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Xue-Qing Zhang ^a; Janjira Intra ^a; Aliasger K. Salem ^a

^a Division of Pharmaceutics, College of Pharmacy, University of Iowa, Iowa City, Iowa, USA

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Comparative study of poly (lactic-co-glycolic acid)-poly ethyleneimine-plasmid DNA microparticles prepared using double emulsion methods

XUE-QING ZHANG*, JANJIRA INTRA*, & ALIASGER K. SALEM

Division of Pharmaceutics, College of Pharmacy, University of Iowa, Iowa City, Iowa, USA

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Abstract

Controlled release of plasmid DNA (pDNA) from biodegradable poly lactic-co-glycolic acid (PLGA) microparticles has the potential to enhance transgene expression. However, barriers to this approach include limited encapsulation efficiency, pDNA damage during fabrication and confinement of the microparticles inside phagolysosomal compartments. Combining PLGA with poly ethyleneimine (PEI) can improve protection of pDNA during fabrication, increase encapsulation efficiencies and impart the PLGA microparticles with the capacity to escape the phagolysosomal compartments. This study compares three promising formulation methods for preparing PLGA PEI pDNA microparticles and evaluates for buffering capacity, cellular uptake, transfection efficiency and toxicity. In the first method, PLGA PEI pDNA microparticles are prepared by entrapping pDNA in blended PLGA/PEI using the double emulsion water-in-oil-in-water solvent evaporation technique (PA). In a second approach, PEI-pDNA polyplexes are prepared and then entrapped in PLGA microparticles using a double emulsion solvent evaporation method (PB). Microparticles prepared using formulation methods PA and PB are then compared against PLGA microparticles with PEI conjugated to the surface using carbodiimide chemistry (PC); 0.5% PVA is identified as the optimum concentration of surfactant for generating the strongest transfection efficiencies. N:P ratios of 5 and 10 are selected for preparation of each group. Gel electrophoresis demonstrates that all PLGA microparticle formulations have strong pDNA binding capacity. An MTT assay shows that *in vitro* cytotoxicity of PLGA PEI microparticles is significantly lower than PEI alone. PLGA PEI pDNA microparticles mediate higher cellular uptake efficiency and consequently higher transgene expression than unmodified PLGA microparticles in COS7 and HEK293 cells. Preparing PEI-pDNA polyplexes prior to entrapment in PLGA microparticles (PB) results in the highest pDNA loading. This is 2.5-fold higher than pDNA loading in unmodified PLGA microparticles. PLGA PEI pDNA microparticles prepared using method PB generates the strongest transfection efficiencies, which are 500-fold higher than unmodified PLGA pDNA microparticles in HEK293 cells and 1800-fold higher in COS-7 cells. The highest transfection efficiencies generated from microparticles prepared using method PB is achieved using an N:P ratio of 5.

Keywords: PLGA, PEI, microparticle, non-viral gene delivery, formulation

Introduction

Gene therapy has shown significant potential for treatment of a wide variety of diseases ranging from peanut allergies to cancer (Roy et al. 1999, Hung et al. 2001, Salem et al. 2005). Successful application of gene therapy is dependent on optimization and synthesis of the delivery carrier (Luo and Saltzman 2000, Salem et al. 2003, O'Hagan et al. 2004). These carriers must be efficient in transfection, safe for human use, protect the plasmid DNA (pDNA) from enzymatic degradation and should be capable of

delivering pDNA to the target tissue or cells (Luo and Saltzman 2000, Pouton and Seymour 2001).

Poly (D,L-lactide-co-glycolide) (PLGA) is a polymer that is biodegradable, biocompatible (Eldridge et al. 1991) and has shown significant promise for delivery of pDNA and immunostimulatory molecules such as CpG ODN (Walter et al. 1999, 2001, Tinsley-Bown et al. 2000, Walter and Merkle 2002, Panyam and Labhasetwar 2003, O'Hagan et al. 2004, Oster and Kissel 2005, Zhang et al. 2007a, b). A limitation in the use of PLGA microparticles for pDNA delivery is that the preparation process can

Correspondence: Aliasger K. Salem, Division of Pharmaceutics, College of Pharmacy, University of Iowa, Iowa City, Iowa 52242, USA. E-mail: aliasger-salem@uiowa.edu

expose the pDNA to high shear forces, sonication and organic solvents (Walter et al. 1999, Tinsley-Bown et al. 2000, Oster and Kissel 2005, Abbas et al. 2007, Zhang et al. 2007c). These processes can damage and denature the pDNA, thereby inactivating it. PLGA microparticles that are internalized into the phagolysosomes of cells display a limited capacity to escape into the cytoplasm. Finally the acidic microenvironments of the degrading PLGA microparticles can inactivate pDNA (Walter et al. 1999, Tinsley-Bown et al. 2000, Abbas et al. 2007). Several investigators have sought to overcome these limitations. For example, minimizing shear stress during homogenization and preserving the supercoiled structure has been achieved by using a cryopreparation modification of the double emulsion method (Ando et al. 1999). A more common approach has been the utilization of cationic excipients that condense the plasmid DNA protecting it from enzymatic degradation and denaturation during the harsh manufacturing processes involved in microparticle fabrication. Examples of cationic excipients that have been explored for enhancing pDNA formulation in PLGA microparticles include cetyltrimethylammoniumbromide (CTAB) (Singh et al. 2003), cetyldimethylammonium bromide (CDAB), dimethyldioctadecyl ammonium bromide (DDAB) (Wasan et al. 1996), 1,2-dioleoyl-1,3-trimethylammonio propane (DOTAP), cationic DDAB (Wasan et al. 1996), poly(L-lysine) (PLL) (Capan et al. 1999a, b, c, Gebrekidan et al. 2000), polyamidoamine (PAMAM) dendrimers (Zhang et al. 2007c) and chitosan (Ravi Kumar et al. 2004). Recently, PLGA microparticles have been developed that incorporated PEI as a cationic agent that enhances pDNA delivery to cells (Manuel et al. 2001, De Rosa et al. 2003, Nam et al. 2003, Bivas-Benita et al. 2004, Kasturi et al. 2005, 2006, Oster et al. 2005, dos Santos et al. 2006, Moffatt and Cristiano 2006a, b, Sutton et al. 2006). PLGA PEI microparticles have shown significant potential in genetic vaccination and antisense oligonucleotide applications for treatment or prevention of a variety of diseases that include *Listeria Monocytogenes*, glaucoma, lymphoma and prostate cancer (Oster et al. 2005, Kasturi et al. 2006, Moffat and Cristiano 2006b). Branched PEI exhibits a high positive charge density when protonated in aqueous solution and its buffering capacity at the slightly acidic pH values of the endosome facilitates the release of PEI/DNA polyplexes from the endosomal compartment into the cytoplasm (Boussif et al. 1995, De Smedt et al. 2000). As a result, incorporating branched PEI into PLGA microparticles is expected to impart similar properties to hybrid PLGA PEI microparticles. Preparation of PLGA PEI microparticles can be achieved using a number of formulation strategies. For example PLGA/PEI blends can be used to entrap pDNA using a double

emulsion solvent evaporation procedure (Oster et al. 2005). Another approach is to covalently attach PEI to the surface of PLGA microparticles followed by plasmid binding to the cationic microparticle surface (Kasturi et al. 2005). In both cases, the combination of PLGA and PEI lowers the toxicity associated with PEI whilst maintaining much higher transfection efficiencies than PLGA alone. A third approach to preparing PLGA PEI pDNA microparticles is to entrap PEI-pDNA polyplexes in PLGA microparticles. PLGA PEI pDNA microparticles prepared using this method demonstrate higher loading efficiencies and more controlled release profiles than encapsulation of pDNA alone or alternative approaches to formulation such as spray drying (Oster and Kissel 2005). This study compares each of these formulation approaches to PLGA PEI pDNA microparticle preparation. This is achieved by testing for particle size, zeta potential, plasmid DNA entrapment efficiency, buffering capacity, cell uptake, cytotoxicity and transfection efficiency in HEK293 and COS7 cell lines.

Materials and methods

Materials

D,L-Lactide/glycolide copolymers (PLGA, molar ratio: 75/25 LA:GA, inherent viscosity: 0.47 dL g^{-1}) are purchased from Absorbable Polymers International (Pelham, AL). Branched poly ethyleneimine (PEI, Mw 25 kDa) and poly(vinyl alcohol) (PVA, Mw 30–70 kDa) are products of Sigma-Aldrich (St. Louis, MO). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), N-Hydroxysulpho-succinimide (Sulpho-NHS) and the bicinchoninic acid (BCA) protein assay kit are purchased from Pierce Biotechnology Inc. (Rockford, IL). Dulbecco's Modified Eagle's Medium (DMEM) is obtained from Gibco BRL (Grand Island, NY). The luciferase assay system is purchased from Promega (Madison, WI). For cellular uptake studies, FITC labelled PEI (branched, 25 k Da) was prepared by reaction of FITC and branched PEI in 0.1 M sodium carbonate buffer (pH 9) in darkness overnight. FITC-PEI is purified by dialysis (MWCO 7000, Pierce Biotechnology Inc., Rockford, IL) and then lyophilized (Labconco FreeZone 4.5, Kansas City, MI). Spectrofluorometric analysis (Spectramax Microplate reader, Molecular Device) revealed that ~3% of the amino groups of PEI are attached to FITC.

Cell culture

Human embryonic kidney cells (HEK293) and Monkey African green kidney (COS-7) cells are obtained from American Type Culture Collection

(ATCC, Rockville, MD). The cells are maintained in DMEM supplemented with 10% foetal bovine serum (FBS), streptomycin at $100 \mu\text{g ml}^{-1}$, penicillin at 100U ml^{-1} and 4 mM L-glutamine at 37°C in a humidified 5% CO_2 -containing atmosphere.

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 is transformed in *Escherichia coli* DH5 α and amplified in Terrific Broth media at 37°C overnight on a plate shaker set at 300 rpm. The plasmid is purified by an endotoxin-free QIAGEN Giga plasmid purification kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol. Purified DNA is dissolved in saline and its purity and concentration are determined by UV absorbance at 260 and 280 nm using a SpectraMax Plus³⁸⁴ Microplate Spectrophotometer (Molecular device).

Preparation of PLGA PEI pDNA microparticles

PLGA pDNA microparticles. PLGA pDNA microparticles are prepared using water-in-oil-in-water (w/o/w) double emulsion, solvent evaporation technique. Briefly, 100 mg of 75:25 PLGA is dissolved in 5 ml of dichloromethane (DCM). VR1255 pDNA in 0.5% (w/v) PVA solution is prepared at a concentration of 4mg ml^{-1} . 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 is mixed with the PLGA/DCM solution for 20 s to form the first emulsion. This emulsion is 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 mixture is stirred overnight during which time the DCM solvent is evaporated. The microparticles are then washed four times with deionized water and lyophilized (Labconco FreeZone 4.5, Kansas City, MI). The supernatant is collected and analysed spectrophotometrically at 260 nm using a SpectraMax Plus³⁸⁴ Microplate Spectrophotometer (Molecular device) for pDNA content. Plasmid DNA encapsulated in the PLGA microparticles is calculated by subtracting the pDNA content in the supernatant from the initial concentration of pDNA added. Microparticles are stored at -20°C until use. For particle uptake studies *in vitro*, PLGA microparticles loaded with Rhodamine 123 are prepared using a single emulsion evaporation methodology. Briefly, 100 mg of 75:25 PLGA and 2 mg Rhodamine 123 (Sigma) are dissolved in 5 mL DCM. This is then rapidly added to 50 mL of 0.5% (w/v) PVA in deionized water with stirring at 13 500 rpm.

The rhodamine labelled particles are washed and collected as described above.

PLGA PEI pDNA microparticles (PA5 and PA10). Microparticles are prepared by a modified w/o/w double emulsion, solvent evaporation procedure. Briefly, 1.3 mg or 2.6 mg of PEI is dissolved in 5 ml of DCM containing 100 mg of 75:25 PLGA. The amount of PEI used produces N/P ratios of 5 or 10 (which is defined as the molar ratio of primary amino groups in PEI to phosphate groups in DNA). Then 500 μl of the 0.5% (w/v) PVA solution containing 2 mg of VR1255 pDNA is mixed with the PLGA/PEI solution using the microtip probe sonicator set at level 2 for 30 s to form the first emulsion. This emulsion is then rapidly added to 50 ml of 0.5% (w/v) PVA solution that is homogenized at 13 500 rpm for 30 s. The mixture is stirred overnight during which time the DCM solvent is evaporated. The microparticles are then washed four times with deionized water and lyophilized. Microparticles PA5 and PA10 are stored at -20°C until use.

PLGA PEI pDNA microparticles (PB5 and PB10). PEI/pDNA complexes at N/P ratio of 5 or 10 are prepared by mixing 2 mg of VR1255 with 1.3 mg or 2.6 mg of PEI in 500 μl of 0.5% (w/v) PVA solution, respectively. The mixture is vortexed for 20 s and incubated for 30 min at room temperature. Then 500 μl of PEI/pDNA complexes solution with N/P ratio of 5 or 10 is mixed with 5 ml of DCM containing 100 mg of 75:25 PLGA using the microtip probe sonicator set at level 2 for 30 s to form the first emulsion. This emulsion is then rapidly added to 50 ml of 0.5% (w/v) PVA solution and homogenized at 13 500 rpm for 30 s. The mixture is stirred overnight during which time the DCM solvent evaporated. The microparticles prepared using the PB method are then washed four times with deionized water and lyophilized. Microparticles are stored at -20°C until use.

PLGA PEI pDNA microparticles (PC). A modified EDC/NHS chemistry is used to conjugate PEI to the surface of PLGA microparticles to obtain cationic microparticles. Blank PLGA microparticles are prepared with 5 ml of DCM containing 200 mg of PLGA-COOH and 500 μl of the 0.5% (w/v) PVA solution using a w/o/w double emulsion, solvent evaporation technique; 100 mg of the PLGA-COOH microparticles are suspended in 10 ml of 0.1 M MES (2-(N-morpholino) ethane sulphonic acid) buffer, pH 5.1; 1 ml of EDC solution (60 mM) in 0.1 M MES buffer and 1 ml of Sulpho-NHS solution (60 mM) in 0.1 M MES buffer are added dropwise to the PLGA microparticle suspension. EDC activation is carried out for 2 h at room temperature. A 10 molar excess of 25 kDa branched PEI is dissolved in 5 ml of 0.1 M

MES buffer. Activated PLGA microparticles are added dropwise to the PEI solution with magnetic stirring and incubated for another 4h at room temperature. PEI-conjugated PLGA microparticles are washed twice in 1 M NaCl to remove physically adsorbed PEI and twice with deionized water. The resulting microparticles are lyophilized and stored at -20°C until use. Fluorescamine (4-phenylspiro [furan-2(3H), 1'-phthalan]-3, 3'-dione) is used for colourimetric quantification of primary amines and is used to quantify the amount of PEI conjugated to the microparticles (Manuel et al. 2001). PEI-conjugated microparticles are hydrolysed in 0.1 N NaOH overnight and the PEI content is measured using spectrofluorometric analysis (Spectramax Microplate reader, Molecular Device). Fluorescamine reacts with primary amines in PEI to form pyrrolinones, which are excited at 390 nm and have an emission peak at 475–490 nm. The PEI amounts (w/w) in microparticles are estimated using standard curves of PEI.

Plasmid DNA loading on cationic microparticles (PC). VR1255 pDNA is loaded on the surface of PLGA PEI microparticles (PC) as described by Singh et al. (2000). Briefly, pDNA is incubated with the PLGA PEI microparticles prepared using methodology PC (pH adjusted to 6.5) at a concentration of $20\ \mu\text{g pDNA/mg particles}$ at 4°C for 6 h. The resulting particles are centrifuged at 13 200 rpm for 5 min on a microcentrifuge and washed twice with the loading buffer to obtain PLGA PEI pDNA microparticles. The supernatant is collected and analysed spectrophotometrically at 260 nm using a SpectraMax Plus³⁸⁴ Microplate Spectrophotometer (Molecular device) for pDNA content. Plasmid DNA loading on the cationic microparticles is calculated by subtracting the pDNA content in the supernatant from the initial concentration of pDNA added.

Microparticle size and surface morphology analysis

Microparticle size and zeta potentials are measured using the Zetasizer Nano ZS (Malvern, Southborough, MA). Briefly, the particles are suspended in deionized water at a concentration of $1\ \text{mg ml}^{-1}$. The size is measured at 25°C at a 173° scattering angle. The mean hydrodynamic diameter is performed by cumulative analysis. The zeta potential determination is based on electrophoretic mobility of the microparticles in the aqueous medium, which are performed using folded capillary cells in automatic mode. Microparticle morphology is assessed by Scanning Electron Microscopy (SEM, Hitachi S-4000). Air-dried microparticles are placed on adhesive carbon tabs mounted on SEM specimen stubs. The specimen stubs are coated with $\sim 5\ \text{nm}$ of gold by ion beam evaporation before examination in the SEM operated at 5 kV accelerating voltage.

Buffering ability of PLGA PEI microparticles

The ability of PLGA PEI microparticles to resist acidification is tested using the acid titration assay as described by Tang et al. (1992). Briefly, $10\ \text{mg ml}^{-1}$ PLGA PEI microparticles are suspended in 150 mM NaCl. The pH is first adjusted to ~ 9.0 and then titrated in small increments with 0.1 N HCl until a pH of 3.0 is reached. The slope of the pH vs. HCL added graph provides an indication of the intrinsic buffering capability of the delivery vehicles.

Gel electrophoresis analysis

The PLGA pDNA and PLGA PEI pDNA microparticles are prepared as described above and then loaded on a 0.8% (w/v) agarose gel stained with ethidium bromide ($0.5\ \mu\text{g ml}^{-1}$) for 40 min at 80 V. The gel is visualized on an UV transilluminator (Spectroline TE-312S, Spectronics Corporation, Westbury, NY) to document the mobility of the pDNA.

Cytotoxicity evaluation using the MTT assay

Cytotoxicity of the PLGA pDNA and PLGA PEI pDNA microparticles is evaluated using the MTT (3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) assay. PEI-pDNA polyplexes alone are used as a control. COS7 and HEK293 cells are seeded in a 96-well plate at a density of 1×10^4 cells/well. Twenty-four hours later, cells are incubated with 200 μl of complete DMEM containing PLGA pDNA microparticles, PLGA PEI pDNA microparticles or PEI-pDNA at various concentrations. After 4 h of incubation, the medium in each well is replaced with 100 μl of fresh complete medium. MTT solution in PBS is added to each well and incubated with cells for an additional 2 h. Cells are lysed with 100 μl of the extraction buffer (20% SDS in 50% DMF, pH 4.7) overnight. The optical density of the lysate is measured at 550 nm using a Spectramax plus³⁸⁴ Microplate Spectrophotometer (Molecular Device). Values are expressed as a percentage of the control to which no microparticles are added.

Cellular uptake of microparticles

PLGA PEI pDNA microparticles are prepared with FITC-PEI using formulation methodologies PA, PB and PC. HEK293 cells are seeded into a 12-well plate at a density of 1×10^6 cells/well 24 h before transfection. After 24 h incubation, the medium is replaced with fresh DMEM containing 10% FBS. 0.5 mg/well PLGA/Rhodamine 123, FITC labelled microparticles prepared using methods PA, PB and PC are incubated with HEK293 cells for 16 h. Then the cells are washed by PBS three times to remove free fluorescent labelled microparticles and the microparticles adsorbed on the cells surface. Samples are then

assessed using flow cytometry (Becton Dickinson). Dot plots are gated on FSC/SSC properties of HEK293 cells to exclude free fluorescent labelled microparticles. Data are analysed using Cell-Quest^{Pro} software. All samples are tested in triplicate.

Evaluation of luciferase expression in COS7 and HEK293 cells

Cells are seeded into a 24-well plate at a density of 8×10^4 cells/well of COS7 and HEK293 cells 24 h before transfection; 0.2 mg/well PLGA pDNA and PLGA PEI pDNA microparticles are 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 is chosen from an estimated pDNA loading and a target pDNA dose of 1 µg/well. After 44 h incubation, cells are treated with 200 µl of lysis buffer (Promega). The lysate is subjected to two cycles of freezing and thawing, then transferred into tubes and centrifuged at 13 200 rpm for 5 min. Twenty microlitres of supernatant are added to 100 µl of luciferase assay reagent (Promega) and samples are measured on a luminometer for 10 s (Lumat LB 9507, EG&G Berthold, Bad Wildbad, Germany). The relative light units (RLU) are normalized against protein concentration in the cell extracts, measured by a BCA protein assay kit (Pierce). Luciferase activity is expressed as relative light units (RLU/mg protein in the cell lysate). The data are reported as mean ± standard deviation for triplicate samples. Every transfection experiment is repeated at least twice.

Statistical analysis

Group data are reported as mean ± SD. Differences between groups are analysed by one way analysis of variance with a Tukey post-test analysis. Levels of significance are accepted at the $p < 0.05$ level. Statistical analyses are performed using Prism 3.02 software (Graphpad Software, Inc., San Diego, CA).

Results and discussion

Formulations of hybrid PLGA-PEI microparticles

This study prepared PLGA PEI pDNA microparticles using three different formulation methods. In the first method (denoted as PA), PLGA and PEI are blended. This blend is then dissolved into DCM and pDNA is entrapped into the mixture using the double emulsion water-in-oil-in-water solvent evaporation technique. PLGA PEI microparticles prepared using this approach have shown significant potential in vaccinations against *Listeria Monocytogenes* (Oster et al. 2005). The second approach to PLGA PEI pDNA microparticle formulation is to prepare

PEI-pDNA polyplexes and entrap them in PLGA microparticles using the double emulsion water-in-oil-in-water solvent evaporation technique (denoted as PB). The final approach is to conjugate PEI to the surface carboxylic acid (COOH) groups present on PLGA microparticles using EDC/NHS chemistry. Plasmid DNA is then bound to the surface of the microparticles using electrostatic interactions (PC) (Kasturi et al. 2005). The PC approach has been reported to significantly reduce the toxicity associated with PEI (Kasturi et al. 2005) and has shown subsequent potential as a vaccine for prevention of lymphoma (Kasturi et al. 2006). Figure 1 shows schematically the approaches for preparing each of the PLGA PEI pDNA microparticle formulations. A number of studies have shown that the surfactant concentration used in PLGA pDNA microparticles can have a significant impact on transfection efficiencies (Capan et al. 1999a, Hsu et al. 1999). For this reason, initial surfactant optimization studies are carried out. PLGA pDNA and PLGA PEI pDNA microparticles are prepared in solutions with PVA concentrations of 0.2%, 0.5% and 1.0% (w/v). The luciferase expression mediated by these particles is evaluated in HEK293 cells. The optimal transfection efficiency is observed in cells transfected with formulations prepared in 0.5% (w/v) PVA solution (Figure 2); 0.5% (w/v) PVA solution is therefore selected to prepare the PLGA microparticle formulations in the following experiments. These results are consistent with previous observations, in which a 0.5% PVA solution resulted in a lower pDNA loading than a 5% PVA solution, but generated higher transfection efficiencies (Prabha and Labhasetwar 2004). For PLGA PEI microparticles prepared using methodology PC, the conjugation of PEI is quantified using a fluorescamine assay. This indicates that the amount of PEI present on the microparticle surface is ~3.8 µg per milligram of microparticles. This conjugation efficiency is consistent with previous observations (Kasturi et al. 2005).

Particle size, Zeta potential and morphology of microparticles

Figure 3(a) shows the particle size of the PLGA and PLGA PEI formulations selected for transfection experiments. Previous studies have shown how the stirring rate and other formulation parameters can be used to control particle size (Zhang et al. 2007a). Microparticles prepared using method PC show a 1.5-fold increase in their average size in comparison with the original PLGA microparticles used for conjugation. The average size increase appears to be due to some cross-linking between microparticles during the EDC/NHS reaction with PEI. Increasing the PEI content of microparticles prepared using methods PA and PB does not significantly change

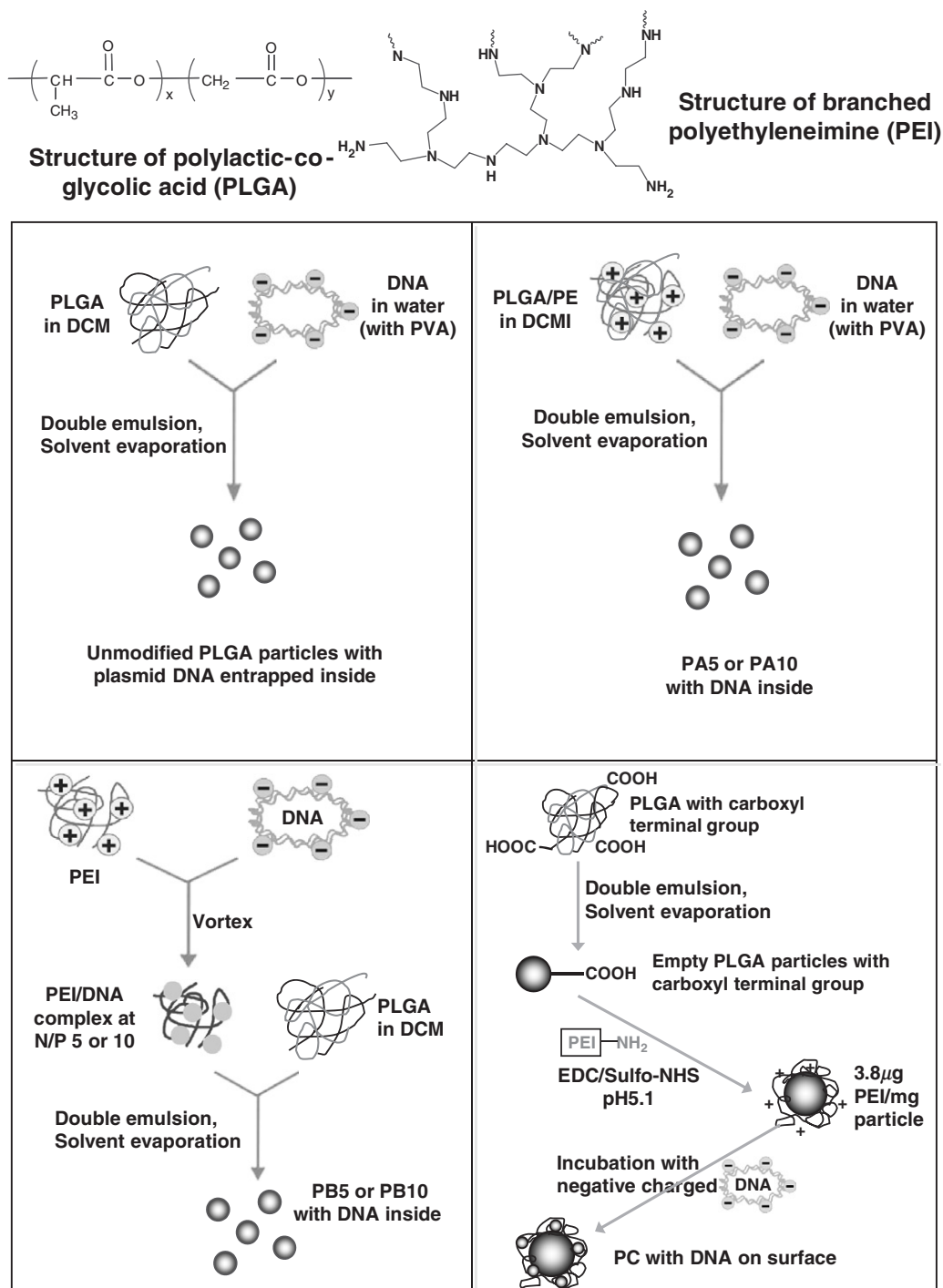


Figure 1. Schematic of the preparation of PLGA and PLGA PEI microparticle formulations using methods PA, PB and PC.

particle size. This result is consistent with previous observations of PLGA PEI microparticle formulations but in contrast to the reported effects of alternative cationic excipients such as CTAB that display a concentration-dependent effect on increasing particle size (Oster et al. 2005, Ungaro et al. 2005). Figure 3(b) shows the zeta potential of the microparticles. Blank PLGA microparticles with carboxylic acid groups display a net negative surface charge of ~ -50 mV. After the introduction of PEI,

the surface charge of all formulations of PLGA PEI microparticles becomes positive with net values ranging from +40 to +50 mV. When PEI concentrations are increased to 10% and above (w/w), multiple pores are formed on the microparticle surface (Oster et al. 2005). However, for the concentrations of PEI used in these studies, SEM analysis (Figure 3(c)) shows that all the microparticles have a smooth morphology and spherical shape.

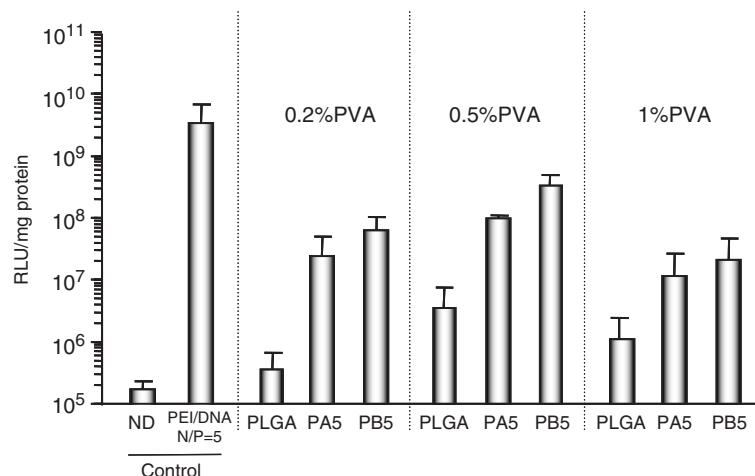


Figure 2. Luciferase activity of HEK293 cells that have been treated with unmodified PLGA, PA5 and PB5 microparticles prepared with varying concentrations of PVA solution (0.2%, 0.5%, 1.0% (w/v)). Transfection was performed by incubating these formulations with HEK293 cells for 4 h (reporter gene: VR1255; pDNA: 1 μ g/well). Data represented as the mean \pm standard deviation ($n = 3$).

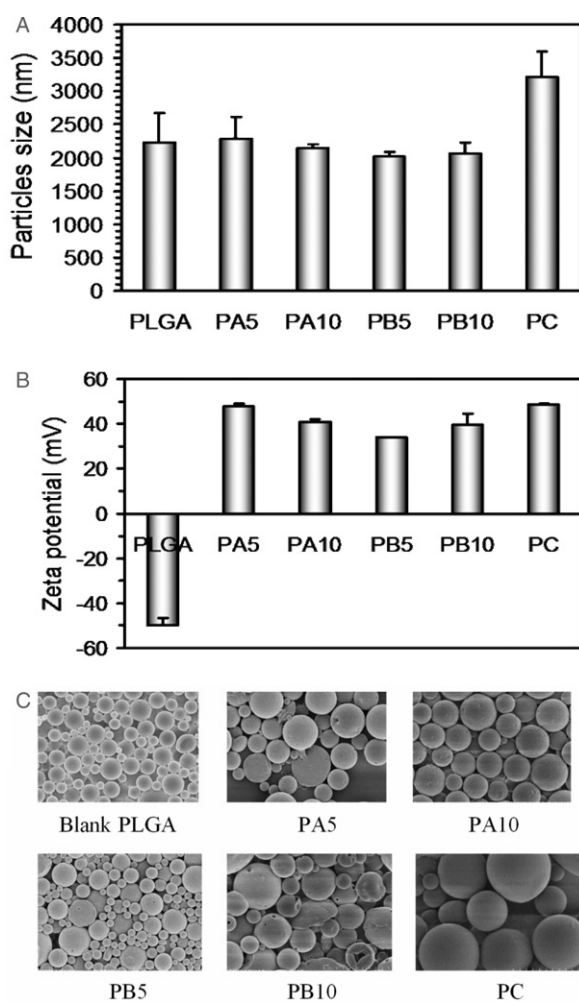


Figure 3. Size (a) and zeta potential (b) of PLGA PEI microparticles (averages representative of three measurements \pm SD). SEM images (c) show the morphology of all the PLGA microparticles is smooth and spherical in appearance.

PEI component imparts buffering properties to PLGA microparticles

Combining PEI with PLGA provides the microparticles with secondary and tertiary amines necessary to enhance their phagolysosomal pH buffering capacity, which could then lead to increased phagolysosomal escape of the microparticles into the cytoplasm. This proposed method of release into the cytoplasm is referred to as the proton sponge hypothesis (Boussif et al. 1995, Kasturi et al. 2005). The buffering capacity of PLGA PEI pDNA microparticles prepared using each method is assessed by measuring the change in the pH of a particle suspension (10 mg ml^{-1}) upon addition of increasing amounts of 0.1 N HCL. Significant buffering capacity is imparted on PLGA microparticles after introduction of PEI, especially for PLGA PEI microparticles prepared using methods PA and PB (Figure 5). This is indicated by the shift and decrease in the slope of the titration curves. PLGA PEI microparticles prepared using method PC show weaker buffering capacity than PLGA PEI microparticles prepared using methods PA and PB. This is presumably due to the lower PEI content present per microparticle. This suggests that buffering capacity of the PLGA PEI microparticles is directly correlated to PEI content.

Plasmid DNA can be efficiently loaded on cationic PLGA PEI microparticles

As shown in Table I, efficient loading of pDNA is achieved in PLGA PEI microparticles prepared using all three formulation methodologies. In all cases, pDNA loading efficiency in PLGA PEI microparticles is higher than unmodified PLGA microparticles. For example PLGA PEI pDNA microparticles prepared

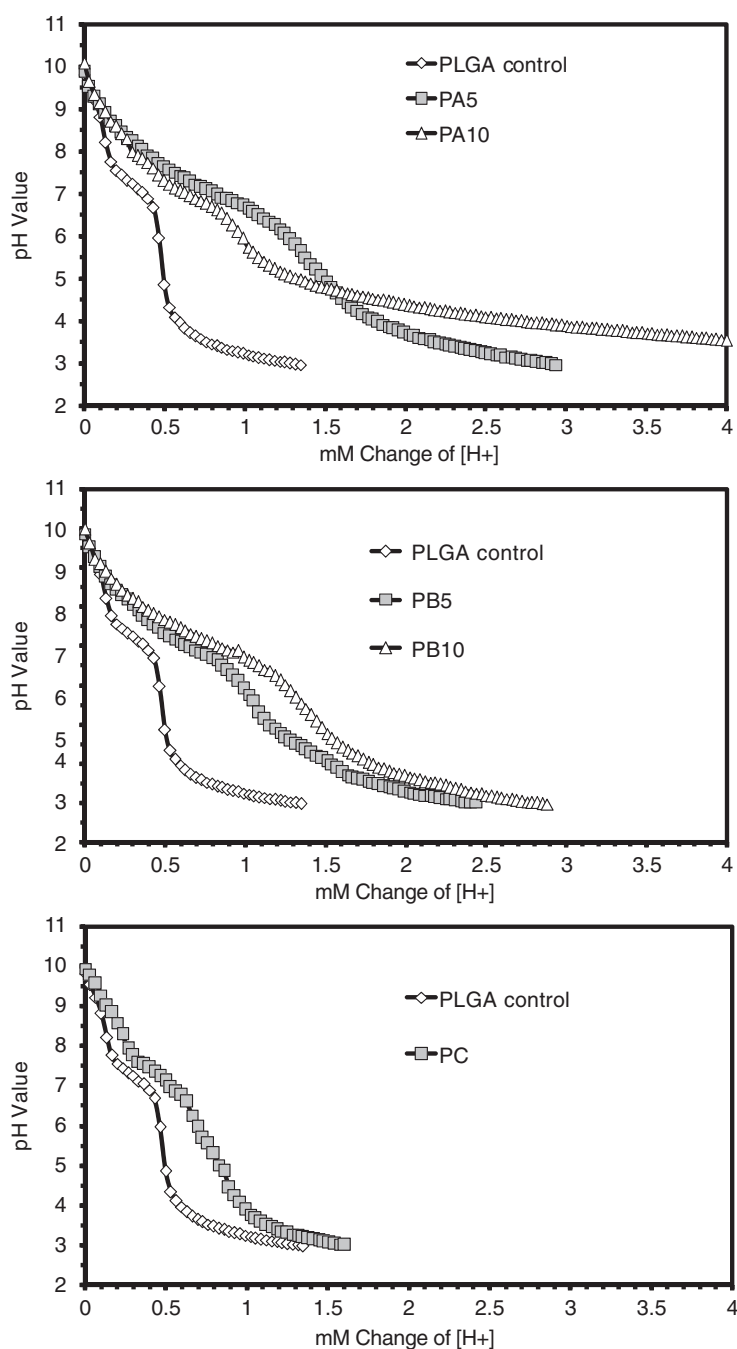


Figure 4. Acid titration experiments with 0.1 N HCL to demonstrate the buffering capacity of PLGA and PLGA PEI microparticles. The data shows significantly increased buffering by PLGA PEI microparticles prepared using formulation methodologies PA and PB compared to unmodified PLGA microparticles.

Table I. Plasmid DNA loading efficiency of PLGA and PLGA PEI microparticles (averages representative of three measurements \pm SD).

	DNA-loading efficiency (μg DNA/mg particles)
PLGA	3.5 ± 0.1
PA5	4.2 ± 0.4
PA10	6.3 ± 0.5
PB5	8.9 ± 0.4
PB10	8.7 ± 0.3
PC	4.3 ± 0.2

using the PB method displays a 2.5-fold higher pDNA loading in comparison to unmodified PLGA microparticles. It is possible that the increased loading of PEI-pDNA polyplexes in PLGA microparticles in comparison to pDNA alone is due to the lower hydrophilicity of the complex, which results in enhanced dispersion in the PLGA matrix (Oster and Kissel 2005). Formulation method PB generates pDNA loading efficiencies that are 1.5–2-fold higher than either the PA or PC methods. This highlights the critical role that the formulation method has in

optimizing pDNA loading efficiency of PLGA PEI microparticles.

Evaluation of the ability of PLGA PEI microparticles to entrap pDNA

PLGA pDNA and PLGA PEI pDNA microparticles are prepared as described earlier and pDNA binding capacity of these microparticles is analysed by agarose gel electrophoresis, as shown in Figure 5. All the PLGA microparticle formulations (including unmodified PLGA microparticles, PA5, PA10, PB5 and PB10 microparticles) completely inhibit pDNA migration on the gel, suggesting a strong pDNA binding capacity. For PLGA PEI pDNA microparticles prepared using method PC, migration of a small amount of DNA is observed on the gel. This suggests that a portion of the pDNA bound to the microparticles is weakly bound. This is likely to be due to the low conjugation degree of PEI on the PLGA microparticle surface. Further increases in the PEI content of microparticles prepared using the PC

method is limited by the number of carboxylic acid groups present on the surface of the microparticle.

PLGA particles were non-cytotoxic gene delivery vehicles

In vitro cytotoxicity is evaluated in COS7 and HEK293 cells with increasing doses of PLGA microparticle concentrations (from 7.8~250 µg of particles per millilitre of DMEM). PEI alone is used as a control. As shown in Figure 6, PEI-containing cationic PLGA microparticles show a moderately higher cytotoxicity than unmodified PLGA microparticles, but they are significantly less toxic in comparison with PEI alone ($p < 0.001$). For PEI, concentrations above 15 µg per ml medium results in lower than 50% cell viability. In comparison, for PLGA microparticles and PLGA PEI microparticles prepared using methods PA, PB and PC, more than 50% of the cells are viable at concentrations as high as 250 µg per ml medium. This shows that cationic PLGA PEI microparticles retain the low cytotoxicity

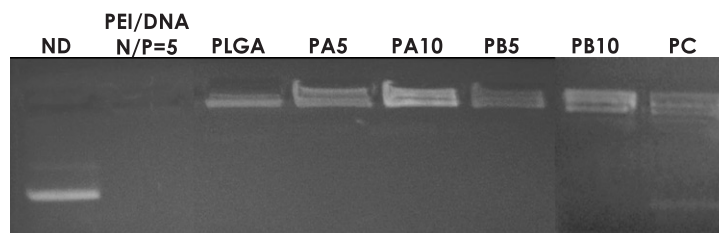


Figure 5. The ability of PLGA microparticles to entrap pDNA was analysed on agarose gel stained with ethidium bromide. Naked pDNA was used as control.

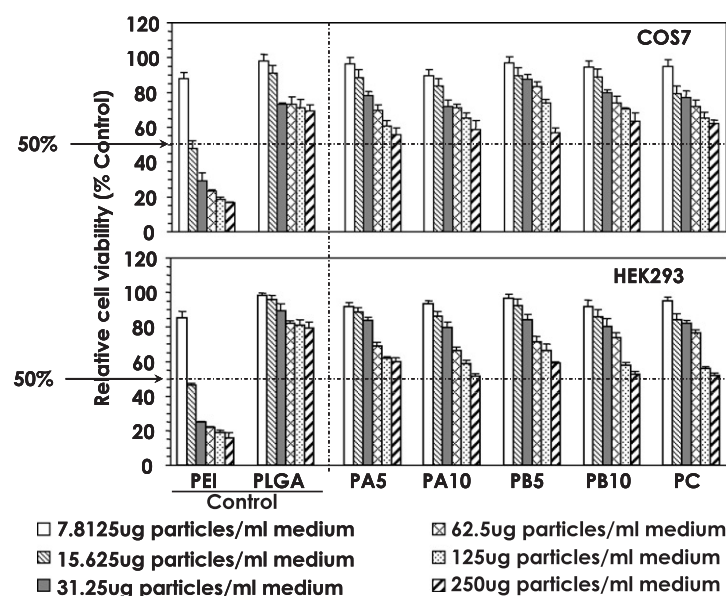


Figure 6. Cytotoxicity of PLGA PEI pDNA microparticles in COS7 and HEK293 cells in comparison with free PEI and unmodified PLGA microparticles. Cell viability was measured using the MTT assay as described in the experimental section. Data is represented as the mean \pm SD ($n = 6$).

properties of PLGA microparticles. No significant difference in the toxicity of the PLGA PEI microparticles is observed between formulation methods PA, PB and PC.

Cationic microparticles PA and PB could be efficiently taken up by HEK293 cells

Figure 7 shows cellular uptake data of fluorescence-labelled microparticles incubated with the HEK293 cells. The percentage of HEK293 cells that take up

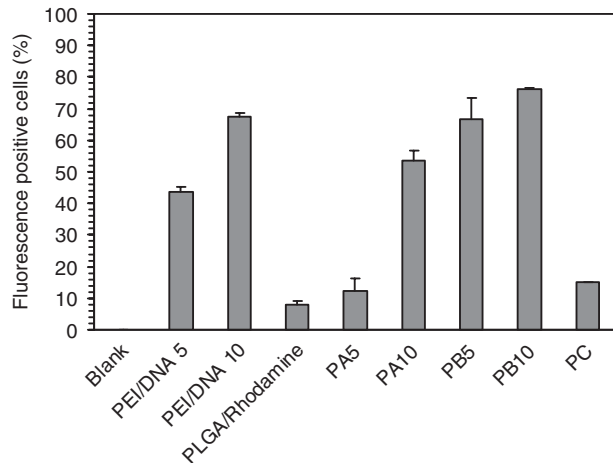


Figure 7. Flow cytometry data of fluorescence-labelled PLGA microparticles that have been incubated with HEK293 cells. The cellular uptake of the PLGA formulations is presented as the percentage of fluorescence-labelled cells ($n = 3$ per group). PLGA and PLGA PEI microparticle formulations were added at doses of 0.5 mg/well in HEK293 cells.

PEI-containing PLGA microparticles is significantly higher than PLGA/Rhodamine 123 microparticles. Although the cationic PLGA PEI microparticle formulations are of a similar size ($\sim 2 \mu\text{m}$) to the unmodified PLGA microparticles ($p > 0.05$), the positively charged surface of cationic particles clearly facilitates the attachment of the microparticles to cells followed by internalization. PLGA PEI pDNA microparticles prepared by the PB formulation method display a 6–7-fold higher cell uptake in comparison to PLGA PEI pDNA microparticles prepared by methods PA and PC. Increasing the N:P ratio is observed to enhance cell uptake. This result confirms that a high positive charge density on microparticles enhances their cellular uptake and highlights the importance that preparation methods exert on PLGA PEI pDNA microparticle uptake by cells.

PLGA PEI pDNA microparticles enhance transfection efficiencies in COS7 and HEK293 cells in comparison to PLGA pDNA microparticles

Gene transfection mediated by PLGA pDNA and PLGA PEI pDNA formulations is evaluated in COS7 and HEK293 cells (Figure 8). All cationic microparticles show significantly higher transgene expression than unmodified PLGA microparticles ($p < 0.001$). These results are consistent with the acid titration and cellular uptake results. All of the PEI-containing PLGA microparticle formulations show higher pDNA-loading ability, stronger endolysosomal pH buffering capacity and higher cellular uptake efficiency and, therefore, mediated higher

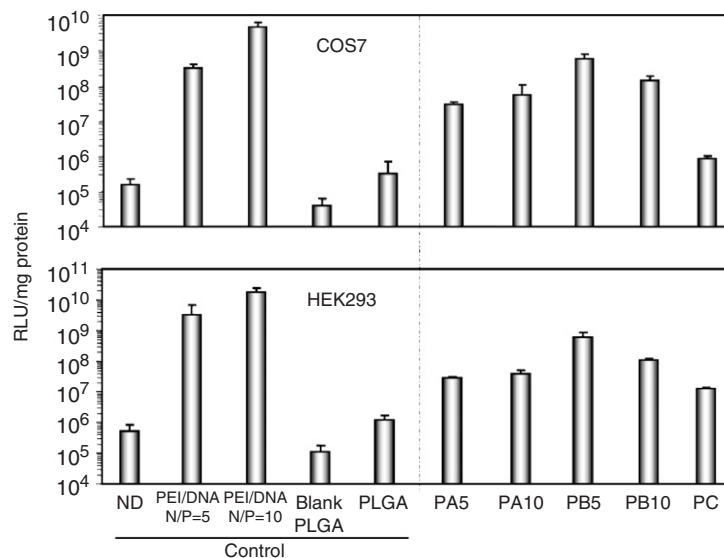


Figure 8. PLGA pDNA and PLGA PEI pDNA microparticle mediated gene transfection in COS7 and HEK293 cells. Microparticles incubated at concentrations that provided a target pDNA dose of 1 μg /well. Cell harvesting and luciferase assays were performed 48 h after transfection as described in the materials and methods section. Data is represented as mean \pm SD ($n = 3$).

luciferase expression compared with unmodified PLGA microparticles. The transfection efficiency of microparticles prepared using formulation method PB is higher than that of microparticles prepared using methods PA and PC. This is probably due to their higher pDNA-loading efficiency and higher cellular uptake efficiency. PB5 is the optimal particle formulation method for *in vitro* transfection in both cell lines. Microparticles prepared using the PB5 method show the highest transfection efficiency. This is 1800-fold higher than PLGA pDNA microparticles in COS7 cells ($p < 0.001$) and 500-fold higher than PLGA pDNA microparticles in HEK293 cells ($p < 0.001$). PLGA PEI pDNA microparticles prepared using the PC method mediates the lowest luciferase expression in both cell lines. Because of the low PEI-conjugation efficiency, fewer PEI molecules are present on the surface of microparticles prepared using method PC than microparticles prepared using methods PA and PB. Therefore, microparticles prepared using method PC shows weaker endolysosomal pH buffering capacity, lower pDNA-loading efficiency and poorer DNA association ability. All of these physicochemical properties of microparticles prepared using method PC lead to its low cellular uptake efficiency and poor transfection ability as a gene delivery vehicle. It should be noted that the pDNA binding, buffering capacity and transfection efficiency of PLGA PEI microparticles prepared using method PC can be substantially improved by using a 70k PEI instead of a 25k PEI (Kasturi et al. 2005). For microparticles prepared using the PC method, the use of a 70k PEI remains a viable option because of the low quantity of PEI conjugated per mg of microparticles (Kasturi et al. 2005). In all other PLGA PEI formulation methods, however, a 70k PEI would be expected to significantly increase toxicity in comparison to 25k PEI. Although microparticles prepared using the PA and PC formulation methods are less effective than PEI pDNA complexes alone, they are also significantly less toxic in comparison with PEI. In COS7 cells, microparticles prepared using method PB generated similar transfection efficiencies as PEI alone when used at an N:P ratio of 5 ($p > 0.05$) whilst maintaining lower toxicity than PEI alone ($p < 0.001$).

Conclusion

Plasmid DNA delivered using biodegradable polymeric microparticles has shown significant promise in vaccination applications (Hanes et al. 1997, O'Hagan et al. 2004). Microparticle-mediated delivery of pDNA results in more efficient and direct delivery to target cells than naked DNA (O'Hagan et al. 2004). A common approach to encapsulating pDNA within microparticles has been to use emulsion-based

techniques. However, barriers to this approach include limited encapsulation efficiency, plasmid damage during the emulsion process and confinement of the microparticles inside phagolysosomal compartments. To avoid these problems, cationic PEI molecules can be introduced to the PLGA microparticle formulation. Three promising approaches to preparing PLGA PEI pDNA microparticles were compared. After the introduction of PEI to PLGA microparticles, all of the modified microparticle formulations show a net positive surface charge, enhanced buffering capacity in endolysosomal pH environment and higher pDNA-loading efficiency. The MTT assay demonstrates that all the cationic PLGA PEI microparticle formulations generate significantly lower cytotoxicity than free PEI. This suggests that the cytotoxicity of the PEI component is minimized by PEI-pDNA entrapment in PLGA, physical blending of PLGA/PEI or covalent immobilization of PEI to solid PLGA microparticles. Furthermore, the addition of the PEI component significantly enhances the cellular uptake efficiency of PLGA microparticles. As a consequence, all the cationic PLGA PEI pDNA microparticle formulations mediate significantly higher transgene expression levels than unmodified PLGA microparticles ($p < 0.001$). These results are consistent with previous observations on the advantages of combining PLGA and PEI as a delivery vehicle for pDNA (Bivas-Benita et al. 2004, Huang et al. 2005, Kasturi et al. 2005, 2006, Oster and Kissel 2005, Oster et al. 2005, Sutton et al. 2006). This study shows for the first time that entrapping PEI-pDNA polyplexes in PLGA microparticles in comparison to alternative approaches to preparing PLGA PEI pDNA microparticles produces the highest cellular uptake and transfection efficiency whilst maintaining a comparable cytotoxicity profile.

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