAPS\(_{55\%}/p\)EGFP complexes exhibit their maximum efficiency values at a N/P ratio of 60. Up to \(~56\%\) transfection efficiency was achieved by the two complexes, which was statistically significantly higher than the 21\% transfection efficiency obtained from PEI 25K in the absence of serum, at its optimal N/P ratio of 10. In Figure 7B, the comparison of transfection efficiency for PEI-betaine conjugates and PEI 25K is also presented. Similarly, the transfection efficiencies for the conjugates are also much higher than that for PEI 25K. In addition, we found the sample PEI-DMAAPS\(_{55\%}\) displayed very poor gene transfection efficiencies of \(~1\%\) (with some typical flow cytometry profiles and fluorescence images shown in Figure S10), and this may be due to its poor buffering capacity caused by the overly high graft ratio of betaine monomers on its surface.

One of the major problems of the cationic vector-mediated gene delivery is that the transfection is prominently inhibited in the presence of serum. The study of gene vectors concerning serum-conditioned transfection can serve as a fundamental predictive model for their in vivo efficiency evaluation. In this context, we investigated the effects of serum on the transfection efficiency of the complexes based on PEI-DMAAPS\(_{23\%}\) and PEI-DMAAPS\(_{55\%}\), as revealed in Figures 7 and S11. Compared to PEI 25K, the PEI-betaine conjugates exhibit much higher transfection efficiencies in the presence of high serum concentrations. In particular, for the pristine PEI 25K, its transfection efficiency decreased drastically in 10% serum compared to that in the absence of serum, while for the two PEI-DMAAPS vectors, no significant difference could be observed between the efficiencies in the presence and absence of 10% serum, as shown in Figure 7. As revealed in Figure S11, the pristine PEI 25 K exhibits very low transfection efficiency (less than 5\%) in 30 and 50% serum. On the other hand, at 30% serum, there are only \(~32\%\) and \(~10\%\) decreases in transfection efficiency for PEI-DMAAPS\(_{23\%}\) and PEI-DMAAPS\(_{55\%}\), respectively. In the case of 50% serum, in spite of a decline in transfection level, PEI-DMAAPS\(_{23\%}\) and PEI-DMAAPS\(_{55\%}\) still show 6 and 10 times higher transfection efficiency than pristine PEI 25 K. These results clearly indicate that the incorporation of DMAAPS onto PEI can significantly improve serum-tolerant transfection activity for the polycation vectors, suggesting that PEI-betaine conjugates hold a promising potential in vivo application.

To visualize the transfection effect, the EGFP expression in HeLa and HepG2 cells was observed with an inverted fluorescent microscope. Figure 8 gives some typical fluorescence images of HeLa cells transfected by PEI-DMAAPS\(_{23\%}/p\)EGFP, PEI-DMAAPS\(_{55\%}/p\)EGFP, and PEI 25K/pEGFP complexes. The expression could not be detected when the transfection was mediated by naked pEGFP, which was used as a negative control (data not shown). As shown in Figure 8, fluorescence images could be observed when the transfections were mediated by PEI 25K; however, the cells mediated by PEI-DMAAPS\(_{23\%}\) and PEI-DMAAPS\(_{55\%}\) show more bright green fluorescent spots than that by PEI 25K. It appears that the amount of green cells mediated by PEI-DMAAPS\(_{23\%}\) and PEI-DMAAPS\(_{55\%}\) were similar. Furthermore, it can be seen
from the figure that the fluorescence was unaffected by the presence of 10% serum for PEI-DMAAPS 23% and PEI-DMAAPS 55% except for a couple of observations for HepG2 cell line. The results presented in Figure 8 were largely in accordance with the flow cytometry results (Figure S12), and this gives additional evidence that betaine-modified PEI was more effective as a gene carrier than PEI 25K.

The enhanced gene transfection efficiency of the two PEI-DMAAPS samples, we suppose, is due to the improvement of the surface properties of the vector as well as the PEI-DMAAPS samples, we suppose, is due to the improvement of more enhanced biocompatibility. We suppose this new surface modification strategy may also be suitable for fabricating some other materials with enhanced biocompatibility.

**CONCLUSIONS**

In summary, we have developed a facile and effective approach to significantly improve the gene delivery performance of PEI. By incorporating the sulfobetaine monomers onto PEI chains via a simple Michael addition chemistry, we obtained a new PEI-based gene vector featuring low protein adsorption, low cytotoxicity, and high gene transfection efficiency. In addition, the vectors display a satisfactory serum-tolerant property. In this study, incorporating betaine monomer (not polybetaine) onto PEI may ensure higher packing density of sulfobetaine groups on the PEI surface. This approach is of low-cost and facile in terms of synthesis and is much preferred in biological applications. We suppose this new surface modification strategy may also be suitable for fabricating some other materials with enhanced biocompatibility.

**ASSOCIATED CONTENT**

1H NMR spectrum and mass spectrum for the synthesized DMAAPS, PEI-DMAAPS 23%, PEI-DMAAPS 55%, and PEI-DMAAPS 95%. Protein adsorption for the complexes. Cell viability profile for the complexes. Buffering capacity of PEI 25K and three PEI-betaine conjugates determined by performing acid–base titration from pH 10 to pH 3. Comparison of agarose gel electrophoresis retardation assay for PEI-DMAAPS/pEGFP complex with or without addition of xylene cyanol dye. Diameter distribution for PEI/pEGFP and three PEI-betaine/pEGFP polyplexes as determined by DLS. Flow cytometry profiles and fluorescence images of PEI-DMAAPS 95%/pEGFP complex in HeLa at three N/P ratios. Percent transfection efficiency for PEI 25K (at N/P = 10) and two PEI-DMAAPS/pEGFP complexes in HeLa at N/P = 30 with 30% and 50% serum. Typical flow cytometry profiles for pEGFP transfection in HeLa and HepG2 cell lines mediated by PEI 25K, PEI-DMAAPS 55% and PEI-DMAAPS 95%. And size, size polydispersity (PDI), and zeta potential measurement of typical PEI-DMAAPS/pDNA polyplexes. This material is available free of charge via the Internet at http://pubs.acs.org.

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**Notes**

The authors declare no competing financial interest.

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**REFERENCES**


[Figure 8. Fluorescence microscope images for HeLa and HepG2 cells treated with PEI 25K/pEGFP, PEI-DMAAPS23%/pEGFP and PEI-DMAAPS55%/pEGFP. The corresponding flow cytometry profiles for the images are listed in Figure S9.]