

Conjugation of Polyamidoamine Dendrimers on Biodegradable Microparticles for Nonviral Gene Delivery

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We report on the preparation and characterization of poly(D,L-lactide-co-glycolide) (PLGA) microparticles with surface-conjugated polyamidoamine (PAMAM) dendrimers of varying generations. The buffering capacity and zeta-potential of the PLGA PAMAM microparticles increased with increasing generation level of the PAMAM dendrimer conjugated. Conjugation of the PAMAM dendrimer to the surface of the PLGA microparticle removed generation-dependent cytotoxicity in HEK293 and COS7 cell lines. PLGA PAMAM pDNA microparticles displayed similar cytotoxicity profiles to unmodified PLGA pDNA microparticles in COS7 cells. A generation three PAMAM dendrimer conjugated to PLGA microparticles significantly increased transfection efficiencies in comparison to unmodified PLGA microparticles.

1. INTRODUCTION

Delivery of plasmid DNA (pDNA) using biodegradable microparticles has shown significant potential for improving the efficacy of transgene expression (1). These microparticles are commonly prepared from poly(D,L-lactide-co-glycolide) (PLGA), a polymer that is biodegradable and biocompatible and has FDA approval for use in the clinic (1–8). Delivery of pDNA using PLGA microparticles can be achieved using two approaches. In the first approach, pDNA is entrapped into the matrix of the microparticle, allowing for sustained release. Drawbacks to this approach include inactivation of the pDNA during the harsh manufacturing conditions of the microparticles. These conditions include exposure to organic solvents and shear forces associated with the homogenization and sonication steps (3, 5, 8). Several approaches have sought to overcome these limitations. These approaches include incorporating cationic excipients that condense and protect the pDNA during entrapment or minimizing shear stress during homogenization by using a cryopreparation modification of the double emulsion method (9). In the second approach, pDNA is adsorbed onto the surface of the microparticles after they have been prepared (3, 10–13). This method prevents pDNA damage due to microparticle preparation techniques. This approach has found the most promise in genetic vaccination applications (11, 13, 14). In addition, microparticles can be loaded with drugs or proteins that complement or enhance the efficacy of the surface-bound pDNA. To bind the pDNA to the surface of the microparticles, a cationic agent is typically incorporated into the formulation method to prepare microparticles that have a net positive charge (3, 10–13). This facilitates binding of the negatively charged pDNA to the surface of the microparticles by electrostatic interactions. Approaches to date for preparing cationic microparticles have included the use of cationic surfactants such as cetyltrimethylammoniumbromide (CTAB) (15) or cationic polymers such as polyethyleneimine (PEI) that are adsorbed, blended, or covalently attached to the microparticles (3, 10, 13, 16, 17). In this study, we prepare PLGA microparticles that have cationic polyamidoamine (PAMAM) dendrimers covalently attached to the surface of the PLGA microparticles. PAMAM dendrimers have several advantages

over linear cationic polymers (18, 19). Similar to polymers such as PEI, PAMAM dendrimers have both primary and secondary amines that generate significant buffering capacity (18, 19). This imparts the carriers with the ability to release from the endosomes into the cytoplasm via the proton sponge mechanism (19, 20). In comparison to PEI, however, dendrimers are nanoscale, spherical, monodisperse polymers that display lower cytotoxicity and reduced structural density in the intramolecular core (19). As the generation of the PAMAM dendrimer increases, the transfection efficiency increases (21, 22). A drawback to PAMAM dendrimers, however, is their generation-dependent cytotoxicity (21–23). As the generation of the PAMAM molecule increases, its cytotoxicity increases proportionally. In this study, we show, for the first time, that conjugation of PAMAM dendrimers onto the surface of PLGA microparticles imparts the microparticles with generation-dependent buffering capacity and net positive charge while removing generation-dependent cytotoxicity. PLGA microparticles with PAMAM conjugated to the surface produce significantly higher transgene expression than unmodified PLGA microparticles. These results will have considerable potential for a wide variety of applications in microparticle-based nonviral DNA, RNA, and oligonucleotide delivery.

2. MATERIALS AND METHODS

2.1. Materials. D,L-Lactide/glycolide copolymer (PLGA, molar ratio with acid terminal group 50:50, inherent viscosity 0.54 dL/g) was purchased from Absorbable Polymers International (Pelham, AL). PAMAM dendrimers from generations 3–6 (G3, G4, G5, and G6, ethylenediamine core), PEI (25 kDa), and poly(vinyl alcohol) (PVA, MW 30–70 kDa) were purchased from Sigma-Aldrich (St. Louis, MO). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), *N*-hydroxysulfosuccinimide (Sulfo-NHS), and bicinchoninic acid (BCA) protein assay kit were purchased from Pierce Biotechnology Inc. (Rockford, IL). Dulbecco's Modified Eagle's Medium (DMEM) was obtained from Gibco BRL (Grand Island, NY). The luciferase assay system was purchased from Promega (Madison, WI). For cellular uptake studies, FITC (fluorescein isothiocyanate, Sigma-Aldrich)-labeled dendrimers G3–G6 were prepared by reaction of FITC and PAMAM dendrimers in 0.1

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M sodium carbonate buffer (pH 9) at 4 °C in darkness overnight. FITC-conjugated dendrimers were purified by dialysis (MWCO 7000, Pierce Biotechnology Inc., Rockford, IL) and then lyophilized (Labconco FreeZone 4.5, Kansas City, MO). The level of fluorescein in the dendrimers was determined by measuring their absorbance at 498 nm using standard curves for FITC. Spectrofluorometric analysis (Spectramax M5 Microplate reader, Molecular Device) revealed that approximately 3% of the dendrimer amino groups were attached with FITC.

2.2. Cell Culture. Human embryonic kidney cells (HEK293) and monkey African green kidney fibroblast-like cell line (COS7) were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The cells were maintained in DMEM supplemented with 10% FBS, streptomycin at 100 µg/mL, penicillin at 100 U/mL, and 4 mM L-glutamine at 37 °C in a humidified 5% CO₂-containing atmosphere.

2.3. Amplification and Purification of Plasmid DNA. VR1255 plasmid is a 6.4-kb cDNA encoding firefly luciferase driven by the cytomegalovirus (CMV) promoter/enhancer. The plasmid was transformed in *Escherichia coli* DH5α and amplified in Terrific Broth media at 37 °C overnight with a shaking speed of 300 rpm. The plasmid was purified by an endotoxin-free QIAGEN Giga plasmid purification kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol. Purified pDNA was dissolved in saline, and its purity and concentration were determined by UV absorbance at 260 and 280 nm.

2.4. Preparation of PLGA pDNA and PLGA PAMAM pDNA Microparticles. **2.4.1. PLGA pDNA Microparticles** PLGA microparticles were prepared using a water in oil in water (w/o/w) double emulsion, solvent evaporation technique. Briefly, 100 mg of 50:50 PLGA was dissolved in 5 mL of dichloromethane (DCM). VR1255 pDNA in 0.5% (w/v) PVA solution was prepared at a concentration of 4mg/mL. Using a microtip probe sonicator set at level 2 (Sonic Dismembrator model 100, Fisher Scientific, Pittsburgh, PA), 500 µL of the PVA solution containing 2 mg of VR1255 pDNA was mixed with the PLGA/DCM solution for 20 s to form the first emulsion. This emulsion was then rapidly added to 50 mL of 0.5% (w/v) PVA solution with stirring at 13,500 rpm for 30 s using an IKA Ultra-Turrax T25 basic homogenizer (IKA, Wilmington, NC). The second emulsion was stirred overnight during which time the DCM solvent was evaporated. The microparticles were then washed 3 times with deionized water and then lyophilized (Labconco FreeZone 4.5, Kansas City, Missouri). The supernatant was collected and analyzed spectrophotometrically at 260 nm using a SpectraMax Plus³⁸⁴ microplate spectrophotometer (Molecular Device) for pDNA content. Plasmid DNA entrapped in the PLGA microparticles was calculated by subtracting the pDNA content in the supernatant from the initial concentration of pDNA added. Microparticles were stored at -20 °C until use. For *in vitro* microparticle uptake studies, PLGA microparticles loaded with Rhodamine 123 were prepared using a single emulsion evaporation methodology. Briefly, 100 mg of 50:50 PLGA and 2 mg of Rhodamine 123 (Sigma-Aldrich, St. Louis, MO) were dissolved in 5 mL of DCM. This was then rapidly added to 50 mL of 0.5% (w/v) PVA in deionized water with stirring at 13,500 rpm for 30 s. After evaporation overnight, the rhodamine-labeled microparticles were washed and collected as described above.

2.4.2. PLGA PAMAM pDNA Microparticles. A modified EDC/NHS chemistry was used to conjugate PAMAM dendrimers to the surface of PLGA microparticles to obtain cationic microparticles. Blank PLGA microparticles were prepared with 500 µL of the 0.5% (w/v) PVA solution and 5 mL of DCM containing 200 mg of 50:50 PLGA-COOH using a w/o/w double emulsion, solvent evaporation technique as described above. 100 mg of the PLGA-COOH microparticles were then

suspended in 10 mL of 0.1 M MES (2-(*N*-morpholino)ethane-sulfonic acid) buffer, pH 5.1. 1 mL of EDC solution (60 mM) in 0.1 M MES buffer and 1 mL of Sulfo-NHS solution (60 mM) in 0.1 M MES buffer were added dropwise to the PLGA microparticle suspension. EDC activation was carried out for 2 h at room temperature. A 10 molar excess of dendrimers G3, G4, G5, G6 or 25 kDa branched PEI was dissolved in 5 mL of 0.1 M MES buffer, respectively. Activated PLGA microparticles were added dropwise to the G3, G4, G5, G6 dendrimer or 25 kDa branched PEI solutions with magnetic stirring and incubated for another 4 h at room temperature. Dendrimer-conjugated PLGA microparticles were washed twice in 1 M NaCl to remove physically adsorbed polymer and twice with deionized water. The resulting microparticles, which were thereafter named PG3, PG4, PG5, PG6, and P-PEI were lyophilized and stored at -20 °C until use. Fluorescamine (4-phenylspiro[furan-2(3H),1'-phthalan]-3,3'-dione) has been used for colorimetric quantification of primary amines and to quantify dendrimers associated with microparticles (24). Microparticles PG3, PG4, PG5, PG6, or P-PEI were hydrolyzed in 0.1 N NaOH overnight, and the dendrimer content was measured using spectrofluorometric analysis (Spectramax M5 microplate reader, Molecular Device). Fluorescamine reacts with primary amines in dendrimers to form pyrrolinones, which are excited at 390 nm and have peak emission at 475–490 nm. Quantification of dendrimer conjugated to the PLGA microparticles (w/w) was estimated using standard curves of dendrimer with corresponding generation.

2.5. Microparticle Size and Surface Morphology Analysis. Microparticle size and zeta potential measurements were conducted using the Zetasizer Nano ZS (Malvern, Southborough, MA). Briefly, the microparticles were suspended in deionized water at a concentration of 1 mg/mL. The size measurements were performed at 25 °C at a 173° scattering angle. The mean hydrodynamic diameter was determined by cumulative analysis. The zeta potential determinations were based on electrophoretic mobility of the microparticles in the aqueous medium, which was performed using folded capillary cells in automatic mode. Microparticle morphology was assessed by scanning electron microscopy (SEM, Hitachi S-4000). Briefly, air-dried microparticles were placed on adhesive carbon tabs mounted on SEM specimen stubs. The specimen stubs were coated with approximately 5 nm of gold by ion beam evaporation before examination in the SEM operated at 5 kV accelerating voltage.

2.6. Buffering Capacity of PLGA pDNA and PLGA PAMAM pDNA Microparticles. The ability of PLGA PAMAM microparticles (PG3 to PG6) to resist acidification was tested using the acid titration assay as described by Tang et al. (24) Briefly, 10 mg/mL PG3, PG4, PG5, or PG6 microparticles were suspended in 150 mM NaCl, respectively. The pH was first adjusted to ~9.0 and then titrated in small increments with 0.1 N HCl until a pH of 3.0 was reached. The slope of the pH versus HCl added graph provides an indication of the intrinsic buffering capacity of the delivery vehicles.

2.7. Plasmid DNA Loading on Dendrimer-Conjugated Cationic Microparticles. VR1255 pDNA was loaded on the surface of cationic microparticles PG3, PG4, PG5, or PG6 as described by Singh et al. (11) Plasmid DNA was incubated with PG3, PG4, PG5, or PG6 suspensions in PBS (pH adjusted to 6.5) at a concentration of 20 µg pDNA/mg microparticles at 4 °C for 6 h, respectively. The resulting microparticles were centrifuged at 13,200 rpm for 5 min on a microcentrifuge and washed twice with the loading buffer to obtain PG3 pDNA, PG4 pDNA, PG5 pDNA, or PG6 pDNA microparticles. The supernatant was collected and analyzed spectrophotometrically at 260 nm using a SpectraMax Plus³⁸⁴ microplate spectrophotometer (Molecular Device) for pDNA content. Plasmid DNA

loading on the cationic microparticles was calculated by subtracting the pDNA content in the supernatant from the initial concentration of pDNA added.

2.8. Cytotoxicity Evaluation using the MTT Assay. The cytotoxicity of the PLGA pDNA, PLGA PAMAM pDNA microparticles, PLGA PEI pDNA microparticles, and PEI pDNA was evaluated using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay (25). COS7 and HEK293 cells were seeded in a 96-well plate at a density of 1×10^4 cells/well. Twenty-four hours later, cells were incubated with 200 μ L of complete DMEM containing PLGA pDNA or PLGA PAMAM pDNA microparticles at various concentrations. After 4 h of incubation, the medium in each well was replaced with 100 μ L of fresh complete medium. Twenty-five microliters of 5 mg/mL MTT solution in PBS was added to each well and incubated with cells for additional 2 h. Cells were lysed with 100 μ L of the extraction buffer (20% SDS in 50% DMF, pH 4.7) overnight. The optical density of the lysate was measured at 550 nm using a Spectramax Plus³⁸⁴ microplate spectrophotometer (Molecular Device). Values were expressed as a percentage of the control to which no microparticles were added.

2.9. Cellular Uptake of PLGA pDNA and PLGA PAMAM pDNA Microparticles. Dendrimer-conjugated PLGA microparticles PG3, PG4, PG5, and PG6 were prepared using FITC-labeled dendrimers. HEK293 cells were seeded into 12-well plates at a density of 1×10^6 /well 24 h before transfection. After 24 h incubation, the medium was replaced with fresh DMEM containing 10% FBS. 0.5 mg/well PLGA/Rhodamine 123, FITC-labeled PG3, PG4, PG5, or PG6 microparticles with and without loaded pDNA were incubated with HEK293 cells for 16 h. Then, the cells were vigorously washed with PBS three times to remove free fluorescent-labeled microparticles and the microparticles adsorbed on the cells surface. Samples were then assessed using flow cytometry (Becton Dickinson). Dot plots were gated on FSC/SSC properties of HEK293 cells to exclude free fluorescent-labeled microparticles. Data was analyzed using *Cell-Quest^{Pro}* software. All samples were tested in triplicate. To confirm that fluorescence would be detectable in the acidic environment of the endosomes, FITC was dissolved into a 150 mM NaCl aqueous solution. When the solution was adjusted to pH 4–5 using 0.1 N HCl, fluorescence could still be clearly detected in comparison to FITC at pH 7.4. To confirm that FITC-labeled particles had been internalized, cells were viewed under a confocal microscope (Bio-Rad Radiance 2100MP). Orthogonal sections from stacked images confirmed that particles were predominantly located inside the cell and not just on the surface (data not shown).

2.10. Evaluation of Luciferase Expression in COS7 and HEK293 Cells. COS7 and HEK293 cells were seeded into 24-well plates at a density of 8×10^4 /well 24 h before transfection. 0.2 mg/well PLGA pDNA and PLGA PAMAM pDNA microparticles were added to the cells in transfection medium (serum-free) and incubated for 4 h at 37 °C, followed by further incubation in serum-containing medium for 44 h. The concentration of the microparticles was selected on the basis of a target pDNA loading of 5 μ g/mg microparticles and an equivalent pDNA dose of 1 μ g/well. After the incubation, cells were treated with 200 μ L of lysis buffer (Promega). The lysate was subjected to two cycles of freezing and thawing, then transferred into tubes and centrifuged at 13,200 rpm for 5 min. Twenty microliters of supernatant was added to 100 μ L of luciferase assay reagent (Promega), and samples were measured on a luminometer for 10 s (Lumat LB 9507, EG&G Berthold, Bad Wildbad, Germany). The relative light units (RLU) were normalized against protein concentration in the cell extracts, measured by a micro BCA protein assay kit (Pierce). Luciferase activity was expressed as relative light units (RLU/mg protein in the cell lysate).

The data were reported as mean \pm standard deviation for triplicate samples. Every transfection experiment was repeated at least twice.

2.11. Statistical Analysis. Group data were reported as mean \pm SD. Differences between groups were analyzed by one-way analysis of variance with a Tukey post-test analysis. Levels of significance were accepted at the $P < 0.05$ level. Statistical analyses were performed using *Prism 3.02* software (Graphpad Software, Inc., San Diego, CA.)

3. RESULTS

3.1. PAMAM Dendrimers Can Be Conjugated To the Surface of PLGA Microparticles Using EDC/NHS Chemistry. Figure 1 schematically shows our approach for preparing unmodified PLGA pDNA microparticles and dendrimer-conjugated PLGA microparticles. PLGA pDNA microparticles were prepared using a w/o/w double emulsion, solvent evaporation technique. 0.5% (w/v) PVA solution was chosen to prepare PLGA microparticles based on results of a previous optimization study on microparticle fabrication processes. Rhodamine 123 is soluble in DCM. Therefore, PLGA microparticles loaded with Rhodamine 123 were prepared using a single emulsion technique that used identical stirring and PVA concentration conditions to the PLGA pDNA microparticles.

For dendrimer-conjugated PLGA microparticles, PAMAM dendrimers from generations 3–6 were covalently attached to the surface of PLGA microparticles using EDC/NHS chemistry. Each of the PAMAM dendrimers from G3 to G6 was conjugated to PLGA microparticles prepared from the same batch. The conjugation of dendrimers was quantified using a fluorescamine assay, which indicated that the amounts of G3, G4, G5, and G6 present on the microparticle surfaces were 5.0, 5.5, 4.6, and 5.4 μ g per milligram of microparticles, respectively (Table 1).

3.2. Microparticles Size, Zeta Potential, and Morphology of Microparticles. Figure 2a shows the microparticle size of the PLGA and PLGA PAMAM microparticles. Unmodified PLGA microparticles had an average size of 2.2 μ m. After conjugation of PAMAM dendrimers onto the PLGA microparticles surface, the average PLGA microparticle size ranged from 2.3 to 3.2 μ m. Figure 2b shows the zeta potential of these PLGA microparticles before and after loading with pDNA. Blank PLGA microparticles with carboxyl groups displayed a net negative surface charge of approximately -50 mV. After the conjugation of PAMAM dendrimers, the surface charge of PLGA microparticles turned positive with values ranging from $+20$ to $+40$ mV. After pDNA was electrostatically bound to the PAMAM-conjugated microparticle surface, the zeta potential was reduced to values ranging from $+0.5$ to $+13.5$ mV. Furthermore, both before and after pDNA binding, the surface charge of the dendrimer-conjugated PLGA microparticles increased in the order PG6 > PG5 > PG4 > PG3. SEM images (figure 2c) demonstrated that all the PLGA microparticles had a smooth morphology and spherical shape.

3.3. Increases in Buffering Capacity of PLGA PAMAM Microparticles Are Dependent on the Generation of the Conjugated PAMAM Dendrimer. Covalent attachment of PAMAM to PLGA microparticles is expected to provide the microparticles with tertiary and secondary amines that impart endolysosomal pH buffering capacity. This could lead to increased phagoendosomal escape of the carrier microparticles (proton sponge mechanism) (21). The buffering capacity of the dendrimer-conjugated PLGA microparticles was assessed by measuring the change in pH of a microparticle suspension (10 mg/mL) upon addition of increasing amounts of 0.1 N HCL. As shown in figure 3, buffering capacity of PAMAM PLGA microparticles is directly correlated with the generation level. The higher the generation level of the PAMAM

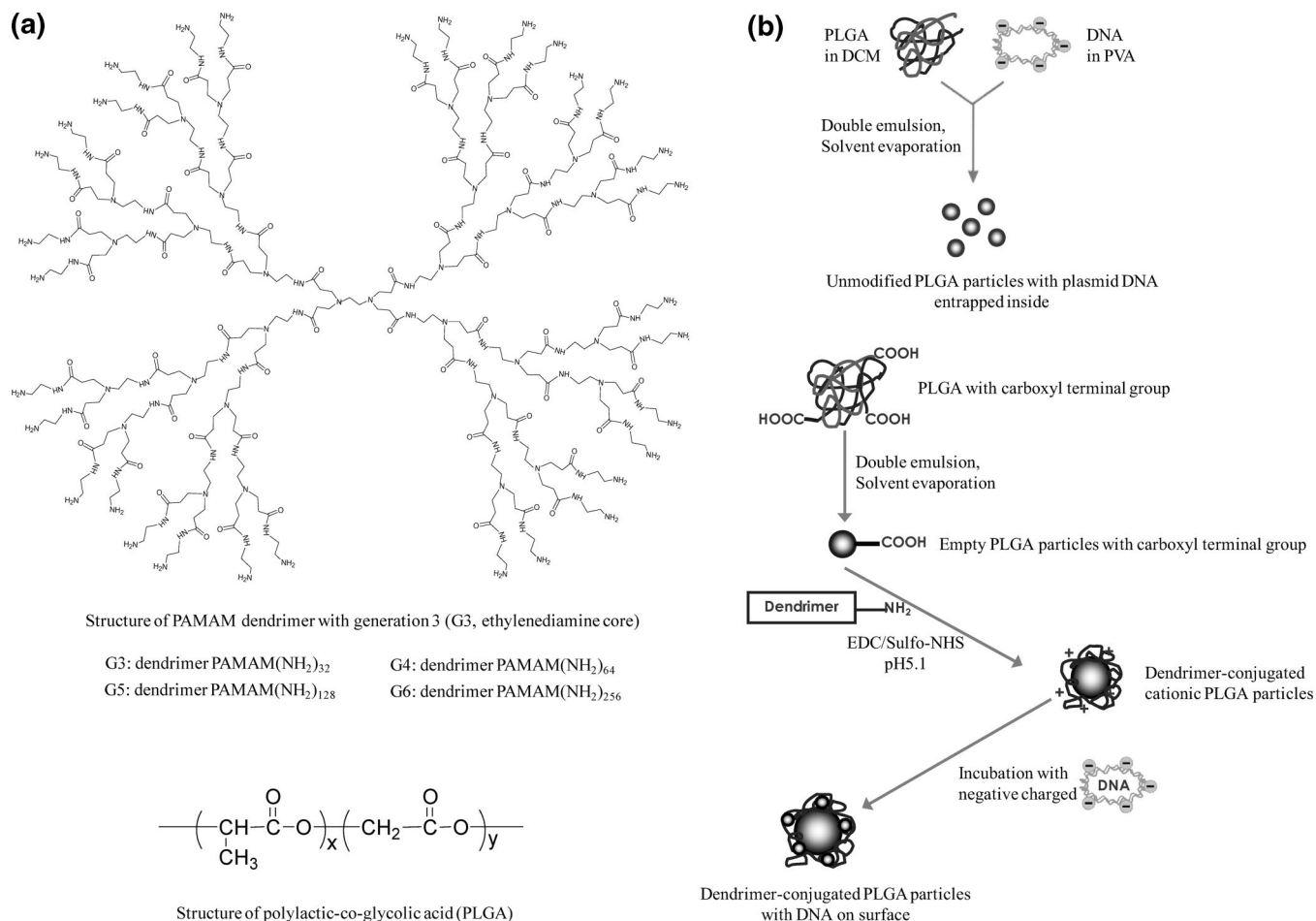


Figure 1. (a) Structure of poly(lactic-co-glycolic acid) (PLGA) and PAMAM dendrimers with ethylenediamine core. (b) Schematic of preparation of dendrimer-conjugated PLGA microparticles and unmodified PLGA microparticles.

Table 1. PAMAM Dendrimer Conjugation Efficiency and pDNA Loading Efficiency of PLGA and PAMAM-Conjugated PLGA Microparticles^a

	dendrimer-conjugation efficiency (μg dendrimer/mg particles)	DNA-loading efficiency (μg pDNA/mg particles)
PLGA/pDNA		3.5 ± 0.1
PG3	5.0	4.0 ± 0.1
PG4	5.5	4.2 ± 0.5
PG5	4.6	4.1 ± 0.1
PG6	5.4	4.8 ± 0.2

^a Averages representative of 3 measurements \pm standard deviation.

dendrimer conjugated to the PLGA microparticle, the more effective it is at buffering against pH changes. This is indicated by the shift and decrease in the slope of the titration curves.

3.4. PAMAM Conjugation to PLGA Microparticles Significantly Increases pDNA Loading. Covalent attachment of cationic PAMAM dendrimers to the surface of PLGA microparticles significantly increases the efficacy of pDNA binding in comparison to PLGA microparticles entrapping pDNA using a double emulsion, solvent evaporation technique. Plasmid DNA loading on the surface of PG3 microparticles was 15% higher than pDNA entrapment in unmodified PLGA microparticles. Increasing the generation of the PAMAM dendrimer conjugated to the PLGA microparticle from 3 to 6 increased pDNA loading from $4.0 \mu\text{g}$ to $4.8 \mu\text{g}$ pDNA/mg particles, respectively. This was 37% higher than the pDNA loading in PLGA microparticles using the double emulsion, solvent evaporation technique and 540% higher than pDNA

adsorbed onto the surface of unmodified PLGA microparticles ($\sim 1 \mu\text{g}$ pDNA/mg particles).

3.5. PAMAM Conjugation to PLGA Microparticles Removes Generation-Dependent Toxicity. *In vitro* cytotoxicity was evaluated in COS7 and HEK293 cells with increasing doses of PLGA pDNA and PLGA PAMAM pDNA microparticle concentrations (from 7.8 to 250 μg of microparticles per milliliter of DMEM). As shown in figure 4, PLGA PAMAM pDNA microparticles had similar cytotoxicity profiles to unmodified PLGA pDNA microparticles in COS7 cells. Cytotoxicity of PLGA PAMAM dendrimers was moderately higher than unmodified PLGA pDNA microparticles in HEK293 cells. No significant difference in the toxicity of PLGA PAMAM pDNA microparticles was observed in either cell line for increasing generation levels of the PAMAM dendrimer conjugated. No significant difference was observed in cytotoxicity between PLGA PAMAM pDNA microparticles and PLGA PEI pDNA microparticles. PLGA PAMAM microparticles were significantly less toxic than PEI alone.

3.6. PAMAM Dendrimer-Conjugated PLGA Microparticles Could Be Efficiently Taken Up by HEK293 Cells. Figure 5 shows cellular uptake data of fluorescence-labeled microparticles incubated with HEK293 cells. PLGA PAMAM pDNA microparticles displayed 6–7-fold higher uptake in cells than unmodified PLGA microparticles. No significant difference in the cell uptake of PLGA PAMAM pDNA microparticles was observed for increasing generation levels of the conjugated PAMAM dendrimer.

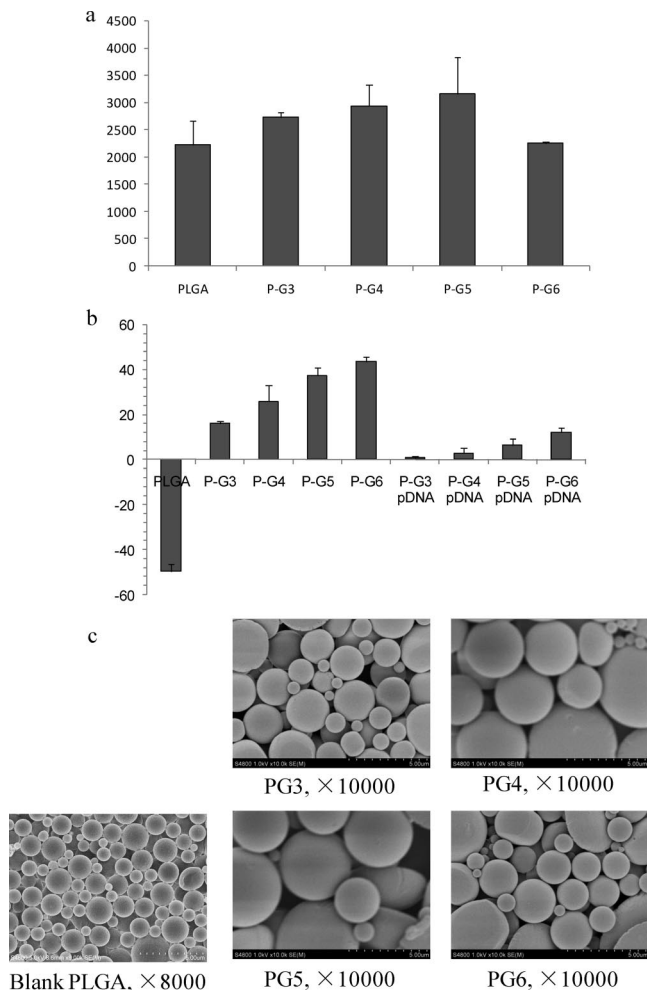


Figure 2. Size (a) and zeta potential (b) of PLGA PAMAM microparticles before and after pDNA binding. Data are represented as the mean \pm standard deviation ($n = 3$). SEM images (c) show that the morphology of all the PLGA particles is smooth and spherical in appearance.

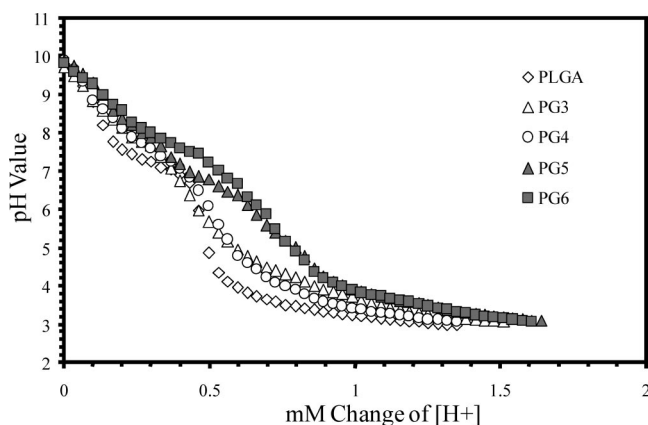


Figure 3. Acid titration experiments with 0.1 N HCL demonstrate the buffering capacity of PAMAM-conjugated and unmodified PLGA microparticles. The data show significantly increased buffering by PAMAM-conjugated PLGA microparticles in comparison to unmodified PLGA microparticles.

3.7. Dendrimer Conjugation Enhanced the Gene Transfection Mediated by PLGA Microparticles in COS7 and HEK293 Cells. Gene transfection mediated by PLGA pDNA and PLGA PAMAM pDNA microparticle formulations was evaluated in COS7 and HEK293 cells (Figure 6). All PLGA

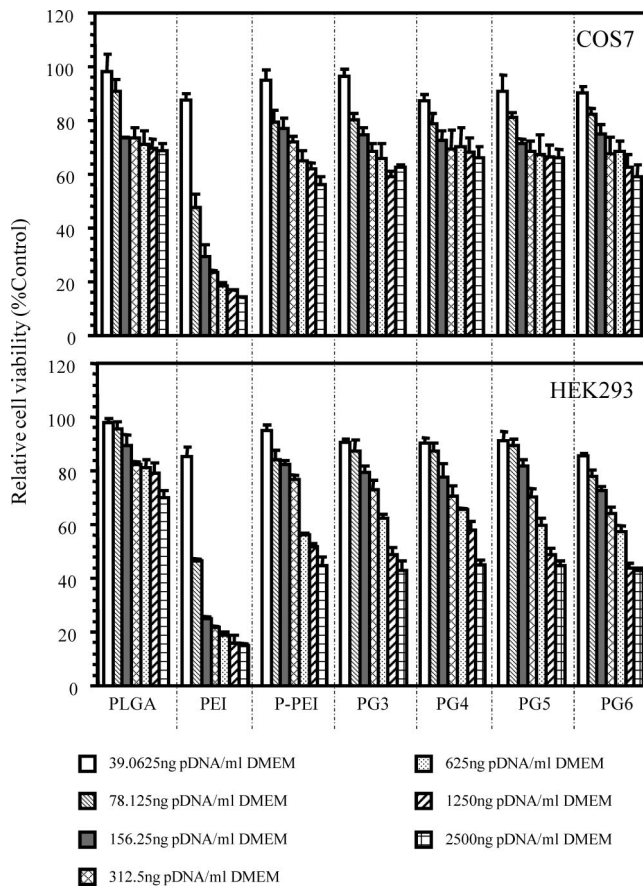


Figure 4. Cytotoxicity of dendrimer-conjugated PLGA pDNA microparticles in COS7 and HEK293 cells in comparison with PEI-conjugated PLGA pDNA microparticles, unmodified PLGA pDNA microparticles, and PEI alone. PEI amounts were selected on the basis of an estimated pDNA dose using an N/P ratio of 10:1. Cell viabilities were evaluated by MTT assay as described in the Materials and Methods section. Data is represented as the mean \pm standard deviation ($n = 6$).

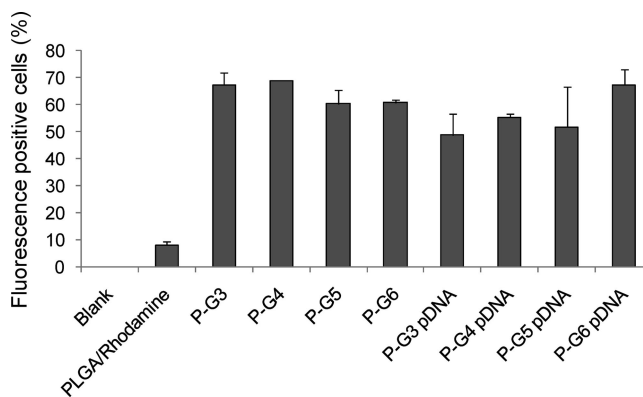


Figure 5. Flow cytometry data of fluorescence-labeled PLGA microparticles that have been incubated with HEK293 cells. The cellular uptake of the PLGA formulations is presented as a percentage of fluorescence-labeled cells ($n = 3$ per group). PLGA microparticle formulations were added at doses of 0.5 $\mu\text{g}/\text{well}$ in HEK293 cells. The concentrations of PLGA particles were chosen from an estimated pDNA loading of 5 $\mu\text{g}/\text{mg}$ particles and a target pDNA dose of 2.5 $\mu\text{g}/\text{well}$.

PAMAM microparticles showed significantly higher transgene expression than unmodified PLGA microparticles. In contrast with zeta potential and buffering capacity measurements, transfection efficiencies with PLGA PAMAM pDNA microparticles were not dependent on the generation of conjugated

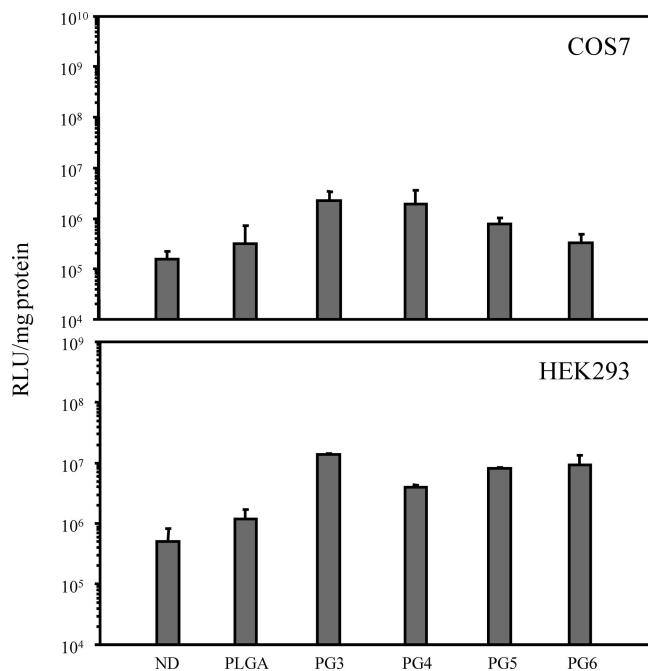


Figure 6. PLGA pDNA and PLGA PAMAM pDNA microparticle mediated gene transfection in COS7 and HEK293 cells. Microparticles were incubated with cells at a concentration of 0.2 mg/well with a target pDNA dose of 1 μ g/well. Cell harvesting and luciferase assays were performed 48 h after transfection as described in the Materials and Methods section. Data are represented as mean \pm standard deviation ($n = 3$). All the dendrimer-conjugated PLGA microparticles exhibited efficient gene delivery.

PAMAM. PG3 pDNA microparticles mediated the highest luciferase expression in both cell lines, which was 11-fold or 7-fold higher than unmodified PLGA pDNA microparticles in HEK293 or COS7 cells, respectively.

4. DISCUSSION

Delivery of pDNA from PLGA microparticles has shown significant potential in nonviral gene delivery. Entrapping pDNA in PLGA microparticles exposes the pDNA to potentially inactivating solvents and shear forces. (3, 5, 8). In addition, the local acidic microenvironment of PLGA microparticles has been reported to nick and subsequently inactivate pDNA (5, 8). Loading pDNA onto microparticles after they have been prepared overcomes several of these barriers for efficient pDNA delivery. However, PLGA microparticles are typically prepared using poly(vinyl alcohol) (PVA) surfactants. PVA surfactants impart a negative surface charge on the microparticles because of physical entrapment in the surface of the microparticles (11, 12, 26, 27). As a result, pDNA, which has a net negative charge, does not bind efficiently to the PLGA microparticle surface. Several investigators have sought to overcome this limitation by preparing cationic microparticles that bind pDNA electrostatically. Methodologies that have been developed for preparing cationic PLGA microparticles include the use of cationic agents such as cetyltrimethylammoniumbromide (CTAB) (15), cetyldimethylethylammonium bromide (CDAB), dimethyldioctadecylammonium bromide (DDA) (28), 1,2-dioleoyl-1,3-trimethylammonio propane (DOTAP), cationic DDAB (28), poly(L-lysine) (PLL), (29–32) chitosan (33) and polyethyleneimine (PEI) (10, 34). Preparation of the cationic microparticles has included blending, surface coating, and covalent attachment of these agents (3, 10, 13, 16, 17). Each method has its attributes and drawbacks. However, covalent attachment of cationic agents to the surface of microparticles is particularly advantageous

because it minimizes toxicity associated with noncovalently attached cationic polymers or surfactants. This method also provides a method for more reliable loading levels—a prerequisite for the pharmaceutical industry (10). A significant advantage of these systems is the ability to instantaneously deliver DNA/RNA-based material followed by sequential delivery of complementary agents such as antigens, chemokines, or cytokines entrapped in the PLGA microparticle matrix (10, 11). In this study, we evaluated the impact of conjugating PAMAM dendrimers onto the surface of PLGA microparticles for enhanced pDNA delivery. SEM analysis showed that covalent attachment of PAMAM dendrimers to the surface of PLGA microparticles resulted in a smooth and spherical appearance at all generation levels of the PAMAM dendrimer. No statistical difference was observed in particle size after PAMAM conjugation ($P > 0.05$). Covalent attachment of G3 PAMAM dendrimers onto PLGA microparticles (PG3) increased the zeta potential from -50 mV to $+20$ mV ($P < 0.001$). The zeta potential of the PAMAM dendrimer-conjugated microparticle significantly decreased to values ranging from $+0.5$ mV to 13.5 mV after pDNA binding. This is most likely because pDNA has a net negative charge that neutralizes the positive charges associated with PAMAM dendrimer when binding electrostatically. Increasing the generation of the PAMAM dendrimer conjugated to the PLGA microparticle surface increased the zeta potential proportionally both before and after plasmid binding. For example, PG6 microparticles displayed zeta potentials of approximately $+40$ mV before pDNA binding and $+12.2$ mV after pDNA binding. This presumably relates to the fact that dendrimers at higher generations have more surface amine groups and a higher positive charge density. This change from negative to positive zeta potential increased the ability of pDNA to bind to the surface of the microparticles. PLGA microparticles with PAMAM conjugated to the surface displayed on average 5-fold higher pDNA loading levels in comparison to unmodified PLGA microparticles ($P < 0.001$). PG6 microparticles had significantly higher pDNA loadings than PG3, PG4, and PG5 microparticles ($P < 0.05$). No significant difference in pDNA loading was observed among PG3, PG4, and PG5 ($P > 0.05$). The presence of secondary and tertiary amines on the PAMAM dendrimer structures enabled the PAMAM-modified PLGA microparticles to resist acidification in acid titration experiments ($P < 0.001$ in comparison to unmodified PLGA microparticles). This ability to buffer against pH changes is believed to contribute to endophagosomal escape into the cytoplasm as described by the proton sponge hypothesis (20, 24). Increases in the generation level of the PAMAM conjugated to the PLGA microparticles correlated to increases in buffering capacity. The buffering capacity of PLGA PAMAM microparticles also correlated to zeta potential values. Dose-dependent toxicity measurements were made using the MTT assay (21). PLGA PAMAM microparticles could be incubated at concentrations as high as 500 μ g of particles per milliliter of DMEM with more than 50% of the cells still viable. Several investigators have reported that PAMAM dendrimers alone display generation-dependent toxicity. For example, Duncan and colleagues reported that PAMAM dendrimers display concentration- and generation-dependent cytotoxicity and hemolysis as observed by changes in red cell morphology (22). The mechanism by which PAMAM dendrimers induce cytotoxicity is apoptosis involving a caspase-dependent pathway (35). Of notable interest in this study is that no significant differences in cytotoxicity were observed for PG3 pDNA, PG4 pDNA, PG5 pDNA, and PG6 pDNA microparticles. PLGA PAMAM microparticles retained the low cytotoxicity properties of unmodified PLGA microparticles in COS7 cells ($P > 0.05$). In the HEK293 cell line, cytotoxicity of PLGA PAMAM microparticles was mod-

erately higher than unmodified PLGA microparticles. This result suggests that cytotoxicity is cell-type dependent, and for certain applications that target specific tissues or cells, PAMAM conjugation to PLGA microparticles could completely remove the undesirable toxicity associated with PAMAM dendrimers alone. These results are consistent with the reduced cytotoxicity that is observed when PEI (25 kDa) is attached to PLGA microparticles in comparison to PEI alone (Figure 4) (10). Flow cytometry studies showed that conjugation of PAMAM to the PLGA microparticles significantly enhanced cell uptake by 6–7-fold in comparison to unmodified PLGA microparticles ($P < 0.001$). Changes in particle size were not significant between each group ($P > 0.05$) and were therefore not responsible for this increase in cell uptake. The zeta potential of PLGA PAMAM microparticles was significantly higher than unmodified PLGA microparticles even after pDNA binding. Cationic microparticles demonstrate an enhanced interaction with the negatively charged cellular membranes that is most likely responsible for the enhanced cell uptake. However, increasing the generation of the PAMAM dendrimer conjugated to the PLGA microparticles had no impact on increasing cell uptake further.

Gene transfection experiments were carried out on two model cell lines commonly used to evaluate transfection efficiency. Conjugation of PAMAM dendrimers to PLGA microparticles significantly enhanced luciferase expression in comparison to unmodified PLGA microparticles in both COS7 and HEK293 cell lines. G3 dendrimers covalently attached to PLGA microparticles generated the highest transgene luciferase expression. In contrast to dendrimers alone, however, increasing the generation of the dendrimer conjugated to the PLGA microparticle did not increase luciferase transgene expression. This result was consistent in both HEK293 and COS7 cell lines. This observation is important with respect to the manufacture of the PLGA PAMAM microparticles because G3 PAMAM dendrimers are easier to synthesize (23) and four times cheaper than G6 PAMAM dendrimers when purchased commercially (Dendritech). In addition, lower generation number PAMAM dendrimers have longer circulation times (22). Lower-generation PAMAM dendrimers that would eventually become free from the microparticle are also substantially less cytotoxic than higher-generation PAMAM dendrimers (21–23, 35, 36). Lin and colleagues have shown that G2 dendrimer conjugated to mesoporous silica nanoparticles can generate transfection efficiencies that are significantly higher than commercial transfection reagents because of a sedimentation effect previously described by Luo et al. (37) Given that we have observed generation-dependent increases in the buffering capacity and zeta potential for PLGA PAMAM microparticles, we would have expected corresponding transfection efficiency increases. However, in our studies, increases in zeta potential and buffering capacity do not correlate to further increases in transfection efficiency. This is consistent with a number of studies that have shown that zeta potential of dendrimers and transfection efficiency do not necessarily correlate (38, 39). The increase in transfection efficiency of PLGA PAMAM pDNA microparticles in comparison to PLGA pDNA microparticles is therefore related to the increased pDNA loading and increased interaction of microparticles with the cell surface. The existence of some buffering capacity may enhance transfection, but this study suggests that further promotion of the proton sponge effect does not further enhance transfection efficiency with this delivery system.

Conjugating PAMAM dendrimers to the surface of PLGA microparticles reveals a number of options for further exploration. The PAMAM dendrimers impart the PLGA microparticles with a much greater proportion of reactive surface functional

groups. These could be used for efficient pegylation (40, 41), conjugation of cell targeting ligands to the microparticle surface (42), or a combination of both (43). Loading of the PLGA microparticles with radioisotopes or fluorescent dyes also provides the potential for combined imaging and delivery of biomolecules (23).

5. CONCLUSION

We have prepared PLGA microparticles with PAMAM dendrimers conjugated to the surface. The buffering capacity and zeta potential of the microparticles increases with increasing generation level of the conjugated PAMAM dendrimer. Conjugation of the PAMAM dendrimer to the surface of the PLGA microparticle removes generation-dependent toxicity. PLGA PAMAM microparticles displayed similar cytotoxicity profiles to unmodified PLGA microparticles. A G3 PAMAM dendrimer conjugated to PLGA microparticles significantly increased transfection efficiencies in comparison to unmodified PLGA microparticles. Further increases in the generation level of the PAMAM did not increase transfection efficiency. Plasmid DNA entrapped in PLGA microparticles has shown *in vivo* efficacy in animal models and phase II clinical trials (44–47). Therefore, the development of PLGA polymers with optimized cationic surfaces should have significant potential for enhancing the response of these systems further.

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