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Characterization of the transgene expression generated by branched and linear polyethylenimine-plasmid DNA nanoparticles *in vitro* and after intraperitoneal injection *in vivo*

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ABSTRACT

Polyethylenimine (PEI) is a cationic polymer that has shown significant potential for delivering genes *in vitro* and *in vivo*. Mixing cationic PEI with negatively charged plasmid DNA (pDNA) results in the spontaneous electrostatic formation of stable nanoparticle complexes. The structure of PEI can be branched or linear. In this study, we show that branched PEI has a stronger electrostatic interaction with pDNA than linear PEI, which accounts for greater compaction, higher zeta potentials and smaller nanoparticle sizes at equivalent pDNA concentrations. For both linear and branched PEI, increasing the concentration of pDNA mixed in the same volume and at the same nitrogen to phosphate (N:P) ratio results in larger average particle sizes. Increasing the N:P ratio increases luciferase activity generated by branched PEI-pDNA nanoparticles and linear PEI-pDNA nanoparticles in HEK293, COS7 and HeLa cell lines. Increasing the N:P ratio at which branched PEI-pDNA nanoparticles are prepared also increases luciferase expression in HepG2 cells but does not increase luciferase expression generated by linear PEI-pDNA nanoparticles. In all of the cell lines, branched PEI-pDNA nanoparticles prepared at N:P ratios of 10 and above generated significantly higher luciferase activity than linear PEI-pDNA nanoparticles. Luciferase activity was highest in the HEK293 cells and luciferase expression in each of the cell lines followed the order of HEK293>COS7>HepG2>HeLa. Intraperitoneal (IP) injection of PEI-pDNA nanoparticles is attractive because it is simple, reproducible and often leads to a depot effect of nanoparticle complexes residing in the peritoneum. The IP route of administration avoids PEI-pDNA nanoparticle accumulation in the lung and the nanoparticles do not pass through the blood-brain barrier.

In this study, using bioluminescent imaging (BLI), we show that changing the PEI structure and dose of the PEI-pDNA nanoparticles has a significant impact on the strength and duration of transgene expression after IP injection *in vivo* but increasing the N:P ratio does not. Increasing the dose and N:P ratio for all the PEI-pDNA nanoparticle formulations injected IP did not reduce mice survival and all mice remained in good health as determined by the Body Condition Scoring (BCS) technique.

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1. Introduction

Gene therapy aims to treat diseases by delivering genes or DNA to target cells or tissues to produce therapeutics proteins or suppress defective genes [1–3]. Successful application of gene therapy is dependent on optimization of the delivery carrier [2–6]. Gene therapy can be achieved using viral or non-viral vectors [7,8]. Viral vectors generate significantly higher transgene expression than non-viral vectors [7,8]. Viral vectors, however, also have several limitations. These include the requirement of cell mitosis for most retroviruses, immunogenicity of adenoviruses, safety concerns with HIV-like viruses and packaging constraints of adeno-associated viruses (AAV) [4]. Promising results with some viral vectors such as AAV have

progressed into human clinical trials, but the long-term safety concerns of viral vectors still exist and therapeutic levels of expression remain transient in the clinic [9,10]. In contrast, non-viral vectors display substantially reduced immunogenicity. Advantages to non-viral vectors include ease of scale-up, storage stability, and improved quality control [4].

One of the most efficient non-viral vectors *in vitro* and *in vivo* is the cationic polymer, polyethylenimine (PEI) [11–25]. PEI is a water-soluble polymer in which the repeat unit of PEI is two carbon atoms followed by a nitrogen atom. Under physiological conditions, approximately 20% of the nitrogens are protonated [18,26,27]. The positive charge of the PEI results in effective binding to the negatively charged plasmid DNA (pDNA), and this condensation protects the pDNA from digestion in serum and as the nanoparticle complex enters cells [28,29]. Once in the endosomal compartment, PEI can act as a buffer or “proton sponge” to induce osmotic swelling and cause

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release from the endosome. This is necessary to avoid degradation of the plasmid DNA when the endosome fuses with the lysosome [29].

PEI comes in two structural forms: linear and branched (Fig. 1) [13,30,31]. Several studies have shown that linear PEI generates stronger transgene expression *in vivo* when injected intravenously or administered via the lung in comparison to branched PEI at equivalent molecular weights [24,31–35]. In contrast, other studies have shown that branched PEI generates stronger transgene expression than linear PEI *in vitro* in selected cell lines [18]. Some studies have shown that linear PEI is more efficient than branched PEI *in vitro* and *in vivo* [31, 34]. No study, to date, has compared linear and branched PEI after intraperitoneal (IP) injection *in vivo* or compared these results to transgene expression *in vitro*.

Linear PEI has shown strong potential for gene and siRNA delivery when injected intraperitoneally (IP) [36]. Intraperitoneal injection of PEI is attractive because organs in the peritoneal cavity are normally covered with peritoneum and subperitoneal connective tissue whereas tumor nodules on the peritoneal surfaces are not [37]. The peritoneum lining and underlying fibrous components significantly reduce the entry of nanoparticles through the surface of non-targeted organs. Intraperitoneal injections have been shown to be simple, reproducible and often lead to a depot effect of nanoparticle complex residing in the peritoneum for several hours [37,38]. In addition, a number of studies have shown that polymers of similar chemistry but different structure induce changes in transgene activity that can readily be correlated between IP and intravenous (IV) injections [6]. The IP route of administration is considered promising for treatment of a variety of diseases including cancer. Ovarian, pancreatic, and gastric cancers are particularly attractive targets for IP injections [6,37,39–46].

In this study, for the first time, we comprehensively compare the transgene expression generated by IP injection of branched or linear PEI-pDNA nanoparticles using bioluminescent imaging (BLI). BLI is a modern transgene expression imaging technique from which the results strongly correlate to transgene expression results measured using traditional tissue and organ harvesting methodologies [47]. We also compare the transgene expression generated by linear and branched PEI-pDNA nanoparticles in a series of cell lines that are commonly used as transfection hosts for evaluating non-viral vector efficiency. Finally, we compare the transgene expression generated by branched or linear PEI-pDNA nanoparticles *in vitro* and after IP injection *in vivo*. The results from this study highlight the differences in transgene expression generated by PEI-pDNA nanoparticles *in vitro* and *in vivo* and provide a robust time-dependent profile of transgene expression generated by linear or branched PEI-pDNA nanoparticles after IP injection in a murine model.

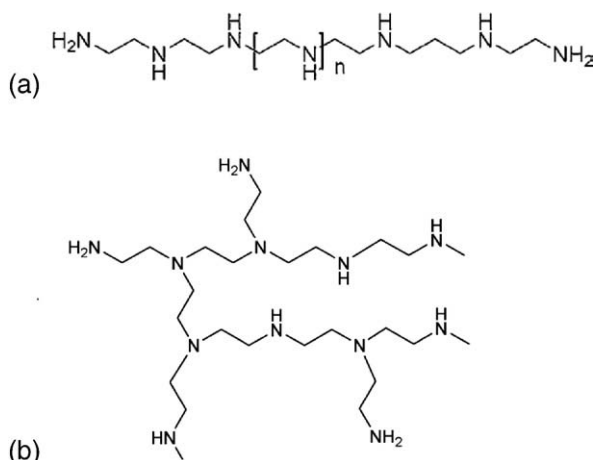


Fig. 1. Chemical structure of (a) linear and (b) branched polyethylenimine.

2. Materials and methods

2.1. Materials

Branched polyethylenimine (PEI, MW 25 kDa) was purchased from Sigma-Aldrich (St. Louis, MO). Linear polyethylenimine (PEI, MW 25 kDa) was obtained from Polysciences Inc. (Warrington, PA). Luciferin was obtained from Xenogen (Hopkinton, MA).

2.2. Cell culture

Human Embryonic Kidney cells (HEK293), Monkey African Green Kidney cells (COS7), Human Liver Hepatocellular Carcinoma cells (HepG2) and Human Cervix Adenocarcinoma cells (HeLa) were purchased from American Type Culture Collection (ATCC, Rockville, MD). The cells were maintained in DMEM supplemented with 10% Fetal Bovine Serum (FBS), streptomycin at 100 µg/ml, penicillin at 100 U/ml, and 4 mM L-glutamine at 37 °C in a humidified 5% CO₂-containing atmosphere.

2.3. Amplification and purification of pDNA

The firefly luciferase gene was used as a reporter gene to monitor gene expression. The pDNA (VR1255) is a 6.4-kb cDNA encoding firefly luciferase driven by the cytomegalovirus (CMV) promoter/enhancer. The pDNA was transformed in *Escherichia coli* DH5α and amplified in Terrific Broth media at 37 °C overnight on a plate shaker set at 300 rpm. The pDNA was then purified by an endotoxin-free QIAGEN Giga plasmid purification kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol. Purified pDNA was dissolved in Tris-EDTA solution, and its purity and concentration were determined by UV absorbance at 260 and 280 nm.

2.4. Preparation of PEI-pDNA nanoparticles

Branched or linear PEI-pDNA nanoparticles were formulated in 5% glucose solution at various ratios of PEI nitrogen to pDNA phosphate (N/P ratio). Different amounts of pDNA were diluted and branched or linear PEI was then transferred into the pDNA solution. The solution was vortex mixed for 20 s and left at room temperature for 30 min.

2.5. Particle size and zeta potential analysis

Nanoparticle size measurements were conducted using the Zetasizer Nano ZS (Malvern, Southborough, MA). Briefly, the nanoparticles were suspended in deionized water at a concentration of 1 mg/ml. The size measurements were performed at 25 °C at a 173° scattering angle. The mean hydrodynamic diameter was determined by cumulative analysis. The zeta potential determinations were based on electrophoretic mobility of the nanoparticles in the aqueous medium, which were performed using folded capillary cells in automatic mode.

2.6. Evaluation of luciferase expression in COS7, HEK293, HeLa and HepG2 cells

Cells were seeded into a 24-well plate at a density of 8×10^4 /well of COS7, HeLa, HepG2 and HEK293 cells 24 h before transfection. One hundred µl of PEI-pDNA nanoparticle solution containing 1 µg of pDNA was 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. After 44 h incubation, cells were treated with 200 µl of lysis buffer (Promega, Madison, WI). The lysate was subjected to two cycles of freezing and thawing, then transferred into tubes and centrifuged at 13,200 rpm for 5 min. Twenty µl of supernatant was added to 100 µl of luciferase assay reagent (Promega, Madison, WI)

Table 1

Characterization of the size of PEI-pDNA nanoparticle complexes

Group	Amount of DNA ($\mu\text{g}/\text{mouse}$)	PEI structure	N:P ratio	Size (nm)
1	1	Branched	5	77.9 \pm 11.5
2	5	Branched	5	109.1 \pm 2.20
3	10	Branched	5	136.4 \pm 2.84
4	25	Branched	5	155.2 \pm 2.66
5	50	Branched	5	165.3 \pm 4.30
6	100	Branched	5	238.5 \pm 5.26
7	1	Linear	5	106.3 \pm 2.49
8	5	Linear	5	112.3 \pm 8.11
9	10	Linear	5	122.8 \pm 0.85
10	25	Linear	5	138.3 \pm 3.90
11	50	Linear	5	161.9 \pm 3.88
12	100	Linear	5	265.7 \pm 6.40
13	25	Branched	1	96.2 \pm 2.09
14	25	Branched	2	112.5 \pm 1.45
15	25	Branched	3	128.1 \pm 5.35
16	25	Branched	10	131.1 \pm 1.74
17	25	Branched	20	97.5 \pm 1.03
18	25	Linear	1	433.9 \pm 6.76
19	25	Linear	2	581.7 \pm 9.10
20	25	Linear	3	415.1 \pm 3.26
21	25	Linear	10	141.5 \pm 3.26
22	25	Linear	20	179.0 \pm 3.45

N/P ratio is defined as the ratio of primary amino group in PEI to phosphate group (in DNA).

and samples were measured on a luminometer for 10 s (Lumat LB 9507, EG&G Berthold, Bad Wildbad, Germany). The relative light units (RLU) were normalized against protein concentration in the cell extracts, measured by a micro BCA protein assay kit (Pierce). Luciferase activity is expressed as relative light units (RLU/mg protein in the cell lysate). The data was reported as mean \pm standard deviation for triplicate samples. Every transfection experiment was repeated at least twice.

2.7. Evaluation of luciferase expression *in vivo*

All animal experiments were conducted in accordance with the principles and procedures described in the University of Iowa's Guidelines for Care and Use of Experimental Animals. Female Balb/C 6–8 week-old mice were obtained from Charles-River Laboratories and maintained in a pathogen-free environment. Mice were injected IP with pDNA alone or PEI-pDNA nanoparticles prepared with branched PEI or linear PEI at varying N:P ratios and doses. Each PEI-pDNA nanoparticle solution was prepared in 1 ml 5% glucose solution in triplicate. Twenty-four hours later, the mice were injected IP with 200 μl of luciferin (15 mg/ml) and were then anesthetized by isoflurane administered by a gas manifold at a flow rate of 2%. The mice were placed onto a warmed stage in a sealed chamber of an IVIS Imaging 100 Series instrument (Xenogen, Hopkinton, MA). Images of luciferase activity were continuously acquired at a medium binning level until maximal activity was detected. Auto-functions were utilized to determine the minimum (5% of maximum) for the scale at each time-point. The images were analyzed using Live image 2.5 software to provide a quantitative amount of luciferase activity. Region of interest (ROI) from displayed images were drawn around the abdominal and quantified as photons/second (ph/s) and background bioluminescence noted for each experiment.

2.8. Statistical analysis

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

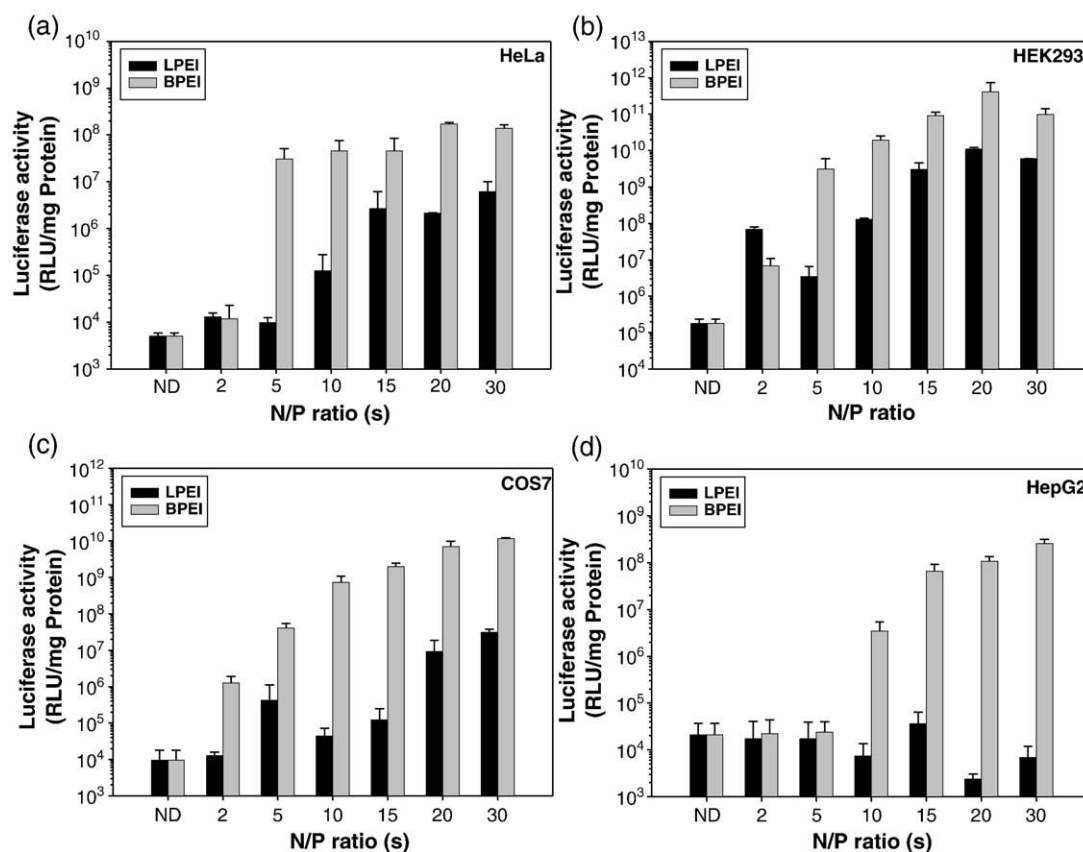


Fig. 2. Transfection mediated by linear polyethylenimine (25 kDa) (LPEI)-pDNA (1 μg) nanoparticle complexes and branched polyethylenimine (25 kDa) (BPEI)-pDNA nanoparticle complexes at various N/P ratios in (a) HeLa, (b) HEK, (c) COS7 and (d) HepG2. Luciferase activity is shown as means \pm STD ($n=3$, error bars represent standard deviation).

3. Results

3.1. Zeta potential and particle size analysis of PEI-pDNA nanoparticles

Mixing cationic PEI with negatively charged pDNA results in the spontaneous electrostatic formation of stable nanoparticle complexes. At a constant N:P ratio of 5, increasing the concentration of pDNA mixed with branched PEI from 1 to 5 μg increases the average particle size from 77.9 nm \pm 11.5 to 109.1 nm \pm 2.2 (Table 1). A further 5-fold increase in concentration to 25 μg increases the particle size to 155.2 nm \pm 2.66. Increasing the concentration of pDNA mixed with branched PEI to 100 μg increased the average nanoparticle size to 238.5 nm \pm 5.26. For linear PEI, at a constant N:P ratio of 5, increasing the concentration of pDNA mixed with linear PEI from 1 to 5 μg resulted in a non-significant increase in particle size from 106.3 nm \pm 2.49 to 112.3 nm \pm 8.11 (Table 1). A further 5-fold increase in concentration to 25 μg increased the particle size to 138.3 nm \pm 3.90. Increasing the concentration of pDNA mixed with linear PEI to 100 μg increased the average nanoparticle size to 265.7 nm \pm 6.40. Branched PEI has a stronger electrostatic interaction with pDNA than linear PEI, which accounts for the greater compaction, higher zeta potentials and smaller nanoparticle sizes at equivalent pDNA concentrations. For both linear and branched PEI, increasing the concentration of pDNA mixed in similar volumes but at the same N:P ratio presumably results in greater aggregation of the nanoparticles and larger average particle sizes (Table 1). For branched PEI, at a fixed pDNA concentration of 25 μg , increasing the N:P ratio from 1 to 2 increases the zeta potential from -33.1 \pm 2.05 to -13.36 \pm 1.88 and the average particle size from 96.2 nm \pm 2.09 to 112.5 nm \pm 1.45. Up to an N:P ratio of 10, the zeta potential and particle size continue to increase to 36.01 \pm 1.33 and 131.1 nm \pm 1.74 respectively. At an N:P ratio of 20, nanoparticles formed with branched PEI and pDNA have a lower particle size of 97.5 nm \pm 1.03 and a zeta potential of 36.21 \pm 0.75. For linear PEI, at a fixed pDNA concentration of 25 μg , increasing the N:P ratio from 1 to 3 does not significantly change the zeta potential or particle size. At an N:P ratio of 10, the particle size drops to 141.5 nm \pm 3.26. At an N:P ratio of 20, nanoparticles formed with linear PEI and pDNA have a lower particle size of 179.0 nm \pm 3.45 and a positive zeta potential of 13.05 \pm 0.56.

3.2. PEI-pDNA nanoparticle mediated transgene expression of luciferase in HeLa cells: effect of N:P ratio

Naked pDNA generated 5.07×10^3 RLU/mg protein mean luciferase activity in HeLa cells (Fig. 2a). Linear PEI-pDNA nanoparticles prepared at an N:P ratio of 2 generated 1.3×10^4 RLU/mg protein mean luciferase activity in HeLa cells. Branched PEI-pDNA nanoparticles prepared at an N:P ratio of 2 generated 1.1×10^4 RLU/mg protein mean luciferase activity in HeLa cells which was not significantly different from the transgene expression generated by linear PEI-pDNA nanoparticles prepared at an N:P ratio of 2 ($P > 0.05$). When the N:P ratio was increased to 5, linear PEI-pDNA nanoparticles generated 9.7×10^3 RLU/mg protein mean luciferase activity in HeLa cells which is not significantly different from luciferase activity generated by linear PEI-pDNA nanoparticles prepared at an N:P ratio of 2 ($P > 0.05$). In contrast branched PEI-pDNA nanoparticles prepared at an N:P ratio of 5 generated 3×10^7 RLU/mg protein mean luciferase activity in HeLa cells which is significantly higher than luciferase activity generated by linear PEI-pDNA nanoparticles prepared at an N:P ratio of 5 and branched PEI-pDNA nanoparticles prepared at an N:P ratio of 2. As the N:P ratio increased to 20, the PEI-pDNA nanoparticles generated increasing mean luciferase activity up to 1.7×10^8 RLU/mg protein in the HeLa cells. From N:P ratios of 5 and upwards, the branched PEI-pDNA nanoparticles generated significantly higher luciferase activity than linear PEI-pDNA nanoparticles. No significant increases in luciferase activity were observed for branched PEI-pDNA nanoparticles prepared at N:P ratios above 20 and no significant increases in

luciferase activity were observed for linear PEI-pDNA nanoparticles prepared at N:P ratios above 15 ($P > 0.05$). The maximal luciferase activity generated by branched PEI-pDNA nanoparticles prepared at an N:P ratio of 20 in HeLa cells was approximately 2400-fold lower than that achieved in HEK293 cells, 60-fold lower than COS7 cells and similar to the luciferase activity generated in HepG2 cells.

3.3. PEI-pDNA nanoparticle mediated transgene expression of luciferase in HEK293 cells: effect of N:P ratio

Naked pDNA generated 1.8×10^5 RLU/mg protein mean luciferase activity in HEK293 cells (Fig. 2b). Linear PEI-pDNA nanoparticles

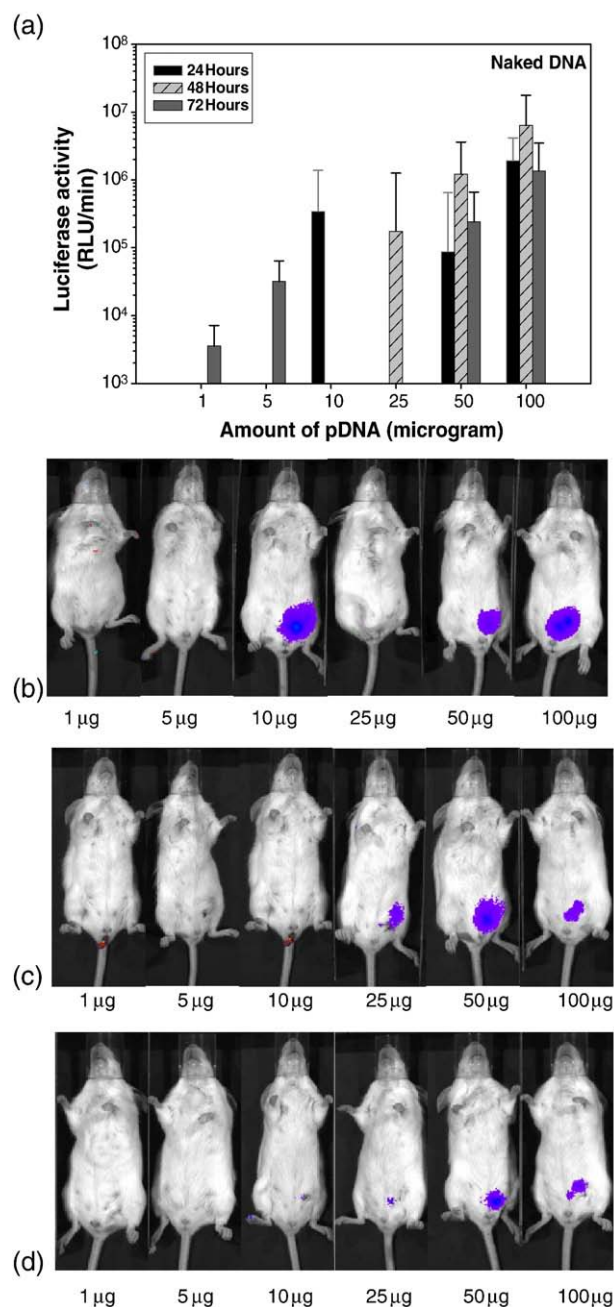
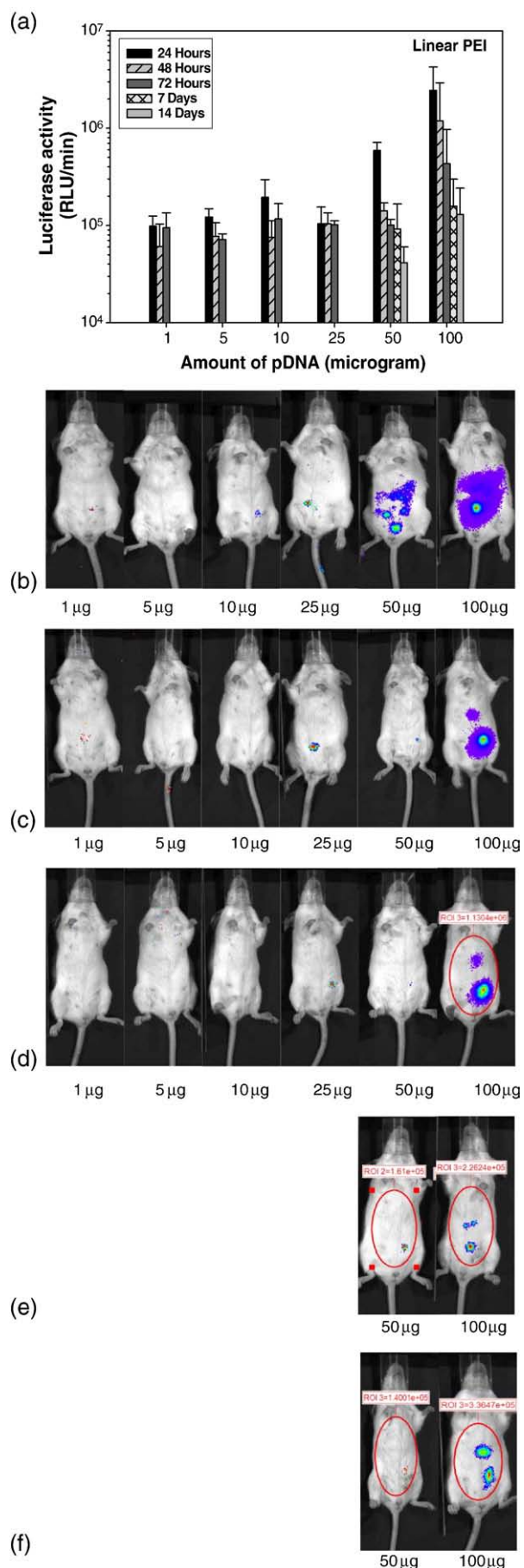


Fig. 3. Dose dependent study of naked DNA. (a) Quantification of the signal produced in Balb/c mice after IP injection of each formulation. Values shown are mean \pm standard deviation ($n = 3$). (b)–(d) Luciferase expression in Balb/c mice measured by IVIS. (b), (c), and (d) represent 24 h, 48 h and 72 h after injection. The number of photons is depicted in a color image superimposed on a video image of the animal. Each mouse presented in the image is representative of at least three mice in each experiment.



prepared at an N:P ratio of 2 generated 6.9×10^7 RLU/mg protein mean luciferase activity in HEK293 cells. Branched PEI-pDNA nanoparticles prepared at an N:P ratio of 2 generated 6.8×10^6 RLU/mg protein mean luciferase activity in HEK293 cells which was 10-fold lower than the mean luciferase activity generated by linear PEI-pDNA nanoparticles prepared at the same N:P ratio. When the N:P ratio was increased to 5, linear PEI-pDNA nanoparticles generated 3.5×10^6 RLU/mg protein mean luciferase activity in HEK293 cells which was 20-fold lower than linear PEI-pDNA nanoparticles prepared at an N:P ratio of 2. In contrast branched PEI-pDNA nanoparticles prepared at an N:P ratio of 5 generated 3.1×10^9 RLU/mg protein mean luciferase activity in HEK293 cells which is significantly higher than linear PEI-pDNA nanoparticles prepared at an N:P ratio of 5 and branched PEI-pDNA nanoparticles prepared at an N:P ratio of 2. Similar results were observed in the HeLa cells.

As the N:P ratio increased to 20, the branched and linear PEI-pDNA nanoparticles generated increasing mean luciferase activity upto 4.1×10^{11} RLU/mg protein in the HEK293 cells. Consistent with results from the HeLa cells, branched PEI-pDNA nanoparticles prepared at N:P ratios of 5 and upwards, generated significantly higher luciferase activity than linear PEI-pDNA nanoparticles ($P < 0.05$). In HEK293 cells, no significant increases in luciferase activity were observed for branched PEI-pDNA nanoparticles prepared at N:P ratios above 20 and no significant increases in luciferase activity were observed for linear PEI-pDNA nanoparticles prepared at N:P ratios above 15 ($P > 0.05$).

3.4. PEI-pDNA nanoparticle mediated transgene expression of luciferase in COS7 cells: effect of N:P ratio

Naked pDNA generated 9.6×10^3 RLU/mg protein mean luciferase activity in COS7 cells (Fig. 2c). Linear PEI-pDNA nanoparticles prepared at an N:P ratio of 2 generated 1.2×10^4 RLU/mg protein mean luciferase activity in COS7 cells. Branched PEI-pDNA nanoparticles prepared at an N:P ratio of 2 generated 1.2×10^6 RLU/mg protein mean luciferase activity in COS7 cells which was 100-fold higher than the transgene expression generated by linear PEI-pDNA nanoparticles prepared at the same N:P ratio. When the N:P ratio was increased to 5, linear PEI-pDNA nanoparticles generated 4.2×10^5 RLU/mg protein mean luciferase activity in COS7 cells which was 35-fold higher than linear PEI-pDNA nanoparticles prepared at an N:P ratio of 2. Similarly, branched PEI-pDNA nanoparticles prepared at an N:P ratio of 5 generated 4×10^7 RLU/mg protein mean luciferase activity in COS7 cells which is significantly higher than luciferase activity generated by linear PEI-pDNA nanoparticles prepared at an N:P ratio of 5 and branched PEI-pDNA nanoparticles prepared at an N:P ratio of 2. As the N:P ratio increased to 20, the branched PEI-pDNA nanoparticles generated increasing mean luciferase activity upto 7×10^9 RLU/mg protein in the COS7 cells. Consistent with results from the HeLa cells and the HEK293 cells, branched PEI-pDNA nanoparticles prepared at N:P ratios of 5 and upwards generated significantly higher luciferase activity than linear PEI-pDNA nanoparticles ($P < 0.001$). Consistent with results from HeLa cells and HEK293 cells, the COS7 cells showed no significant further increases in luciferase activity when treated with branched PEI-pDNA nanoparticles prepared at N:P ratios above 20 ($P > 0.05$).

3.5. PEI-pDNA nanoparticle mediated transgene expression of luciferase in HepG2 cells: effect of N:P ratio

Naked pDNA generated 2×10^4 RLU/mg protein mean luciferase activity in HepG2 cells (Fig. 2d). In contrast to HEK293, HeLa and

Fig. 4. Dose dependent study of linear PEI-pDNA nanoparticle complexes. (a) Quantification of the signal produced in Balb/c mice after IP injection of each formulation. Values shown are mean \pm standard deviation ($n=3$). (b)–(f) Luciferase expression in Balb/C mice measured by IVIS. (b), (c), (d), (e) and (f) represent 24 h, 48 h, 72 h, 7 days and 14 days after injection. The number of photons is depicted in a color image superimposed on a video image of the animal. Each mouse presented in the figure is representative of at least three mice in each experiment.

HepG2 cells, linear PEI-pDNA nanoparticles and branched PEI-pDNA nanoparticles prepared at N:P ratios upto 5 generated no significant difference in luciferase activity in HepG2 cells, when compared to naked pDNA ($P>0.05$). Linear PEI-pDNA nanoparticles resulted in no significant increase in luciferase expression at all N:P ratios ($P>0.05$). This result is in contrast to the improved luciferase activity observed in HEK293, HeLa and COS7 cells as the N:P ratio of the linear PEI-pDNA nanoparticles increased. Branched PEI-pDNA nanoparticles prepared at an N:P ratio of 10, however, increased mean luciferase expression by 170-fold to 3.4×10^6 RLU/mg protein. As the N:P ratio of the branched PEI-pDNA nanoparticles increased to 20, the mean luciferase activity increased to 1.08×10^8 RLU/mg protein ($P<0.001$ compared to linear PEI-pDNA nanoparticles at the same N:P ratio).

3.6. Naked pDNA mediated transgene expression of luciferase after IP injection in Balb/c mice: effect of dose

One μg of pDNA generated 3.57×10^3 RLU/min at 72 h (Fig. 3). Five μg of pDNA generated 3.57×10^3 RLU/min at 72 h. Ten μg of pDNA generated 3.44×10^5 RLU/min at 24 h.

Twenty-five μg of pDNA generated an average 1.75×10^5 RLU/min at 48 h. At fifty μg dosing of pDNA, 8.7×10^4 RLU/min was observed at 24 h. This increased to 1.2×10^6 RLU/min by 48 h followed by a decrease to 2.4×10^5 RLU/min by 72 h. Increasing the dose from 50 μg to 100 μg pDNA increased luciferase expression 20-fold to 1.9×10^6 RLU/min at 24 h. This increased to 6.34×10^6 RLU/min by 48 h followed by a fall to 1.36×10^6 RLU/min by 72 h. No luciferase expression was detected beyond 72 h for mice injected with naked pDNA.

3.7. Linear PEI-pDNA nanoparticle mediated transgene expression of luciferase after IP injection in Balb/c mice: effect of dose

One μg of pDNA complexed with PEI at an N:P ratio of 5 resulted in 9.8×10^4 RLU/min mean luciferase activity at 24 h (Fig. 4). This expression stayed relatively constant upto 72 h ($P>0.05$). After 72 h, no luciferase expression could be detected from mice injected with 1 μg linear PEI-pDNA nanoparticles. This luciferase expression was not significantly different for mice injected IP with upto 25 μg linear PEI-pDNA nanoparticles ($P>0.05$). However, mice dosed with 50 μg linear PEI-pDNA nanoparticles produced a 6-fold increase in mean luciferase activity to 5.9×10^5 RLU/min at 24 h. Doubling the 50 μg dose to 100 μg increased luciferase activity by a further 4-fold at 24 h. Both the 50 μg and 100 μg doses of pDNA-linear PEI nanoparticles generated dose-dependent mean luciferase activity that was still clearly detectable by 14 days. In contrast with naked pDNA injected into mice IP, which produced maximal mean luciferase activity at 48 h or 72 h, IP injected linear PEI-pDNA nanoparticles resulted in the strongest expression levels after 24 h.

3.8. Branched PEI-pDNA nanoparticle mediated transgene expression of luciferase after IP injection in Balb/c mice: effect of dose

Similar to the results from IP injection of linear PEI-pDNA, 1 μg of pDNA complexed with branched PEI at an N:P ratio of 5 resulted in 9.7×10^4 RLU/min mean luciferase activity at 24 h (Fig. 5). In contrast to linear PEI, however, luciferase expression could only be detected upto 48 h. A 5-fold increase in the branched PEI-pDNA nanoparticle dose resulted in a 2-fold increase in luciferase activity at 24 h. After 48 h, no luciferase expression could be detected from mice injected with upto 25 μg branched PEI-pDNA nanoparticles. However, mice dosed with 50 μg branched PEI-pDNA nanoparticles produced a 2-fold increase in mean luciferase activity to 5.8×10^4 at 24 h and luciferase expression at this dose could still be detected at 72 h but not 7 days. Doubling the 50 μg dose to 100 μg did not significantly increase the strength of luciferase activity any further at 24 h but luciferase expression could

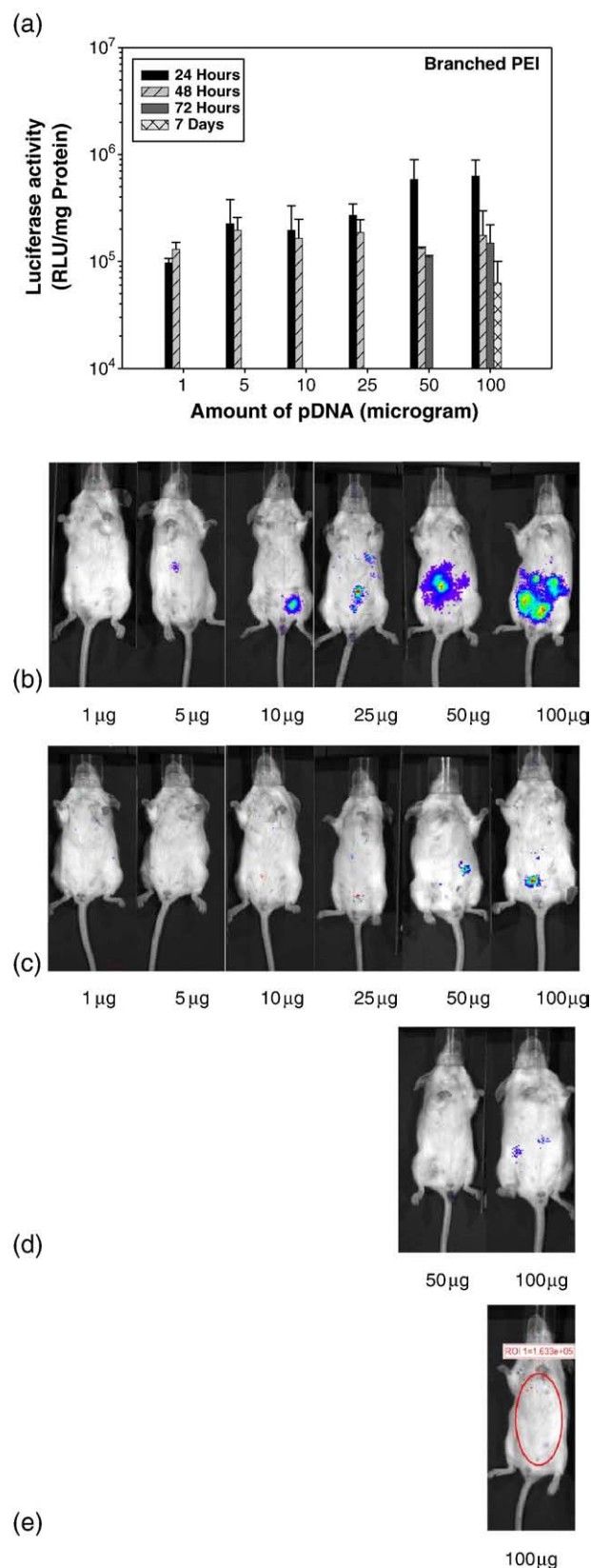
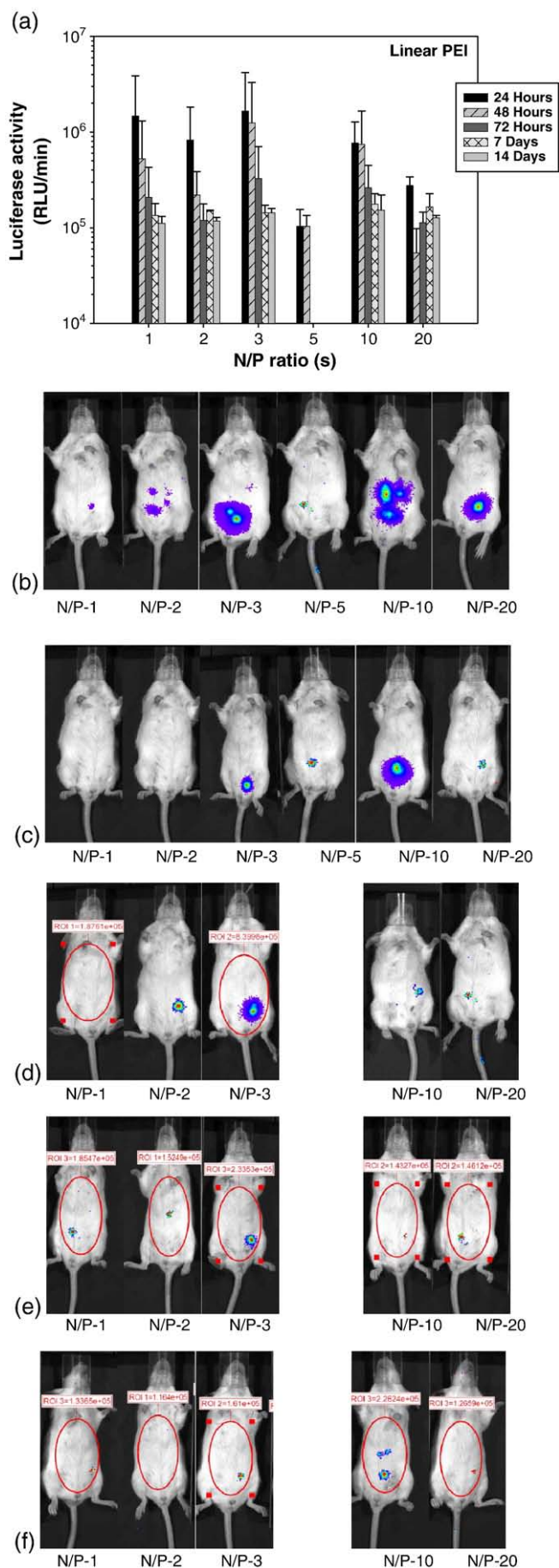


Fig. 5. Dose dependent study of branched PEI-pDNA nanoparticle complexes. (a) Quantification of the signal produced in Balb/c mice after IP injection of each formulation. Values shown are mean \pm standard deviation ($n=3$). (b)–(e) Luciferase expression in Balb/c mice measured by IVIS. (b), (c), (d) and (e) represent 24 h, 48 h, 72 h, and 7 days after injection. The number of photons is depicted in a color image superimposed on a video image of the animal. Each mouse presented in the figure is representative of at least three mice in each experiment.



still be detected at 7 days. For mice injected with branched PEI-pDNA nanoparticles, no luciferase expression could be detected by 14 days at any dose.

3.9. Linear PEI-pDNA nanoparticle mediated transgene expression of luciferase after IP injection in Balb/c mice: effect of N:P ratio

Mice injected with 25 µg linear PEI-pDNA nanoparticles prepared at an N:P ratio of 1 generated 1.48×10^6 RLU/min mean luciferase activity by 24 h (Fig. 6). This dropped 3-fold to 5.3×10^5 RLU/min mean luciferase activity by 48 h and further 2.65 fold by 72 h. Luciferase activity could still be detected upto 1.1×10^5 RLU/min by 14 days. Similar profiles were observed for mice injected with linear PEI-pDNA nanoparticles prepared with N:P ratios upto 20. In contrast to luciferase activity observed *in vitro*, increasing the N:P ratio at which linear PEI-pDNA nanoparticles were prepared did not change luciferase expression significantly ($P > 0.05$).

3.10. Branched PEI-pDNA nanoparticle mediated transgene expression of luciferase after IP injection in Balb/c mice: effect of N:P ratio

Mice injected with branched PEI-pDNA (25 µg) nanoparticles prepared at an N:P ratio of 1 generated 1.85×10^5 RLU/min mean luciferase activity by 24 h (Fig. 7). This was 8-fold lower than luciferase expression generated by linear PEI-pDNA nanoparticles prepared at an N:P ratio of 1. This dropped 2-fold to 8.2×10^4 RLU/min mean luciferase activity by 72 h. No further luciferase expression could be detected beyond 72 h. The maximal luciferase activity was generated from branched PEI-pDNA nanoparticles prepared at an N:P ratio of 2. This was 5.6×10^5 RLU/min luciferase activity at 24 h. Luciferase activity could still be detected upto 2.93×10^5 RLU/min by 14 days. In contrast to linear PEI-pDNA nanoparticles, branched PEI-pDNA nanoparticles prepared at all other N:P ratios did not generate any luciferase expression by day 14.

4. Discussion

PEI has shown significant potential for delivery and protection of siRNA and pDNA [14,38]. The structure of the PEI significantly impacts the transfection efficiency [20]. A number of studies have shown that, in selected cell lines, branched PEI generates stronger transgene expression than linear PEI [18,31]. In contrast, when PEI is injected intravenously or via the lungs, linear PEI, generates stronger transgene expression than branched PEI [31, 34]. *In vitro* studies show that increasing the N:P ratio of the PEI-pDNA nanoparticles increases luciferase activity and toxicity.

In this study, we show that increasing the concentration of PEI-pDNA nanoparticle concentration results in aggregation and larger particle sizes but that this does not reduce the transfection efficiency *in vivo*. We prepared PEI-pDNA nanoparticles in salt-free conditions with 5% glucose because these preparation conditions have been shown to generate the strongest transgene expression *in vivo* [34]. Linear PEI-pDNA nanoparticles show lower zeta potentials than branched PEI-pDNA nanoparticles when prepared at the same N:P ratio. Branched PEI-pDNA nanoparticles prepared at the same N:P ratio as linear PEI-pDNA nanoparticles are smaller in size and more compact. The higher zeta potential and smaller size of the branched PEI-pDNA nanoparticles translate into higher transfection efficiencies *in vitro* but not *in vivo*. We selected four cell lines that are commonly

Fig. 6. Effect of linear PEI complexed with 25 µg of pDNA at different N/P ratios on gene expression in Balb/C. (a) Quantification of the signal produced in Balb/c mice after IP injection of each formulation. Values shown are mean \pm standard deviation ($n=3$). (b)–(f) Luciferase expression in Balb/C mice measured by IVIS. (b), (c), (d), (e) and (f) represent 24 h, 48 h, 72 h, 7 days and 14 days after injection. The number of photons is depicted in a color image superimposed on a video image of the animal. Each mouse presented in the figure is representative of at least three mice in each experiment.

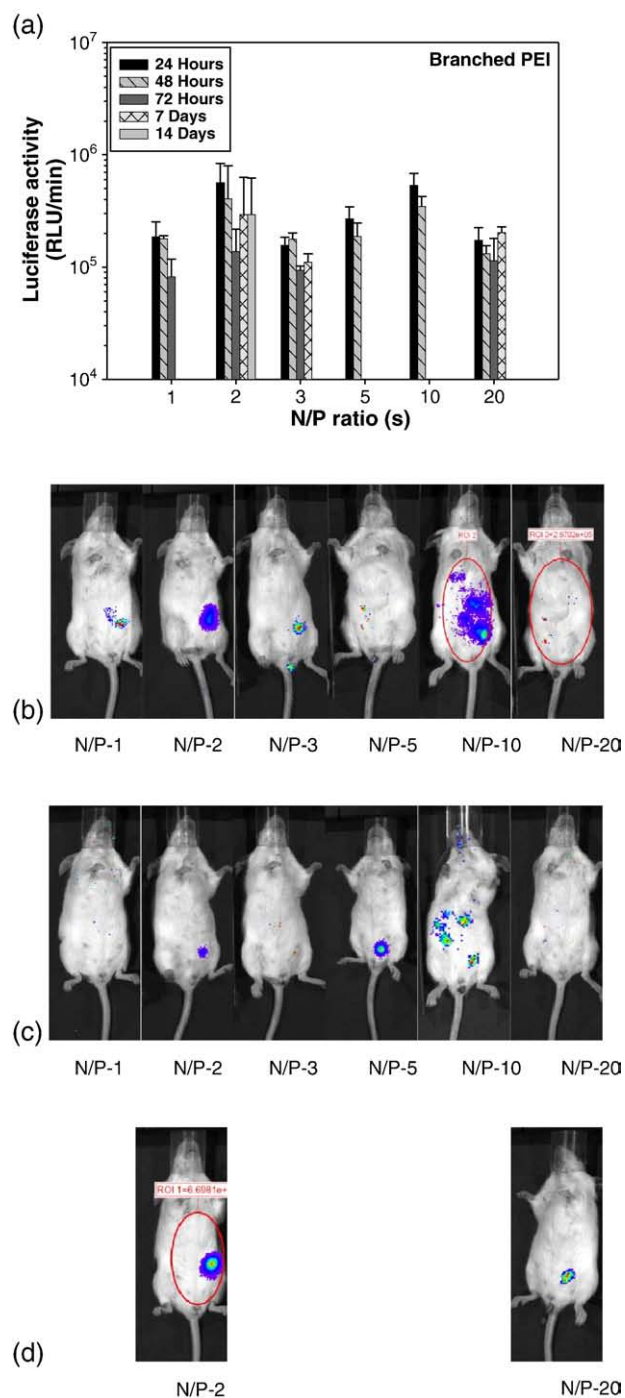


Fig. 7. Effect of branched PEI complexed with 25 µg of pDNA at different N/P ratios on gene expression in Balb/C. (a) Quantification of the signal produced in Balb/c mice after IP injection of each formulation. Values shown are mean±standard deviation ($n=3$). (b)–(d) Luciferase expression in Balb/C mice measured by IVIS. (b), (c) and (d) represent 24 h, 48 h and 72 h after injection. The number of photons is depicted in a color image superimposed on a video image of the animal. Each mouse presented in the figure is representative of at least three mice in each experiment.

used as transfection hosts but rarely compared side by side when evaluating non-viral gene delivery systems. Specifically, we show that increasing the N:P ratio increases luciferase activity for branched PEI-pDNA nanoparticles and linear PEI-pDNA nanoparticles in HEK293, COS7 and HeLa cell lines. Increasing the N:P ratio for branched PEI-pDNA nanoparticles also increases luciferase expression in HepG2 cells but does not increase luciferase expression generated by linear PEI-pDNA nanoparticles. In all of the cell lines, branched PEI-pDNA

nanoparticles prepared at N:P ratios of 10 and above generated significantly higher luciferase activity than linear PEI-pDNA nanoparticles. These results are based on PEI-pDNA nanoparticles prepared in salt-free conditions. It should be noted that Wightman et al. have shown that preparing linear and branched PEI-pDNA nanoparticles in salt containing buffer reversed these results with linear PEI-pDNA nanoparticles generating stronger luciferase activity than branched PEI-pDNA nanoparticles [34]. Choosakoonkirang et al., have previously reported that a minimum N:P ratio of 4 and above is necessary for transfection of COS7 and CHO-K1 cells [18]. Transfection results from PEI-pDNA nanoparticles prepared at N:P ratios less than 4 were not presented in their studies. Our results are consistent with the observations of Choosakoonkriang et al. for linear PEI. However, we show that branched PEI-pDNA nanoparticles can significantly improve transfection efficiencies in COS7 cells even when prepared at an N:P ratio of 2. Although linear and branched PEI display similar rates of internalization, the higher charge associated with branched PEI in comparison to linear PEI is reported to enhance extracellular binding by 5-fold [13]. In addition, branched PEI is internalized by cholesterol dependent pathways whilst the internalization of linear PEI appears to be independent of clathrin and cholesterol pathways [13]. Luciferase activity was highest in the HEK293 cells and luciferase expression in each of the cell lines followed the order of HEK293>COS7>HepG2>HeLa. These results suggest that HEK293 cells are the most sensitive cell line for evaluating cationic polymers as non-viral vectors. It also suggests that HeLa cells may be a suitable starting candidate cell line for testing the development of new non-viral vectors targeted to less transfection permissive cell and tissue targets. These results show that the optimal N:P ratio for PEI-pDNA nanoparticles is cell line dependent and that separate optimization is necessary dependent on the target tissue or cell being transfected.

For IP injection of PEI-pDNA nanoparticles *in vivo*, we have shown that two dominant parameters in increasing luciferase activity are dose and structure of PEI but not increasing N:P ratio. To measure this, we used bioluminescent imaging (BLI), which is a method that in recent years, is increasingly being utilized to determine location and strength of luciferase activity in a variety of animal models [47–51]. Luciferase activity is observed following an IP dose of D-luciferin, which results in bioluminescence that is detected in anesthetized mice by a cooled charge-coupled device camera. BLI is now an established method for quantitatively detecting transgene expression *in vivo* [52]. BLI is rapid, sensitive and does not generate false positives [47,52]. In addition, Rettig et al. have shown that luciferase expression measured by tissue harvesting and luciferase expression measured using BLI are directly correlated. This therefore eliminates the need to harvest tissue and allows for the same reduced number of animals to be assayed over time [47].

In all experiments, branched PEI-pDNA nanoparticles generated significantly lower luciferase activity than linear PEI-pDNA nanoparticles following IP injection. For the *in vitro* studies, increasing the N:P ratio at which the PEI-pDNA nanoparticles are prepared increased the transfection efficiency. Increasing the N:P ratio at which PEI-pDNA nanoparticles are prepared for IP injection *in vivo*, in contrast, does not significantly improve the strength or duration of luciferase activity. For example, for branched PEI-pDNA nanoparticles, the strongest and longest duration of luciferase activity was for nanoparticles prepared at an N:P ratio of 2. Branched PEI-pDNA nanoparticles prepared at all other N:P ratios did not generate any luciferase activity at day 14. Linear PEI-pDNA nanoparticles generated higher than 1×10^5 RLU/min mean luciferase activity at all N:P ratios apart from 5 at day 14. However, increasing the N:P ratio at which the linear PEI-pDNA nanoparticles were prepared did not significantly change luciferase activity. These results for IP injections are in contrast to those reported for intraventricular injections and intratracheal intubation where increasing N:P ratio increased transfection efficiencies. This suggests that the N:P ratio may need to be optimized for each different route of administration [20,53].

Our results have shown that increasing zeta potential and decreasing particle size by strengthening the electrostatic interactions between PEI and pDNA can substantially improve transfection efficiencies *in vitro*. In contrast, for *in vivo* gene delivery, there is an optimal balance between having interactions between the PEI that are strong enough to pack the pDNA so it is protected from enzymatic degradation whilst simultaneously having interactions that are weak enough to allow for eventual release of the pDNA. Bertschinger et al. have shown that linear PEI-pDNA particles are much more efficiently disassembled than branched PEI-pDNA nanoparticles in the presence of molecules typically found *in vivo*. These molecules include heparin, RNA, non-genomic DNA and proteins. This is important because compacted PEI-pDNA nanoparticles that enter the nucleus cannot function as a template for transcription and even low levels of PEI have been reported to disrupt efficient transcription of DNA [54]. Another factor that may reduce the *in vivo* efficacy of branched PEI, is that it has been reported to induce pro-inflammatory chemokine receptors. Linear PEI in comparison does not [11]. One parameter in these studies that requires further investigation is the effect of number of doses. Previous studies have shown that re-dosing mice after 7 days with linear PEI-pDNA nanoparticles results in significantly reduced luciferase activity in comparison to the first dose. This reduced expression has been attributed to a variety of factors including CpG sequences in the pDNA stimulating immune responses [55–57] and possibly the effect of anaesthesia on the mouse [31]. Kawakami et al. have suggested that the buffering capacity of the PEI in the endosome abrogate the pDNA (CpG)-induced activation of NFκB [30]. Increasing the time after which the second dose of PEI-pDNA nanoparticles is given has been reported to be the dominant parameter in improving the luciferase activity generated by the second dose [31].

In our studies, despite the reported toxicity that is associated with PEI, including deterioration in lung function when lungs are exposed to PEI, [30,31] increasing dose or N:P ratio of the PEI-pDNA nanoparticles did not result in reduced mice survival *in vivo*. The toxicity that arises with PEI administration is associated with charged PEI-pDNA nanoparticles interacting with blood components such as erythrocytes, causing aggregation in the lung capillaries and subsequent lung embolism [34,58,59]. Injecting PEI-pDNA nanoparticles intravenously or intratracheally results in gene expression that is stronger in the lung than liver, heart, spleen or kidneys [30, 34]. Because the peritoneal cavity acts as barrier to PEI-pDNA nanoparticles entering the lung or bypassing the blood-brain barrier, PEI-pDNA nanoparticles injected via this route are expected to demonstrate significantly reduced toxicity [6,36–38]. Consistent with these expectations, in all of the formulations we tested, mice tolerated the PEI-pDNA nanoparticles and remained in good health as determined by the Body Condition Scoring (BCS) technique [60]. The mice appeared well conditioned. The vertebrae and dorsal pelvis were not prominent but palpable with slight pressure indicating a state of good health in the mice. No adverse injection site reactions such as infection, redness or wounding were observed. These observations suggest that the intraperitoneal route of administering PEI-pDNA nanoparticles is safe for the doses and compositions tested.

5. Conclusion

Polyethylenimine is a cationic polymer that has shown promising potential as a non-viral carrier for gene delivery. The structure of PEI can be branched or linear. PEI binds to pDNA electrostatically to form nanoparticles. In this study, we show that branched PEI-pDNA nanoparticles generate stronger luciferase activity than linear PEI-pDNA nanoparticles *in vitro*. In contrast, we show that linear PEI-pDNA nanoparticles generate stronger and more sustained luciferase activity when injected intraperitoneally *in vivo*. Further differences between *in vitro* and *in vivo* results include the impact of N:P ratio on the preparation of PEI-pDNA nanoparticles. Increasing the N:P ratio at

which PEI-pDNA nanoparticles are prepared increased luciferase activity *in vitro*. The optimal N:P ratio is dependent on the cell line. Of the cell lines tested, HEK293 cells are the most sensitive whilst HeLa cells were the least transfection permissive. For *in vivo* experiments, increasing the N:P ratio did not significantly increase the luciferase activity generated by linear or branched PEI-pDNA nanoparticles after IP injection. These studies highlight the need for non-viral delivery systems to be optimized *in vitro* and *in vivo* for specific cell and tissue targets prior to utilization in therapeutic applications. The IP route of administration has been demonstrated to be relatively non-toxic for all the PEI-pDNA nanoparticle formulations tested in this study, can be optimized by dose and polymer structure and should have significant potential for future non-viral gene delivery applications.

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