Published in final edited form as:

J Control Release. 2012 January 30; 157(2): . doi:10.1016/j.jconrel.2011.09.071.

Polyplex-Microbubble Hybrids for Ultrasound-Guided Plasmid DNA Delivery to Solid Tumors

Shashank Sirsi¹, Sonia Hernandez², Lukasz Zielinski³, Henning Blomback³, Adel Koubaa³, Milo Synder³, Shunichi Homma⁴, Jessica J. Kandel⁵, Darrell J. Yamashiro^{2,5}, and Mark A. Borden^{1,*}

¹Department of Mechanical Engineering, University of Colorado, Boulder, CO 80309

²Department of Pediatrics and Pathology, Columbia University, New York, NY 10032

³Department of Chemical Engineering, Columbia University, New York, NY 10027

⁴Department of Cardiology, Columbia University, New York, NY 10032

⁵Department of Surgery, Columbia University, New York, NY 10032

Abstract

Microbubble ultrasound contrast agents are being developed as image-guided gene carriers for targeted delivery *in vivo*. In this study, novel polyplex-microbubbles were synthesized, characterized and evaluated for systemic circulation and tumor transfection. Branched polyethylenimine (PEI; 25 kDa) was modified with polyethylene glycol (PEG; 5 kDa), thiolated and covalently attached to maleimide groups on lipid-coated microbubbles. The PEI-microbubbles demonstrated increasingly positive surface charge and DNA loading capacity with increasing maleimide content. The *in vivo* ultrasound contrast persistence of PEI-microbubbles was measured in the healthy mouse kidney, and a two-compartment pharmacokinetic model accounting for free and adherent microbubbles was developed to describe the anomalous time-intensity curves. The model suggested that PEI loading dramatically reduced free circulation and increased nonspecific adhesion to the vasculature. However, DNA loading to form polyplex-microbubbles increased circulation in the bloodstream and decreased nonspecific adhesion. PEI-microbubbles coupled to a luciferase bioluminescence reporter plasmid DNA were shown to transfect tumors implanted in the mouse kidney. Site-specific delivery was achieved using ultrasound applied over the tumor area following bolus injection of the DNA/PEI-microbubbles. *In vivo* imaging showed over 10-fold higher bioluminescence from the tumor region compared to untreated tissue. *Ex vivo* analysis of excised tumors showed greater than 40-fold higher expression in tumor tissue than non-sonicated control (heart) tissue. These results suggest that the polyplex-microbubble platform offers improved control of DNA loading and packaging suitable for ultrasound-guided tissue transfection.

Keywords

theranostic; ultrasound contrast agent; delivery vehicle; gene delivery; tumor; SKNEP-1; polyethylenimine; PEI; polyethylene glycol; PEG

*Corresponding Author address: Mark Andrew Borden, PhD, Department of Mechanical Engineering, University of Colorado, 1111 Engineering Drive, Boulder, CO 80309-0427, Phone: 303.492.7750, Fax: 303.492.3498, mark.borden@colorado.edu.

1. Introduction

Over the last decade, microbubble contrast agents have been cited frequently as promising vehicles for targeted drug delivery and controlled release [1–12]. Microbubbles are gas-filled spheres, typically between 1–10 μm in diameter, which circulate in the bloodstream when injected systemically. Microbubbles undergo cavitation, or volumetric oscillations, when insonified at ultrasonic frequencies. Stable cavitation is marked by microbubble persistence over the acoustic pulse train and generally results in relatively mild viscous effects, such as microstreaming. Inertial cavitation may occur at higher acoustic powers and involves rapid microbubble collapse and fragmentation to produce shock waves, water jets and other intense, highly localized effects. Both forms of microbubble cavitation can create pores in the endothelial layer (sonoporation) to aid in drug and gene delivery.

Sonoporation is an effective method of promoting extravasation of large macromolecules, such as plasmid DNA, to improve delivery to tissue beyond the vasculature [13–17]. The tradeoff comes as stable cavitation creates less “collateral damage” to nearby tissue, while inertial cavitation provides a greater extent of extravascular delivery. Sonoporation is suited for site-specific drug delivery, since permeabilization of vasculature and delivery of cargo only occurs at sites where ultrasound is applied and microbubbles are present. By spatially and temporally controlling the application of ultrasound energy, gene uptake can be targeted to specific regions. Since microbubble cavitation also results in an acoustic emission, sonoporation can be guided and tracked by ultrasound imaging. Image-guided sonoporation is particularly appealing for tumor-targeted drug and gene therapy.

Systemic gene delivery is still largely inefficient due to rapid clearance of nucleic acids from the bloodstream via the mononuclear phagocyte system (MPS) and enzymatic degradation [18, 19]. Several novel microbubble formulations have been developed in order to improve systemic gene transfection efficiency by loading plasmid DNA or oligonucleotides onto the microbubble surface [11, 12, 20]. Deposition methods include (i) direct physical incorporation of the DNA into the microbubble shell during fabrication [21, 22], (ii) the use of cationic lipids incorporated into the microbubble shell to electrostatically bind DNA [23], (iii) deposition of single [24] or multi-layers [25] of cationic polymer on the microbubble shell to electrostatically bind DNA, (iv) covalent linking of DNA-nanoparticle carriers [26] and (v) use of complementary DNA strands to load nanoparticles [27]. Physical attachment to the microbubble surface can protect nucleic acids from enzymatic degradation, improve the circulation profile and ultimately increase the drug payload to tissue following ultrasound-mediated microbubble destruction. Most of these microbubble formulations demonstrated either high DNA loading capacity or high transfection efficiency. However, effective *in vivo* gene transfection using microbubble carriers requires both a high nucleic acid payload and sophisticated packaging to promote intracellular delivery and trafficking to the nucleus.

In this study, we present a method of coupling plasmid DNA to the surface of lipid-based microbubbles using a chemically modified cationic polymer, polyethylenimine (PEI). PEI is a well-established transfection agent due to its high cationic charge, which enables the polymer to bind and condense DNA, inhibit enzymatic degradation, prolong the *in vivo* lifetime, promote endocytic uptake in cells, and facilitate endosomal escape of DNA into the cytoplasm by the so-called “proton-sponge” effect [28–31]. Since it was introduced, PEI has been cited as one of the most potent *in vitro* non-viral DNA transfection agents [32–34]. However, efforts towards using PEI-based vectors to deliver DNA *in vivo* have been less successful. Due to the high cationic charge of the polymer backbone, PEI-based vectors are rapidly cleared from circulation and potentially cytotoxic in high doses. The biocompatibility can be dramatically improved by the addition of non-ionic polyethylene

glycol (PEG) to ameliorate the surface charge and reduce complement activation [30, 35, 36]. Other methods of reducing toxicity have been employed, such as cross linking low-molecular-weight PEI molecules to make biodegradable PEI-based vectors [37–40]. Despite improvements in biocompatibility, the circulation profile and target organ uptake of PEI-based DNA vectors remains relatively poor, presumably due to rapid accumulation in the lung, liver, spleen and kidney [41]. Ultimately, alternative methods need to be explored for improving the safety and efficacy of PEI-based vectors.

Recent reports have demonstrated that microbubble-induced sonoporation can improve the transfection efficiency of PEI-based synthetic gene vectors *in vitro* [42, 43] and *in vivo* [44, 45]. These studies used co-injection of DNA/PEI polyplexes with microbubbles or passive adsorption of PEI to the microbubble surface. Our approach is different in that it employs covalent, stoichiometric linking of the PEI to the microbubble surface, in order to ensure firm coupling between the polyplex vector and the microbubble carrier. Our formulation was designed to deliver DNA to target tumor cells *in vivo* through a combined mechanism of microbubble-induced sonoporation and PEI-enhanced extra/intra-cellular trafficking (Fig. 1).

Below, we demonstrate a methodology for covalently coupling PEI polymers to lipid-coated microbubbles to create polyplex-microbubble hybrids. The PEI was modified with PEG to improve biocompatibility and thiolated (-SH) for covalent binding to PEG-tethered maleimide groups on the microbubble shell. The microbubbles were size-selected to improve their circulation persistence and echogenicity [46] and sonoporation capability [47]. We hypothesized that (1) PEI and DNA loading onto microbubbles can be controlled by modulating maleimide concentration in the microbubble shell, (2) the DNA loading capacity is similar to cationic lipid microbubbles, (3) DNA/PEI-microbubbles can circulate systemically and (4) DNA/PEI-microbubbles can transfect tumor tissue *in vivo* with site specificity controlled by the application of ultrasound.

2. Materials and Methods

2.1 Preparation of Modified PEI

Cationic branched polymer polyethylenimine (PEI) with a MW of 25 kDa was purchased from Sigma-Aldrich (St. Louis, MO). Amine-reactive polyethylene glycol succinimidyl ester (NHS-PEG) with a MW of 5 kDa was purchased from Nanocs Inc. (New York, NY). 0.01 M NaCl phosphate buffered saline (PBS) solution was prepared by dissolving salt tablets (Sigma-Aldrich) in purified water (10 M Ω -cm; Millipore) and adjusting pH to 7.4. PBS was filtered through 0.2- μ m pore size polycarbonate filters (VWR, West Chester, PA). PEI was dissolved in PBS (pH adjusted to 8.4) to a concentration of 10 mg/mL. 100 mg of NHS-PEG was dissolved in 300 μ L of dimethylformamide (DMF) purchased from Sigma-Aldrich. The NHS-PEG solution then was added to the PEI solution drop-wise while rigorously mixing for 1 hour. NHS esters on the PEG chains are reactive compounds that form stable amide bonds with amine groups on the PEI structure, thus creating PEG-PEI copolymers when mixed. The resulting solution was dialyzed for 48 hours (water exchanged twice) using dialysis tubing with a MWCO of 14–16 kDa (Spectrum Laboratories, Rancho Dominguez, CA). The dialyzed solution was frozen and lyophilized for 48 hours using a VirTis Benchtop K series freeze drier (Gardiner, NY).

The PEG-PEI polymer was then reacted with 2-iminothiolane (Sigma-Aldrich) in order to introduce free thiol (sulfhydryl) groups for binding to maleimide-expressing microbubbles. Maleimide groups on the microbubble surface react specifically with sulfhydryl groups on the PEI forming a stable thioether linkage when mixed together. PEG-PEI was dissolved to 10 mg/mL in PBS buffer (pH 6.5) containing 5 mM ethylenediaminetetraacetic acid (EDTA;

Sigma-Aldrich). 2-iminothiolane (Trauts reagent; Sigma-Aldrich) was dissolved in PBS buffer to 1 mg/mL and added drop-wise to the PEG-PEI solution at a 50:1 molar ratio while rigorously mixing. The solution was mixed for 1 hour and dialyzed for 48 hours using dialysis tubing with a 4–6 kDa MWCO (Spectrum Laboratories). The resulting solution was subsequently freeze-dried to obtain the final thiolated PEG-PEI polymers (PEG-PEI-SH). The presence of sulfhydryl groups was confirmed using Ellmans reagent (Pierce Biotechnology) using L-Cystein (Sigma-Aldrich) as a standard.

Fluorescent PEG-PEI-SH polymers were made utilizing amine reactive 5-carboxyfluorescein succinimidyl ester (NHS-Fluorescein). PEG-PEI-SH polymers were dissolved in PBS buffer (pH 8.4) to 10 mg/mL. NHS-Fluorescein was dissolved to 10 mg/mL in DMF and added drop-wise to the PEG-PEI-SH solution while rigorously mixing at molar ratio of 5:1. The solution was reacted for an hour and dialyzed for 48 hours using dialysis tubing with a 4–6 kDa MWCO. The resulting solution was subsequently freeze-dried for 48 hours to obtain the fluorescently labeled F-PEG-PEI-SH polymers.

2.2 Preparation of PEI-Microbubbles

The lipids used to encapsulate the microbubbles, 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-PEG2K), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000] (DSPE-PEG2K-Mal), were obtained from Avanti Polar Lipids (Alabaster, AL). Microbubbles were formed by mechanical agitation of the lipid suspension (90 mol% DSPC, 5 mol% DSPE-PEG2K, and 5 mol% DSPE-PEG2K-Mal) in the presence of perfluorobutane (PFB) gas (FluoroMed, Round Rock, TX). DSPE-PEG2K-Mal was varied between 0.5% and 5%, in which case the amount of DSPE-PEG2K was increased so that the DSPE-PEG lipids always comprised 10 mol% of the lipid composition. All lipid stock solutions were dissolved and mixed at the appropriate ratios in chloroform (Sigma-Aldrich) in a sealed 3-mL glass serum vial (Wheaton, Millville, NJ) to 1 mg total lipid per vial. The resulting lipids were dried and re-suspended in 2 mL of 0.01 M PBS buffer containing 10 vol% glycerol and 10 vol% propanediol. The lipid solution was warmed to 60 °C and briefly sonicated to disperse the lipid in a bath sonicator (Branson, Danbury, CT). The air headspace was exchanged with PFB using a custom gas exchange apparatus, and the pressure in the vial was vented briefly to the atmosphere to relieve pressure. The microbubbles were then formed by shaking in a VialMix (Bristol-Myers Squibb, New York, NY). Microbubbles obtained from 5 vials were pooled in a 12-mL syringe. Lipid vesicles and microbubbles less than 4 μm diameter were removed using a centrifugation method similar to Feshitan et al. [48]. The microbubble suspension was repeatedly centrifuged at 90 relative centrifugal force (RCF) for 1 minute using a bucket-rotor centrifuge (Model 5804, Eppendorf, Westbury, NY). For every repetition, the microbubble cake was saved and infranatant was discarded. The final microbubble suspension was diluted in PBS buffer (pH 6.5) containing 1 mM EDTA.

Microbubble size distributions and concentrations were determined by laser light obscuration and scattering (Accusizer 780A, NICOMP Particle Sizing Systems, Santa Barbara, CA). 2- μL samples of each microbubble suspension were diluted into a 30-mL flask under mild mixing. Concentrations and size distributions of each sample were measured in triplicate. The total amount of maleimide in the final sample was estimated from the total surface area calculated by the sizing measurement, the initial DSPE-PEG2k-Mal composition ratio (0.005, 0.02, or 0.05), and an estimated packing density of 0.44 nm² per lipid headgroup.

Microbubbles were coated with PEG-PEI-SH polymers by covalently coupling the maleimide end-groups on the microbubble surface to the thiol groups on the PEG-PEI-SH

polymers. Polymers were dissolved to 10 mg/mL in PBS buffer (pH 6.5) containing 1 mM EDTA. Maleimide bearing microbubbles were added drop-wise to the polymer solution while gently mixing, and the suspension was gently mixed for 24 hours. A molar excess of 10:1 PEI/maleimide ratio was used to prevent aggregation of the microbubbles. Fluorescence microscopy (Olympus IX-71, Center Valley, PA) was used to confirm deposition of the F-PEG-PEI-SH polymer onto the microbubble shell. A small sample of microbubbles was prepared on a glass cover slip and imaged using a digital, high-resolution camera (Hamamatsu Orca-HR, Bridgewater, NJ).

2.3 Flow Cytometric Analysis of Polymer Binding

The binding kinetics of PEG-PEI-SH to the maleimide microbubbles was determined using an Accuri C6 flow cytometer (Ann Arbor, MI). Median fluorescent intensity (MFI) values of the microbubble sample were recorded before and after the addition of F-PEG-PEI-SH polymer. A gating technique developed by Chen and Borden [49] was used to identify regions on the density-scatter plot corresponding to specific size ranges. Separate gating was performed using these regions to measure the MFI of microbubbles at 1–2, 4–5, and 6–8 μm diameters. Experiments were performed starting with 200 μL of size-selected maleimide bubbles (1×10^9 MB/mL) per sample. 2 μL was taken for each measurement at each time-point and diluted in 100 μL of PBS. Baseline MFI readings were performed prior to adding the polymer. F-PEG-PEI-SH was added at a 10:1 PEI:maleimide molar ratio and briefly vortexed. MFI measurements were performed on each sample over 48 hours. Control experiments to determine non-specific binding of the polymer to the microbubble shell were performed by blocking the maleimide reaction with 1,000-fold molar excess of L-cysteine. MFI curves were fit to a total binding-saturation model, described below, and the resulting coefficients were compared between groups and microbubble size-classes.

$$MFI = \frac{B_{max} * t}{K_d + t} + X * t + B \quad (1)$$

where B_{max} is the total maximum specific binding (R.U.), t is time (hours), K_d is the equilibrium binding constant (hours), X is a non-specific binding term (R.U./hour) and B is the initial baseline MFI prior to F-PEG-PEI-SH incubation. The model assumes maleimide is the limiting reagent.

2.4 Measurement of Zeta Potential and DNA Loading Capacity

Polymer-loaded microbubbles were suspended in diluted PBS (2.74 mM NaCl, adjusted to pH 7.2) to a concentration of 10^7 MB/mL. Particle surface charge was measured using a Zetasizer (Malvern Instruments, Southborough, MA). All measurements were made in triplicate.

The DNA loading capacity of the PEI-microbubbles was measured using salmon sperm DNA (Sigma-Aldrich) as described by Borden et al. [25]. Salmon sperm DNA was dispersed to 1 mg/mL by probe sonication for 5 minutes. 500 μL containing 10^9 PEI-loaded microbubbles was added drop-wise to 500 μL of DNA solution while gently mixing. The DNA was allowed to electrostatically couple to the polymer-coated microbubbles while gently mixing for 1 hour. The microbubbles were then concentrated by centrifugation and washed 3 times in a syringe (90 RCF; 1 min; 10 mL washing volume) to remove unbound DNA. The concentration and size distribution of remaining microbubbles was measured using the Accusizer to determine the maximum surface area available for DNA loading, assuming the microbubbles were spheres. The sample then was heated to 65 $^{\circ}\text{C}$ for several hours and briefly bath sonicated until the bubbles were destroyed, evidenced by the solution

becoming clear. The amount of DNA in the sample was measured by UV absorbance at 260 nm using a spectrophotometer (J-815 CD Spectrometer; Jasco Inc.; Easton, MD). Experiments were repeated in triplicate.

2.5 Ultrasound Imaging

All animal studies were performed in accordance with Columbia University institutional animal care and use committee (IACUC) protocols. Contrast-enhanced ultrasound persistence studies were performed in female CD-1 mice 4–6 weeks of age (Charles River Laboratories, Wilmington, MA). Mice were anesthetized using 1–2% isoflurane and placed on a mouse handling table (Visualsonics, Toronto, Ontario, Canada), and the heart rate, respiratory rate and temperature were monitored using a TMH-150 physiological monitoring unit (Visualsonics). Mice were kept under anesthesia for the duration of the experiment. After the mouse was completely anesthetized, the tail vein was catheterized using a modified 27-gauge, ½-inch butterfly catheter (Terumo Medical Corporation, Somerset, NJ). Prior to catheterization, the tubing was removed and replaced with smaller 27-gauge Tygon® tubing (0.015 inner-diameter, Cole-Parmer, Vernon Hills, IL). The mouse was shaved in the kidney region. A Vevo 770 small animal ultrasound imaging scanner with a 30-MHz imaging transducer (707B, Visualsonics) was placed over the kidney of the mouse and coupled using Aquasonic-100 ultrasound transmission gel (Parker Laboratories, Fairfield, NJ). A bolus injection of 50 µL of microbubble solution (2.5×10^7 MB/bolus) was injected while imaging continuously at 16 frames per second (100% power setting). Respiratory gating was used to synchronize data acquisition with the mouse respiratory cycle, in order to reduce motion artifact during image analysis. Respiratory gating lowered the effective acquisition rate to 2 frames per second. Ultrasound imaging was performed between 5 and 20 minutes following injection of the microbubble suspension.

Mice were injected with control, PEI-loaded and DNA/PEI-loaded microbubbles (using sonicated salmon sperm DNA). Each mouse was given three randomized injections per imaging session, with 20 minutes between start points of the injections, and then removed from anesthesia. Experiments were repeated in triplicate at 0.5 mol%, 2 mol%, and 5 mol% DSPE-PEG-Mal compositions. Control microbubbles contained 0% DSPE-PEG-Mal and 10% DSPE-PEG2k.

2.6 Ultrasound Analysis

The raw ultrasound video data was exported from the Vevo 770 machine and processed using a custom program developed in Labview (Version 9, National Instruments, Austin, TX). Multiple regions of interest (ROI) in the kidney were selected (Fig. 2). Three ROIs in the upper portion of the kidney encompassing the cortex region were used to evaluate the change in average video pixel intensity over time caused by the presence of microbubbles (time-intensity curves; TICs). The signals from the three ROI's were averaged to obtain a final TIC. Three more ROIs were selected to encompass hypoechoic areas where the medulla and larger blood vessels were more prominent. A motion analysis algorithm using normalized two-dimensional cross correlation was implemented to evaluate the signal fluctuation caused by circulating microbubbles (supplementary material). The motion analysis algorithm was used to generate a time-fluctuation curve (TFC), which was used to distinguish between freely circulating and adherent microbubbles. The signals from the three regions of interest were averaged to obtain the final TFC. A unique two-compartment model was developed to describe the circulation of polyplex-microbubbles utilizing the time-intensity and time-fluctuation data to delineate freely circulating and adherent microbubbles (Fig. 3). Derivation of the model and more details on the analysis are given in the supplementary material.

2.7 In Vivo Plasmid DNA Transfection

Plasmid DNA was isolated using the Powerprep™ HP Plasmid Maxiprep Kit (Marligen, Ijamsville, MD) and the Nucleic Acid Purification Pack by GIBCO BRL™. The plasmid type was FUW LUC (gift from Dr. Adolfo Ferrando) encoding for the bioluminescent protein luciferase. Luciferase plasmid DNA was dissolved in nuclease free water to 2 mg/mL. UV/VIS spectrometry (Beckman, Fullerton, CA) was used to determine the DNA concentration.

Tumors were formed in female nude NCR mice (Taconic Farms, Germantown NY) injected with a SKNEP-1 human cancer cell line (American Type Culture Collection, Manassas, VA) [50]. For each mouse, 10^6 cells were injected directly into the left kidney through a small incision in the left flank. Tumors were allowed to develop for 5 weeks and were palpated every week to determine size. Five weeks after implantation, the mice were transfected with PEI-microbubbles mixed with the plasmid DNA (10^8 MB with 500 µg DNA in total of 400 µL injection volume).

Each mouse was anesthetized using ketamine/xylazine, and the tail vein was catheterized using a custom 27-gauge, ½-inch butterfly catheter. A Dynatron 125 therapeutic ultrasound machine (Dynatronics) with a 2-cm diameter soundhead was placed over the tumor region. The DNA/PEI-microbubble suspension was injected slowly (0.2 mL/min) while applying continuous ultrasound at 1 MHz, 1 W/cm² and 10% duty cycle. Ultrasound was administered for a total of 10 minutes following the start of injection of the microbubble-DNA solution. Ultrasound was manually turned off every 5 seconds, for 5-seconds duration, to allow replenishment of new microbubbles into the tumor vasculature [49]. After the mouse regained consciousness, it was returned to its cage. Bioluminescence was measured *in vivo* at 2 days post transfection, 5 minutes after a 100 µL intraperitoneal injection of D-Luciferin (Caliper Life Sciences, Hopkinton, MA). All images were taken with an IVIS 200 series bioluminescent *in vivo* imaging system using 1 min exposure times and medium binning.

A group of mice were sacrificed immediately after *in vivo* luciferase imaging, and their tissues were excised to test the specificity of luciferase expression in the tumor. 0.2 grams of tissue (tumor and heart) was collected, weighed and homogenized on ice using a Polytron tissue homogenizer (Elkhart, IN) in 800 mL of passive lysis buffer (Promega, Madison, WI), followed by centrifugation. 40 mL of the supernatant was added to 100 mL of luciferase assay reagent (LARII; Promega, Madison, WI) and read in a TD-20/20 Luminometer. The relative luciferase units were normalized to the tumor weight.

2.8 Statistical Analysis

Students' t-tests were performed to evaluate significant differences between treated and control groups. All statistical analysis was performed using Graphpad™ Prism software (La Jolla, CA).

3. Results and Discussion

3.1 PEI Modification and Loading onto Microbubbles

Branched, 25-kDa PEI was modified with amine reactive NHS-PEG (5 kDa) at a molar ratio of 5:1. Sulfhydryl binding sites were introduced via Trauts reagent onto the PEG-PEI. Analysis with Ellmans reagent indicated that an average of 9.6 ± 3.7 (n=3) sulfhydryl groups/PEG-PEI were introduced during the thiolation process. The resulting PEG-PEI-SH was covalently bound to maleimide groups on the lipid-coated microbubbles.

Polymer grafting onto the microbubble surface did not significantly change the size distribution. Figure 4 shows number- and volume-weighted distributions of a typical size-selected microbubble suspension following conjugation of PEG-PEI-SH. The median diameter was $5.1 \pm 0.3 \mu\text{m}$ by volume and $4.1 \pm 0.2 \mu\text{m}$ by number ($n=3$). This size range was previously found to be optimal for small animal imaging [46] and therapy [47], and hence was chosen for our development of a theranostic microbubble for image-guided gene delivery.

Our conjugation procedure was confirmed by coupling fluorescent F-PEG-PEI-SH to the microbubble surface and directly observing the microbubbles with fluorescence microscopy. Figure 5 shows bright field and fluorescence images from the same field of view of microbubbles loaded with F-PEG-PEI-SH. The bright field image shows the presence of the gas core, as evidenced by the strong optical contrast, spherical shape and diffraction pattern. The presence of F-PEG-PEI-SH was observed on all microbubbles in epi-fluorescence mode. Some microbubbles exhibited surface folds and projections. No fluorescence was observed when using control microbubbles without maleimide, or when the maleimide was blocked using a molar excess of L-cystein (data not shown).

The binding kinetics of F-PEG-PEI-SH to microbubbles was explored using flow cytometry. Figure 6 shows the median fluorescence intensity (MFI) per microbubble measured over 48 hours for microbubbles of 1–2, 4–5, and 6–8 μm diameter. The F-PEG-PEI-SH rapidly bound to the maleimide linkers on the microbubble shell within the first 3 hours, followed by a slower binding phase over the remaining 48 hours. The data was fit to a total binding saturation model (Eq. 1) to describe the trend for each microbubble size class (Table 1). The maximum specific binding (B_{max}), time to reach maximum binding and degree of nonspecific binding (X) each increased with microbubble size. No trend was observed for the equilibrium constant K_d . These results show that the 48-hour incubation was enough to complete the fast binding phase, as defined by the model.

In order to demonstrate that PEI attachment was due to a stable thioether bond, rather than a nonspecific interaction, the maleimide linker was blocked with L-cystein prior to mixing with F-PEG-PEI-SH. In this case, the MFI did not increase above the baseline value at any time-point ($P>0.05$), indicating the absence of electrostatic or other nonspecific adsorption of PEI to the microbubbles. The use of a covalent thioether bond was expected to aid in stabilizing the microbubble/PEI/DNA complex for *in vivo* experiments.

3.2 Surface Charge of PEI-microbubbles and DNA/PEI-microbubbles

The surface charge of microbubbles loaded with PEG-PEI-SH was measured for varying maleimide concentrations and compared to control microbubbles without polymer (Fig. 7A). Zeta potential analysis demonstrated a significant change in the surface chemistry after addition of the polymer. The charge was initially negative owing to the phosphate on the PEG-lipids and the maleimide groups (5 mol%). Following addition of cationic PEG-PEI-SH, the charge was neutralized for 0.5 and 2.0 mol% maleimide and reversed in sign to become cationic at 5 mol% maleimide. All PEI-loaded groups showed significant increases in zeta potential compared to control ($P<0.0001$, $n=3$ per group). The increasing cationic charge was consistent with our hypothesis that PEI loading could be controlled by modulating maleimide content.

Addition of DNA to the PEI-loaded microbubbles reversed the surface charge back to negative values for every group. Covalent attachment prevented PEI from simply desorbing from the surface due to interactions with DNA. This reversal in surface charge therefore indicated that PEI was successful in sequestering DNA from the bulk through electrostatic interactions. This result was confirmed with DNA loading measurements described below.

3.3 DNA Loading onto PEI-microbubbles

Figure 7B shows the total DNA loading capacity per unit surface area of the PEI-microbubbles. The loading capacity increased in proportion with maleimide-lipid concentration. This result was consistent with the zeta potential measurements described above: more maleimide led to greater PEI deposition, which in turn led to greater DNA loading. The highest DNA loading capacity of $0.005 \text{ pg}/\mu\text{m}^2$ was similar (on the same order of magnitude) to previously reported loading capacities of microbubbles formulated with cationic lipids ($0.001 \text{ pg}/\mu\text{m}^2$ [51], $0.004 \text{ pg}/\mu\text{m}^2$ [52], and $0.001\text{--}0.006 \text{ pg}/\mu\text{m}^2$ [25]), and to polymer-coated microbubbles assembled by electrostatic layering using cationic polymers ($0.004 \text{ pg}/\mu\text{m}^2$ [24] and $0.010 \text{ pg}/\mu\text{m}^2$ [25] for single layers). Here, however, we have PEGylated the complex to increase biocompatibility, and the cationic polymer is covalently attached to the microbubble surface in order to form a more stable polyplex linkage.

A theoretical loading efficiency can be calculated based on the available maleimide groups on the microbubble surface with a few reasonable assumptions. Based on the molar composition of the lipid and a 0.44 nm^2 lipid head cross-sectional area [53], the estimated surface density of maleimide groups is $1.14 \times 10^5 \text{ molecules}/\mu\text{m}^2$. Assuming a PEI:maleimide ratio equal to 1:10 (based on the number of measured sulfhydryl groups per PEI), and complete saturation of all PEI amine groups with DNA phosphate groups (using 580 amine groups per PEG-PEI from Lutz et al. [54, 55]), the estimated maximum loading density of DNA onto the microbubble surface is $0.004 \text{ pg}/\mu\text{m}^2$ for 5% maleimide, which is remarkably close to our measured value of $0.005 \pm 0.001 \text{ pg}/\mu\text{m}^2$. Thus, the addition of PEG and proximity to the microbubble surface did not appear to significantly reduce the loading capacity of the PEI.

3.4 In Vivo Pharmacokinetics of PEI-microbubbles

The circulation persistence of the PEI-coated microbubbles with and without DNA loading was evaluated *in vivo* using high frequency ultrasound. The microbubbles were injected intravenously via the tail vein while monitoring the contrast signal in the mouse kidney. No adverse effects from the microbubble injections were observed in the CD-1 mice after anesthesia recovery. Figure 8 shows the grayscale B-mode ultrasound images (column 1), contrast detection (column 2), and B-mode/contrast overlays (column 3) shortly after microbubble injection, when the signal intensity had reached the maximum level. Contrast was detected as an increased scattering signal following reference subtraction using pre-contrast images. Panel A shows a typical ultrasound image snapshot following a bolus injection of control microbubbles. The contrast was distributed throughout the kidney region (denoted by the white border) with greater intensity in the highly vascularized cortex. Panel B shows a representative image for PEI-microbubbles without DNA loading. The contrast signal was much less conspicuous. Finally, Panel C shows an image for PEI-microbubbles loaded with DNA. The contrast was much higher for DNA/PEI-microbubbles compared to PEI-microbubbles, and the contrast intensity and spatial distribution were similar to those for control microbubbles.

Figure 9 shows typical time intensity curves (TICs) generated after a bolus injection (2.5×10^7 microbubbles) for PEI-microbubbles and DNA/PEI-microbubbles with varying degrees of maleimide-lipid in the microbubble shell. The shapes of the TICs were noticeably different for PEI-microbubbles compared to DNA/PEI-microbubbles or control. PEI-microbubbles tended to have a lower maximum signal intensity and slower “wash-in” phase, and this effect increased with increasing PEI content (equivalently, maleimide content). DNA/PEI-microbubbles, on the other hand, exhibited similar TICs to control.

The TICs were fit to a single-compartment model, as described previously [29] (Fig. 3), which allowed determination of the maximum intensity and half-life of the total contrast signal. The mean maximum signal intensity was significantly less for PEI-microbubbles compared to DNA/PEI-microbubbles or control for 2% and 5% maleimide, but not for 0.5% maleimide in the microbubble shell (Fig. 9C). The mean maximum intensity for DNA/PEI-microbubbles was statistically equal to that for control microbubbles. Interestingly, despite the lower signal intensity for PEI-microbubbles, the mean half-life was not statistically different between any of the groups (Fig. 9D). Additionally, we observed less “speckling” in the ultrasound videos for PEI-microbubbles compared to the other groups. These results suggested that PEI-microbubbles were adhering nonspecifically, through electrostatic interactions, to the vascular endothelium. The reduced intensity possibly resulted from adhesion to vasculature upstream of the kidney (e.g., in the tail vein and pulmonary capillary bed), leading to loss of microbubbles en route and fewer entering the kidney. The long half-life could be explained by the slow dissolution of adherent microbubbles.

To investigate this further, we employed a novel two-compartment model to distinguish freely circulating microbubbles from adherent, non-circulating ones using the TICs and time-fluctuation curves (TFCs), which were obtained using the normalized cross-correlation algorithm (supplementary material). Frame-by-frame decorrelation was used to detect changes in the speckle pattern in the region of interest in order to measure the fluctuation of the signal caused by circulating microbubbles. Each ultrasound video was processed to obtain a TFC and TIC. Figure 10 shows examples of the TICs (black) overlaid with the corresponding TFCs (gray). The signal persistence time of the TFCs and TICs appeared to be similar for the control, indicating that circulating microbubbles were the primary contributor to the overall signal enhancement (Fig. 10A). For PEI-microbubbles, however, there was a noticeable difference between the TICs and the TFCs. The TFCs rapidly decreased to baseline (Fig. 10D), or were non-existent (Fig. 10F), while the TICs exhibited prolonged persistence. We reasoned that this discrepancy was due to the cationic charge of PEI-microbubbles, which caused them to adhere through electrostatic interactions with the negatively charged glycocalyx on the vascular endothelium. The DNA/PEI-microbubbles experienced behavior somewhat in between that of control and PEI-microbubbles (Figs. 10C,E,G). For these microbubbles, the TFCs deviated from the TICs, but not to the same extent as for the PEI-microbubbles. For both DNA-loaded and unloaded PEI-microbubbles, the difference between TFCs and TICs increased with increasing maleimide, showing that this effect was modulated by microbubble surface chemistry.

The TICs and TFCs were fit to the two-compartment model (see derivation in supplementary material). Compartment 1 contains freely circulating microbubbles, while compartment 2 contains microbubbles adherent to the kidney vasculature (non-circulating), which slowly dissolve away. Table 2 shows a summary of model coefficients for each group.

The D_o coefficient describes the total contrast agent delivered to the kidney and is closely related to the maximum signal intensity of the TIC (Fig. 11A). The rate constants ($k_1 - k_4$) describe the influx or efflux of contrast agent signal from compartments 1 and 2. The value of k_1 , the influx rate of contrast agent into circulation from the bolus injection, was not significantly different between polymer-modified microbubbles and control. Variations were likely due to the differences in bolus injection speed and in the heart and respiratory rates between animals. However, for 5% maleimide, the average k_1 value for DNA/PEI-microbubbles was 12-fold higher than for PEI-microbubbles ($P < 0.05$). The value of k_2 , the elimination rate of contrast signal from circulation, was significantly lower for PEI-microbubbles (2% and 5% maleimide) compared to the control ($P < 0.05$). These trends in k_1 and k_2 suggest that PEI-microbubbles adherent upstream may be recirculating into the

kidney. PEI-microbubbles also showed an increase in the value of k_3 , the rate at which microbubbles adhere to kidney vasculature, in comparison to control. The mean k_3 value for 2% maleimide PEI-microbubbles was higher than for the control group, but not quite statistically significant ($P=0.08$). For the 5% maleimide PEI-microbubbles, no increase in the time-fluctuation signal was detected above baseline, which suggested that these cationic microbubbles were rapidly becoming adherent after entering the kidney ($k_3 \rightarrow 0$, $k_2 \rightarrow 0$). No significant difference was observed for the dissolution rate of the adherent bubbles (k_4) for any maleimide concentration.

Figure 11B shows the ratio of microbubbles that became adherent compared to those that remained freely circulating, as calculated from the k_2 and k_3 parameters (supplementary material). Control microbubbles gave a very low adhesion ratio (AR ~ 0). On the other hand, adhesion was high for PEI-microbubbles and increased with increasing maleimide content. DNA loading onto the PEI-microbubbles decreased the adhesion ratio, although some adhesion was observed for each group, and the value increased with increasing maleimide content.

In summary, control microbubbles showed almost no adhesion and the resulting TIC was primarily from freely circulating microbubbles. As the amount of PEI conjugation increased, the signal from freely circulating microbubbles diminished and the signal from adherent bubbles became more prevalent. Loading of DNA onto the PEI-microbubbles improved the circulation profile at every maleimide concentration, although the half-life of the control microbubbles in circulation remained significantly greater (~ 8 fold). To illustrate these effects, simulations were performed using the average coefficients obtained by fitting the model to each experimental TFC and TIC set (supplementary material).

Our results show that the DNA/PEI-microbubbles persist on the order of tens of minutes, regardless of whether they are freely circulating or adherent to the vasculature. This is a short persistence time in comparison to some nanocarriers, such as liposomes [56] and filomicelles [57], which have reported circulation times on the order of hours to days. However, the on-demand and site-directed delivery offered by sonoporation precludes the need for the enhanced permeability and retention (EPR) effect to target tumor cells, and thus lessens the restraints for a long-circulating carrier. Below, we test whether the relatively short persistence is sufficient for DNA transfection of tumors *in vivo*.

3.5 In Vivo DNA Transfection

DNA transfections using a luciferase plasmid were performed on SKNEP-1 tumor-bearing mice using the 5 mol% maleimide PEI-microbubbles. No adverse effects in the NCR nude mice were observed after anesthesia recovery using 400- μ L injection volumes of the microbubble formulations. *In vivo* bioluminescence imaging at 48 hours post-transfection showed site-specific luciferase expression in the abdominal area flanking the kidney where the tumor was implanted and the ultrasound transducer was applied (Figure 12A). The photon flux from the tumor area was measured to be over 10-fold higher than the baseline signal from untreated mice (Fig. 12B). The luciferase expression measured *ex vivo* was over 40-fold higher in tumor tissue than in heart tissue in animals that received DNA/PEI-microbubbles and ultrasound (Fig. 12C). The ultrasound transducer was placed in the lower abdominal region; thus heart tissue was not exposed to ultrasound. Heart tissue was used as an internal control to demonstrate lack of luciferase expression where ultrasound was not applied. No bioluminescence was detected above background in the mice exposed to DNA/PEI-microbubbles without ultrasound by either IVIS or in tumor tissue homogenates. These results showed that both microbubbles and ultrasound were necessary to transfect the tumors, and the transfection was isolated to the tissue exposed to ultrasound.

A major benefit of the microbubble-induced sonoporation method is that it does not rely on the EPR effect for tumor cell targeting, which may be highly variable between tumors [58]. Sonoporation provides modulation of the gene carrier biodistribution through increased delivery to tissue perfused with microbubbles in the transducer focus. Our results show that a circulation persistence of only ten minutes was sufficient to achieve tumor-specific gene transfection. We suggest that this is a consequence of the proposed mechanism of (1) microbubble-induced sonoporation to locally release the polyplex/lipid vector and increase vascular permeability and then (2) PEI-enhanced extra/intra-cellular trafficking to increase the efficiency of plasmid DNA transport to the nuclei of tumor cells (see Fig. 1).

4. Conclusions

Polyplex-microbubbles were formed by attaching PEI-PEG-SH to size-selected, maleimide-lipid microbubbles and then loading with plasmid DNA. The polyplex microbubbles were characterized for size distribution, PEI loading kinetics, surface charge, DNA loading, *in vivo* circulation persistence in the mouse kidney, and *in vivo* transfection to kidney tumors. A two-compartment model was developed to describe the anomalous time-intensity curves obtained through contrast-enhanced ultrasound imaging. Fitting of the model to time-intensity and time-fluctuation curves showed that PEI-loaded microbubbles rapidly became adherent to the vasculature, presumably through electrostatic interactions between the cationic microbubbles and the anionic glycocalyx on the endothelium. However, DNA loading onto the PEI-microbubbles increased circulation time and decreased nonspecific adhesion. Successful transfection indicated that the DNA/PEI-microbubbles circulated sufficiently long to provide sonoporation in the tumor tissue. These results demonstrate proof-of-principle that the polyplex-loaded bubbles are viable plasmid DNA transfection agents for targeted delivery to tumors *in vivo*, providing some support for the putative mechanism of microbubble-induced sonoporation and PEI-enhanced extra/intra-cellular trafficking. Results also confirmed our hypotheses that (1) PEI and DNA loading onto microbubbles can be controlled by modulating maleimide concentration in the microbubble shell, (2) the DNA loading capacity is similar to cationic lipid microbubbles, (3) DNA/PEI-microbubbles can circulate systemically and (4) DNA/PEI-microbubbles can transfect tumor tissue *in vivo* with site specificity controlled by the application of ultrasound. More work is necessary to investigate toxicity, biodistribution, intra-tumor distribution, fraction and type of transfected cells, and other necessary criteria for clinical translation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding was provided by NYSTAR James D. Watson Investigator grant C020028 to MAB and St. Baldrick's Foundation grant 139214 to DJY and MAB.

References

1. Unger EC, Hersh E, Vannan M, McCreery T. Gene delivery using ultrasound contrast agents. *Echocardiography*. 2001; 18:355–361. [PubMed: 11415509]
2. Price RJ, Kaul S. Contrast ultrasound targeted drug and gene delivery: an update on a new therapeutic modality. *J Cardiovasc Pharmacol Ther*. 2002; 7:171–180. [PubMed: 12232566]
3. Tsutsui JM, Xie F, Porter RT. The use of microbubbles to target drug delivery. *Cardiovasc Ultrasound*. 2004; 2:23. [PubMed: 15546496]
4. Hernot S, Klibanov AL. Microbubbles in ultrasound-triggered drug and gene delivery. *Adv Drug Deliv Rev*. 2008; 60:1153–1166. [PubMed: 18486268]

5. Sboros V. Response of contrast agents to ultrasound. *Adv Drug Deliv Rev.* 2008; 60:1117–1136. [PubMed: 18486270]
6. Ferrara KW, Borden MA, Zhang H. Lipid-shelled vehicles: engineering for ultrasound molecular imaging and drug delivery. *Acc Chem Res.* 2009; 42:881–892. [PubMed: 19552457]
7. Qin S, Caskey CF, Ferrara KW. Ultrasound contrast microbubbles in imaging and therapy: physical principles and engineering. *Phys Med Biol.* 2009; 54:R27–57. [PubMed: 19229096]
8. Tinkov S, Bekerdjian R, Winter G, Coester C. Microbubbles as ultrasound triggered drug carriers. *J Pharm Sci.* 2009; 98:1935–1961. [PubMed: 18979536]
9. Sheffield P, Trehan A, Boyd B, Wong OL. Microbubbles as ultrasound contrast agents and in targeted drug delivery. *Crit Rev Biomed Eng.* 2008; 36:225–255. [PubMed: 19961426]
10. Bohmer MR, Klivanov AL, Tiemann K, Hall CS, Gruell H, Steinbach OC. Ultrasound triggered image-guided drug delivery. *Eur J Radiol.* 2009; 70:242–253. [PubMed: 19272727]
11. Lentacker I, De Smedt SC, Sanders NN. Drug loaded microbubble design for ultrasound triggered delivery. *Soft Matter.* 2009; 5:2161–2170.
12. Sirsi SR, Borden MA. Microbubble compositions, properties and biomedical applications. *Bub Sci Eng Techn.* 2009; 1:3–17.
13. Deckers R, Moonen CTW. Ultrasound triggered, image guided, local drug delivery. *J Control Release.* 2010; 148:25–33. [PubMed: 20709123]
14. Tinkov S, Winter G, Coester C, Bekerdjian R. New doxorubicin-loaded phospholipid microbubbles for targeted tumor therapy: Part I - Formulation development and in-vitro characterization. *J Control Release.* 2010; 143:143–150. [PubMed: 20060861]
15. Bohmer MR, Chlon CHT, Raju BI, Chin CT, Shevchenko T, Klivanov AL. Focused ultrasound and microbubbles for enhanced extravasation. *J Control Release.* 2010; 148:18–24. [PubMed: 20600402]
16. Klivanov AL, Shevchenko TI, Raju BI, Seip R, Chin CT. Ultrasound-triggered release of materials entrapped in microbubble-liposome constructs: A tool for targeted drug delivery. *J Control Release.* 2010; 148:13–17. [PubMed: 20691227]
17. Lin CY, Liu TM, Chen CY, Huang YL, Huang WK, Sun CK, Chang FH, Lin WL. Quantitative and qualitative investigation into the impact of focused ultrasound with microbubbles on the triggered release of nanoparticles from vasculature in mouse tumors. *J Control Release.* 2010; 146:291–298. [PubMed: 20621645]
18. Nishikawa M, Huang L. Nonviral vectors in the new millennium: delivery barriers in gene transfer. *Hum Gene Ther.* 2001; 12:861–870. [PubMed: 11387052]
19. Read ML, Logan A, Seymour LW. Barriers to Gene Delivery Using Synthetic Vectors. *Non-Viral Vectors for Gene Therapy* (2). 2005; Part 1:19–46.
20. Ferrara KW, Pollard RE, Borden MA. Ultrasound microbubble contrast agents: fundamentals and application to drug and gene delivery. *Annual Rev Biomed Eng.* 2007; 9:415–447. [PubMed: 17651012]
21. Seemann S, Hauff P, Schultze-Mosgau M, Lehmann C, Reszka R. Pharmaceutical evaluation of gas-filled microparticles as gene delivery system. *Pharm Res.* 2002; 19:250–257. [PubMed: 11934230]
22. Frenkel PA, Chen S, Thai T, Shohet RV, Grayburn PA. DNA-loaded albumin microbubbles enhance ultrasound-mediated transfection in vitro. *Ultrasound Med Biol.* 2002; 28:817–822. [PubMed: 12113794]
23. Christiansen JP, French BA, Klivanov AL, Kaul S, Lindner JR. Targeted tissue transfection with ultrasound destruction of plasmid-bearing cationic microbubbles. *Ultrasound Med Biol.* 2003; 29:1759–1767. [PubMed: 14698343]
24. Lentacker I, De Geest BG, Vandenbroucke RE, Peeters L, Demeester J, De Smedt SC, Sanders NN. Ultrasound-responsive polymer-coated microbubbles that bind and protect DNA. *Langmuir.* 2006; 22:7273–7278. [PubMed: 16893226]
25. Borden MA, Caskey CF, Little E, Gillies RJ, Ferrara KW. DNA and polylysine adsorption and multilayer construction onto cationic lipid-coated microbubbles. *Langmuir.* 2007; 23:9401–9408. [PubMed: 17665937]

26. Vandenbroucke RE, Lentacker I, Demeester J, De Smedt SC, Sanders NN. Ultrasound assisted siRNA delivery using PEG-siPlex loaded microbubbles. *J Control Release*. 2008; 126:265–273. [PubMed: 18237813]
27. Lozano MM, Starkel CD, Longo ML. Vesicles Tethered to Microbubbles by Hybridized DNA Oligonucleotides: Flow Cytometry Analysis of This New Drug Delivery Vehicle Design. *Langmuir*. 26:8517–8524. [PubMed: 20229988]
28. Boussif O, Lezoualc'h F, Zanta MA, Mergny MD, Scherman D, Demeneix B, Behr JP. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. *Proc Natl Acad Sci U S A*. 1995; 92:7297–7301. [PubMed: 7638184]
29. Remaut K, Lucas B, Raemdonck K, Braeckmans K, Demeester J, De Smedt SC. Protection of oligonucleotides against enzymatic degradation by pegylated and nonpegylated branched polyethyleneimine. *Biomacromolecules*. 2007; 8:1333–1340. [PubMed: 17358077]
30. Brus C, Petersen H, Aigner A, Czubayko F, Kissel T. Physicochemical and biological characterization of polyethylenimine-graft-poly(ethylene glycol) block copolymers as a delivery system for oligonucleotides and ribozymes. *Bioconjug Chem*. 2004; 15:677–684. [PubMed: 15264853]
31. Brus C, Petersen H, Aigner A, Czubayko F, Kissel T. Efficiency of polyethylenimines and polyethylenimine-graft-poly(ethylene glycol) block copolymers to protect oligonucleotides against enzymatic degradation. *Eur J Pharm Biopharm*. 2004; 57:427–430. [PubMed: 15093589]
32. Vicennati P, Giuliano A, Ortaggi G, Masotti A. Polyethylenimine in medicinal chemistry. *Curr Med Chem*. 2008; 15:2826–2839. [PubMed: 18991638]
33. Basarkar A, Singh J. Nanoparticulate systems for polynucleotide delivery. *Int J Nanomedicine*. 2007; 2:353–360. [PubMed: 18019834]
34. Park TG, Jeong JH, Kim SW. Current status of polymeric gene delivery systems. *Adv Drug Deliv Rev*. 2006; 58:467–486. [PubMed: 16781003]
35. Sung SJ, Min SH, Cho KY, Lee S, Min YJ, Yeom YI, Park JK. Effect of polyethylene glycol on gene delivery of polyethylenimine. *Biol Pharm Bull*. 2003; 26:492–500. [PubMed: 12673031]
36. Petersen H, Fechner PM, Martin AL, Kunath K, Stolnik S, Roberts CJ, Fischer D, Davies MC, Kissel T. Polyethylenimine-graft-poly(ethylene glycol) copolymers: influence of copolymer block structure on DNA complexation and biological activities as gene delivery system. *Bioconjug Chem*. 2002; 13:845–854. [PubMed: 12121141]
37. Forrest ML, Koerber JT, Pack DW. A degradable polyethylenimine derivative with low toxicity for highly efficient gene delivery. *Bioconjug Chem*. 2003; 14:934–940. [PubMed: 13129396]
38. Thomas M, Ge Q, Lu JJ, Chen J, Klibanov AM. Cross-linked small polyethylenimines: while still nontoxic, deliver DNA efficiently to mammalian cells in vitro and in vivo. *Pharm Res*. 2005; 22:373–380. [PubMed: 15835742]
39. Ding Y, Wang J, Wong CS, Halley PJ, Guo Q. Synthesis, characterization and biocompatibility of novel biodegradable cross-linked co-polymers based on poly(propylene oxide) diglycidylether and polyethylenimine. *J Biomater Sci Polym Ed*. 22:457–473. [PubMed: 20566040]
40. Wen Y, Pan S, Luo X, Zhang X, Zhang W, Feng M. A biodegradable low molecular weight polyethylenimine derivative as low toxicity and efficient gene vector. *Bioconjug Chem*. 2009; 20:322–332. [PubMed: 19152330]
41. Malek A, Merkel O, Fink L, Czubayko F, Kissel T, Aigner A. In vivo pharmacokinetics, tissue distribution and underlying mechanisms of various PEI(-PEG)/siRNA complexes. *Toxicol Appl Pharmacol*. 2009; 236:97–108. [PubMed: 19371615]
42. Dang SP, Wang RX, Qin MD, Zhang Y, Gu YZ, Wang MY, Yang QL, Li XR, Zhang XG. A novel transfection method for eukaryotic cells using polyethylenimine coated albumin microbubbles. *Plasmid*. 2011; 66:19–25. [PubMed: 21426917]
43. Qiu Y, Luo Y, Zhang Y, Cui W, Zhang D, Wu J, Zhang J, Tu J. The correlation between acoustic cavitation and sonoporation involved in ultrasound-mediated DNA transfection with polyethylenimine (PEI) in vitro. *J Control Release*. 2010; 145:40–48. [PubMed: 20398711]
44. Lu QL, Liang HD, Partridge T, Blomley MJ. Microbubble ultrasound improves the efficiency of gene transduction in skeletal muscle in vivo with reduced tissue damage. *Gene Ther*. 2003; 10:396–405. [PubMed: 12601394]

45. Chen ZY, Liang K, Qiu RX. Targeted gene delivery in tumor xenografts by the combination of ultrasound-targeted microbubble destruction and polyethylenimine to inhibit survivin gene expression and induce apoptosis. *J Exp Clin Cancer Res*. 2010; 29
46. Sirsi S, Feshitan J, Kwan J, Homma S, Borden M. Effect of microbubble size on fundamental mode high frequency imaging in mice. *Ultrasound Med Biol*. 2010; 36:935–948. [PubMed: 20447755]
47. Choi JJ, Feshitan JA, Baseri B, Wang YST, Borden MA, Konofagou EE. Microbubble-size dependence of focused ultrasound-induced blood-brain barrier opening in mice in vivo. *IEEE Trans Biomed Eng*. 2010; 57:145–155. [PubMed: 19846365]
48. Feshitan JA, Chen CC, Kwan JJ, Borden MA. Microbubble size isolation by differential centrifugation. *J Colloid Interface Sci*. 2009; 329:316–324. [PubMed: 18950786]
49. Bekeredjian R, Chen S, Frenkel PA, Grayburn PA, Shohet RV. Ultrasound-targeted microbubble destruction can repeatedly direct highly specific plasmid expression to the heart. *Circulation*. 2003; 108:1022–1026. [PubMed: 12912823]
50. Rowe DH, Huang JZ, Kayton ML, Thompson R, Troxel A, O'Toole KM, Yamashiro D, Stolar CJH, Kandel JJ. Anti-VEGF antibody suppresses primary tumor growth and metastasis in an experimental model of Wilms' tumor. *J Pediatr Surg*. 2000; 35:30–32. [PubMed: 10646769]
51. Christiansen JP, French BA, Klibanov AL, Kaul S, Lindner JR. Targeted tissue transfection with ultrasound destruction of plasmid-bearing cationic microbubbles. *Ultrasound Med Biol*. 2003; 29:1759–1767. [PubMed: 14698343]
52. Chen S, Ding JH, Bekeredjian R, Yang BZ, Shohet RV, Johnston SA, Hohmeier HE, Newgard CB, Grayburn PA. Efficient gene delivery to pancreatic islets with ultrasonic microbubble destruction technology. *Proc Natl Acad Sci U S A*. 2006; 103:8469–8474. [PubMed: 16709667]
53. Marsh, D. *Handbook of Lipid Bilayers*. CRC; Boca Raton: 1990.
54. Glodde M, Sirsi SR, Lutz GJ. Physiochemical properties of low and high molecular weight poly(ethylene glycol)-grafted poly(ethylene imine) copolymers and their complexes with oligonucleotides. *Biomacromolecules*. 2006; 7:347–356. [PubMed: 16398535]
55. Sirsi SR, Williams JH, Lutz GJ. Poly(ethylene imine)-poly(ethylene glycol) copolymers facilitate efficient delivery of antisense oligonucleotides to nuclei of mature muscle cells of mdx mice. *Hum Gene Ther*. 2005; 16:1307–1317. [PubMed: 16259564]
56. Gabizon A, Shmeeda H, Barenholz Y. Pharmacokinetics of pegylated liposomal doxorubicin - Review of animal and human studies. *Clin Pharmacokinet*. 2003; 42:419–436. [PubMed: 12739982]
57. Christian DA, Cai SS, Garbuzenko OB, Harada T, Zajac AL, Minko T, Discher DE. Flexible Filaments for in Vivo Imaging and Delivery: Persistent Circulation of Filomicelles Opens the Dosage Window for Sustained Tumor Shrinkage. *Mol Pharm*. 2009; 6:1343–1352. [PubMed: 19249859]
58. Fang J, Nakamura H, Maeda H. The EPR effect: Unique features of tumor blood vessels for drug delivery, factors involved, and limitations and augmentation of the effect. *Adv Drug Delivery Rev*. 2011; 63:136–151.

Figure 1.

Cartoon illustrating the suggested mechanism for plasmid DNA transfection to tumor cells *in vivo* using polyplex-microbubbles. (1–2) Polyplex-microbubbles enter the tumor vasculature after being introduced systemically. (3) Ultrasound applied to the tumor region causes inertial cavitation and microbubble fragmentation, resulting in polyplex/lipid release and permeation of the endothelial lining, allowing the DNA vector to extravasate into tumor tissue. (4) Polyplex/lipid vector entry into a tumor cell may be due to (A) physical disruption of the cell membrane to allow passive entry into the cytoplasm, and (B) enhanced clathrin-mediated endocytotic uptake, where PEI facilitates interaction with the cell membrane. In the latter case, polyplex/lipid vectors are taken up into early endosomes (EE) and then trafficked into late endosomes (LE) or lysosomal compartments. PEI is thought to cause osmotic swelling and endosomal rupture (ER) via a proton-sponge effect [28], allowing polyplex entry into the cytoplasm. Plasmid DNA dissociates from the PEI/lipid vector and enters the nucleus of the cell where the genes can be expressed.

Figure 2.

ROI selections for ultrasound images. A typical ultrasound image of a mouse kidney is shown and outlined (dotted white line). Three ROI's were selected in the upper portion of the kidney to obtain time-intensity data (outlined by solid white lines). Three more ROI's were selected in the midportion of the kidney to obtain time-fluctuation data (outlined by dashed white lines).

Figure 3.

Two-compartment pharmacokinetic model. Compartment 1 contains freely circulating microbubbles, and compartment 2 contains adherent microbubbles. The coefficients k_1 through k_4 are pseudo-first-order rate constants that describe the influx or efflux of ultrasound contrast for compartments 1 and 2, as shown.

Figure 4. Typical number-weighted (A) and volume-weighted (B) distributions of microbubbles loaded with PEG-PEI-SH polymer.

Figure 5. Microscopy confirmation of fluorescent PEG-PEI-SH binding to microbubble shells. Brightfield (A) and fluorescence (B) images of microbubbles containing 5% maleimide-lipid taken after mixing with fluorescent PEG-PEI-SH polymers and washing. Scale bars equal 20 μm .

Figure 6.

Flow cytometric analysis of fluorescent PEG-PEI-SH binding to maleimide-containing microbubbles. (A) Density scatter plots from the forward and side scattering of the microbubbles. The gated regions B, C and D correspond to 1–2 μ m, 4–5 μ m, and 6–8 μ m size ranges. (B–D) Median fluorescent intensity (MFI) vs. time of the microbubbles from the gated regions in panel A. Fluorescent readings were taken after mixing the maleimide-bearing microbubbles with (circles) and without (squares) blocking of the maleimide group with L-cysteine.

Figure 7.

Surface charge and DNA loading capacity of PEI-microbubbles. (A) Zeta-potential analysis showing microbubbles without PEG-PEI-SH loading (white), with PEG-PEI-SH loading (gray), and with DNA loading (black) for different maleimide concentrations. (*) Indicates significant difference from the control group ($P < 0.05$). (†) Indicates significant difference between maleimide concentrations ($P < 0.05$).

Figure 8.

Ultrasound B-mode images of the mouse kidney following a bolus injection of 2.5×10^7 microbubbles. Panel A shows results for control microbubbles, Panel B shows results for 5% maleimide PEI-microbubbles without DNA and Panel C shows results for 5% maleimide DNA/PEI-microbubbles. B-mode images (column 1), contrast images (column 2), and B-mode/contrast overlays (column 3) are shown for each group.

Figure 9. Time-intensity curves for PEI-only (A) and DNA/PEI-loaded microbubbles. (C) Maximum intensity and (D) contrast half-lives from analysis of the time-intensity curves using the single-compartment model for control (white), PEI-only (gray) and DNA/PEI-loaded microbubbles (black). (*) indicates significant difference from the control group ($P < 0.05$).

Figure 10.

Representative time-intensity (black) and time-fluctuation curves (grey) for (A) control, (B) 0.5% maleimide PEI-only, (C) 0.5% maleimide DNA/PEI-loaded, (D) 2% maleimide PEI-only, (E) 2% maleimide DNA/PEI-loaded, (F) 5% maleimide PEI-only and (G) 5% maleimide DNA/PEI-loaded microbubbles.

Figure 11.

Maximum signal intensity and adhesion ratio determined from time-intensity and time-fluctuation curves using the two-compartment. (A) The “dose” of contrast D_o . (B) The adhesion ratio calculated from k_2 and k_3 values (Eq. 8 in supplementary material). (*) indicates significant difference from the control group ($P < 0.05$).

Figure 12.

Luciferase expression in tumor-bearing mice transfected with polyplex-microbubbles. (A) Images of luciferase expression in mice 48 hours after transfection with 5% maleimide DNA/PEI-microbubbles and ultrasound, 5% maleimide DNA/PEI-microbubbles without ultrasound, plasmid DNA with ultrasound and untreated mice. (B) Quantification of relative luciferase expression by photon flux. Control experiments using plasmid DNA and ultrasound application without microbubbles did not show a significant difference in the luciferase level in the abdomen compared to untreated mice ($P=0.30$, $n=3$ per group). The luciferase expression in the tumor tissue was significantly higher than the untreated control tissue ($*P<0.05$). Pilot studies using DNA/PEI-microbubbles without ultrasound ($n=1$) did not show luciferase expression. (C) *Ex vivo* quantification of luciferase expression was taken from homogenized tumor tissue (gray). Heart tissue (not exposed to ultrasound) was used as the control. (*) indicates significant difference from control heart tissue ($P<0.01$). Pilot studies using DNA/PEI-microbubbles without ultrasound ($n=1$) and untreated tissue ($n=1$) did not show detectable luciferase expression.

Table 1

PEI Binding Kinetics to the Microbubble Surface

MB Size-Range	B_{max} (R.U.)	K_d (hours)	Time to B_{max} (hours)	X (R.U./hour)	B (R.U.)
1–2 μm	5,700	0.26	1.2	640	280
4–5 μm	23,000	0.14	1.8	680	340
6–8 μm	59,000	0.23	3.1	1,000	1,200
1–2 μm Blocked	N/A	N/A	N/A	9.4	310
4–5 μm Blocked	N/A	N/A	N/A	19	1,100
6–8 μm Blocked	N/A	N/A	N/A	4.8	490

Table 2

Two-Compartment Model Coefficients

	Do (R.U.)	K1 (min ⁻¹)	K2 (min ⁻¹)	K3 (min ⁻¹)	K4 (min ⁻¹)
Control	1100 ± 350	3.0 ± 2.5	0.10 ± 0.05	8.9E-8 ± 7.3E-8	0.04 ± 0.04
0.5% Mal	700 ± 130	2.7 ± 1.4	0.04 ± 0.05	0.27 ± 0.21	0.15 ± 0.04
0.5% Mal DNA Loaded	900 ± 30	3.9 ± 0.2	0.14 ± 0.001	0.04 ± 0.01 *	0.07 ± 0.05
2% Mal	460 ± 120 *	1.5 ± 0.5	0.01 ± 0.01 *	0.37 ± 0.29	0.10 ± 0.06
2% Mal DNA Loaded	1500 ± 160 **	1.5 ± 0.3	0.36 ± 0.18 **	0.31 ± 0.06 *	0.13 ± 0.09
5% Mal	260 ± 85 *	0.4 ± 0.2	~ 0	<i>Inf.</i>	0.20 ± 0.08
5% Mal DNA Loaded	970 ± 380 **	4.7 ± 1.2 **	0.44 ± 0.25 **	0.68 ± 0.13 **	0.07 ± 0.05