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ORIGINAL ARTICLE

M-PEIs nanogels: potential nonviral vector for systemic plasmid delivery to tumor cells

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Successfully systemic gene therapy has been hindered by vector-related limitations, including toxicity and inefficient gene delivery to tumor cells after intravenous administration. In this study, we evaluated the potential of spherical polyethylenimine nanogels (M-PEIs) as a novel vector for intravenous delivery of plasmids to tumor cells. M-PEIs provided a sustained release of plasmids up to 14 days and were also effective in protecting plasmids from enzymatic degradation in serum-conditioned media. M-PEIs showed no obvious cytotoxicity to mammalian cells *in vitro* as well as to liver, heart and kidney in mice after intravenous injection. Importantly, following intravenous administration of M-PEIs/plasmid complexes into human hepatocellular carcinoma xenograft-bearing mice, green fluorescence protein reporter gene expression was predominantly found in the tumor. This study indicates that M-PEIs may be a candidate for systemic delivery of plasmids into tumors.
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Keywords: gene delivery; nonviral vectors; polyethylenimine; nanogels; systemic delivery

Introduction

Currently, gene therapy strategies are being studied for their application in cancer therapy as an alternative to chemotherapeutics and protein drugs. Although there are vectors available with high transfection efficacy in cell culture, efficient delivery of genes to a distant tumor site after intravenous administration is still the major hurdle in achieving successful gene therapy. *In vivo* gene delivery has to overcome additional obstacles such as anatomical size constraints and nonspecific interactions with biological fluids and nontarget cells.[1]

Polymeric nanoparticles for gene delivery have drawn much attention, which can efficiently condense DNA to a size that can easily gain access into cells and maintain the stability and biological activity of DNA.[2,3] Polyethylenimine (PEI) is one of the most successful polymers for its ability of efficient delivery of DNA and siRNAs *in vitro* and *in vivo*.[4–8] However, the potential toxicity or undesired side effects of PEI has severely restricted its use as a gene delivery system.[9–12] Different gene delivery vectors of PEI had been synthesized using different methods, such as jet PEI, linear PEI (22 kDa) and branched PEI (800 and 25 kDa).[13] The insight into the relationship between different PEI structures and their biological performances, such as the DNA compaction, toxicity and transfection efficiency is still limited.

In our previous studies, we first reported a series of M-PEIs nanogels (M-PEIs) prepared by photo-Fenton reaction.[13,14] We investigated the potential for gene transfer by M-PEIs nanogels with different sizes of 38–168 nm *in vitro*. M-PEIs/DNA complexes yielded high transfection efficiency in all of the four cancer cell lines and had no obvious cytotoxicity. But the potential of spherical PEI nanogels as a systemic gene delivery system still needs further investigation.

This study extends to evaluate the potential of M-PEIs for systemic gene transfer. Our study indicates that M-PEIs could be potentially applied as a useful systemic gene delivery system to specifically express a transgene in tumors.

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Materials and methods

Plasmid

The plasmid encoding the green fluorescence protein (GFP) reporter gene (6.9 kb) was purchased from GeneChem (Shanghai, China). All plasmid were purified according to the manufacturer's protocol (Qiagen, Hilden, Germany).

Preparation of M-PEIs/plasmid complexes

M-PEIs nanogels were synthesized as described previously.[13,14] As the most suitable size range of M-PEIs for transfection was 70–90 nm,[13] samples with sizes of 75 nm (Z average size detected by PCS in aqueous solution) were used in this study. M-PEIs/plasmid complexes were prepared by flash mixing of indicated amounts of M-PEIs with plasmid at a weight ratio of 4:1 (by adding 4.8 μg of M-PEIs directly to 1.2 μg of plasmid in 100 μl phosphate-buffered saline (PBS)), followed by a 30 min incubation at room temperature. In a separate tube, naked plasmid was dissolved in PBS.

Serum stability assay

M-PEIs/plasmid complexes or naked plasmid were mixed in a 1:1 ratio with fresh serum to give 50% serum concentration and incubated at 37 °C. The mixture was removed from the sample at an indicated time interval (0, 1, 2, 4, 8, 16, 24, 48 and 72 h) and analyzed by 1% agarose gel electrophoresis in 1 × Tris–borate–EDTA buffer at a constant voltage of 100 V for 1 h. Following electrophoresis, gels were visualized on a UV transilluminator.

In vitro *plasmids release*

M-PEIs/plasmid complexes at a weight ratio of 4:1 (200 μg) were incubated in an equal volume of transfection medium (Dulbecco's modified Eagle medium (DMEM) with pH 7.4) at 37 °C under stirring (100 r.p.m.). At different tim -points, the released plasmids were separated from the complexes by centrifugation at 14 000 *g* for 30 min and analyzed for the amount and integrity using UV spectrophotometer (Shimadzu, Kyoto, Japan) at OD 260 nm.

Cell culture and gene transfection

Human hepatocellular carcinoma cell line HCC-LM3 cells were cultured in DMEM with 10% v/v fetal bovine serum (FBS) in a humidified incubator at 37 °C in 5% CO_2 atmosphere. Transfection of plasmids was performed using M-PEIs in 24-well tissue culture plates at a density of 5×10^4 cells per well as described previously.[13] Briefly, 1.2 μg plasmid was dissolved in 100 μl PBS and then 4.8 μg of M-PEIs was added into plasmid. After vortexing, the plasmid/M-PEIs mixture were allowed to form at room temperature for 30 min and then added to the cells in serum-free DMEM. After 4 h of incubation, the medium was replaced with fresh DMEM medium containing 10% FBS, and the cells were incubated at 37 °C for various time periods before western blotting.

Western blotting

At various times posttransfection, total proteins were extracted in 1 × SDS buffer. Proteins at the same amount were separated by 12% SDS–polyacrylamide gel electrophoresis and transferred onto PDVF membranes (Bio-Rad, Hercules, CA). After probing with antibodies against GFP (Beyotime, Haimen, China), antigen–antibody complex was visualized by enhanced chemiluminescence's reagents Supersignal (Pierce Biotechnology, Rockford, IL).

Cell viability assay

The cytotoxicity of M-PEIs on mammalian cells was assessed using a colorimetric method with 3-(4, 5-dimethylthiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) according to the manufacturer's protocol (Promega, Madison, WI). HCC-LM3 cells were seeded in a 96-well plate at a density of 8000 cells per well in DMEM medium containing 10% FBS. Twenty-four hours postseeding, different amounts of M-PEIs (5, 10 and 20 μg ml^{-1}) or M-PEIs mixed with plasmid (2.5 μg ml^{-1}) were added to the wells. After 48 h incubation, 20 μl MTS solution was added to cells followed by further incubation for 4 h. At the end of incubation, absorbance at 490 nm was measured using EL × 800 Universal Microplate Reader (Bio-Tek, Winooski, VT). The cell viability (%) was calculated according to the following equation: Cell viability (%) $= (OD_{490\ (sample)}/OD_{490\ (control)}) \times 100$. The cells without M-PEIs treatment were taken as control.

Biochemistry assay

Six-week-old male Kunming mice were purchased from Shanghai Experimental Animal Center of Chinese Academic of Sciences (Shanghai, China). All of mice were housed in a ventilated, temperature-controlled and standardized sterile animal room in accordance with established institutional guidance and approved protocols. Animals were randomly divided into two groups (6 mice in each group) and received intravenous injections over a 4-day period as follows: (1) Control group: saline solution; (2) M-PEIs-treated group: M-PEIs saline solution at a dose of 5 mg kg^{-1} body weight daily. All mice of two groups were killed 5 days later for the biochemical studies.

Whole liver, heart and kidney of mice were collected and homogenized in 10-fold volume of physiological saline solution using a motor-driven homogenizer (Ultra-Turrax T25; IKA, Staufen, Germany). The supernatant obtained was used for assays of superoxide dismutase (SOD), glutathione (GSH) and malonaldehyde (MDA) activities using assay kits from Nangjin Jiancheng Bioengineering Institute (Nanjing, China).

Serum biochemistry parameters including blood urea nitrogen (BUN), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were evaluated with Shimadzu CL-7200 automated analyzer (Shimadzu).

Intravenous delivery of M-PEIs/plasmid

Six-week-old male BALB/c nude mice were obtained from Shanghai Experimental Animal Center of Chinese

Academic of Sciences (Shanghai, China). Orthotropic HCC-LM3 tumors were established as described previously.[15] Tumor fragments (1 mm^3 in size) were intrahepatically implanted into mice under sterile conditions. On day 15 after implantation, mice were treated by injection of 20 μg plasmids mixed with or without 80 μg M-PEIs via the tail vein. Mice were killed 6 h after treatment. Tumors and major organs such as liver and lung were collected, embedded in OCT medium (Sakura Finetek, Zoeterwoude, The Netherlands), and frozen in liquid nitrogen. The frozen tissue was cut in 5 μm-thick sections and GFP expression was examined with fluorescent microscope (Olympus, Tokyo, Japan). Pictures were taken at equal exposure times for each tissue. In a separate experiment, total proteins were extracted from tumors and major organs (liver, kidney, lung, heart and spleen) 48 h after treatment. The GFP expression was detected by western blotting.

Statistical analysis

Statistical significance of results was evaluated using two-tailed Student's *t*-tests according to the TTEST function in Microsoft Excel. Results were considered statistically significant at $P<0.05$.

Results

Serum stability of M-PEIs/plasmid complexes

Photo-Fenton reaction was a novel and efficient method to prepare M-PEIs nanogels from PEI prepolymer. The reaction was performed under UV irradiation, which initiated cross-linking of the PEI prepolymer to form M-PEIs particles in homogeneous size and loose spherical structure in aqueous solution. Furthermore, the atomic force microscopy image of M-PEIs/DNA complexes revealed that some DNA molecules were adhered to the surface of M-PEIs due to probably electrostatic interaction and others was embedded into the nanogels owing to the loose structure.[13]

To address the question of M-PEIs/plasmid stability and protection from enzymatic degradation, we performed a serum stability assay. The M-PEIs/plasmid complexes were incubated at 37 °C in 50% of FBS, and the degradation of plasmid was visually observed by gel electrophoresis. Naked plasmids were completely degraded 4 h after incubation with serum. In contrast, plasmids recovered from M-PEIs nanogels exhibited almost no structural loss 72 h after incubation with serum (Figure 1). This result suggested the formation of complexes with M-PEIs efficiently protected the plasmids from enzymatic degradation. The slightly positive surface charge of M-PEIs/plasmid which were likely reducing nonspecific interactions with serum proteins could maintain their stability even in serum conditions.[13] On the other hand, there were many PEI branches in the surface of M-PEIs nanogels, which might embed and protect plasmid from enzymatic degradation.[13] The enhanced stability of M-PEIs/plasmid complexes in the presence of serum supports animal administration where the plasmid is exposed to serum nucleases.

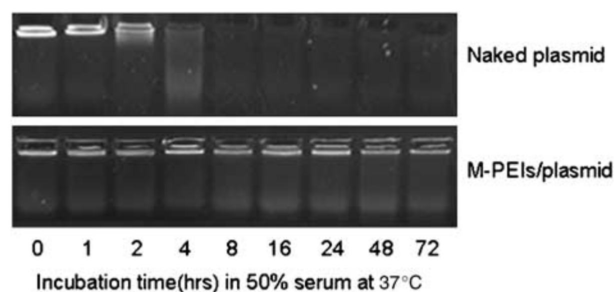

Figure 1 Plasmid protection assay in serum. Degradation of plasmids exposed to serum was measured for polyethylenimine nanogels (M-PEIs)/plasmid complexes and compared with naked plasmid. The M-PEIs/plasmid complexes, or naked plasmid, was incubated in the medium containing 50% FBS from 0 to 72 h and then the remaining intact plasmid was determined by gel electrophoresis.

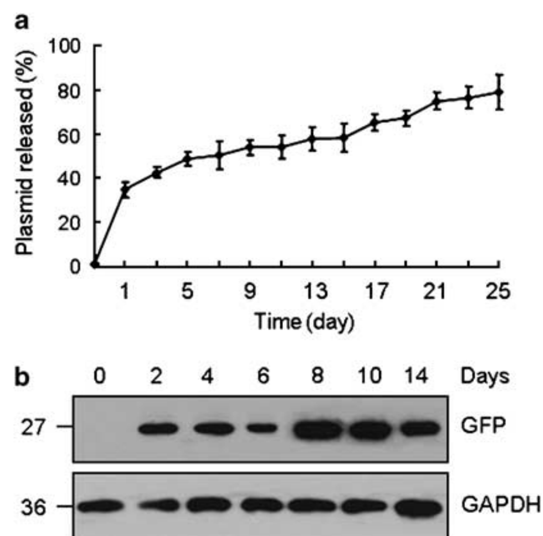

Figure 2 Sustain release of plasmids from polyethylenimine nanogels (M-PEIs)/plasmid complexes. (**a**) *In vitro* release profile of plasmid from M-PEIs/plasmid complexes at a weight ratio of 4:1. Release assay was studied in Dulbecco's modified Eagle medium (DMEM) medium pH 7.4, 37 °C (mean ± s.d., $n=4$). (**b**) At various times posttransfection, total proteins from HCC-LM3 cells were extracted by 1 × SDS buffer and subjected to western Blotting with anti-green fluorescence protein (GFP) antibody.

Release of plasmid from the M-PEIs complexes in vitro

The M-PEIs/plasmid exhibited an initial burst release of plasmid with 34.72% within 1 day (Figure 2a). After 1 day, M-PEIs/plasmid displayed a sustained release pattern. The amount of plasmid released over 25 days reached 78.89%. The results suggested that M-PEIs induced a sustained gene expression. The initial fast release of plasmid might be related to some plasmids adhered on the surface of M-PEIs that would be immediately released during the initial stage. The

sustained release might result from the diffusion of the remainder of the plasmid entrapped in the polymeric particles and release in the erosion of the polymers.[2,13]

We also studied the GFP reporter gene expression in HCC-LM3 cells. M-PEIs-induced GFP expression could be detected 2 days after transfection and became stronger 8 days later. Sustained expression of GFP lasted for 14 days in cell culture (Figure 2b). Thus, M-PEIs were able to deliver plasmids in a functionally active form for a period up to 14 days. The sustained transgene expression appears very promising in view of improving the *in vivo* administration schedule leading to a therapeutic effect.

Cellular toxicity of M-PEIs in vitro

The cytotoxicity of M-PEIs on mammalian cells was examined using MTS assay in HCC-LM3 cells. As shown in Figure 3a, exposure of the cells to free M-PEIs did not cause significant toxicity, as none of the examined samples exhibited survivals below 95% of control when cells were exposed to the M-PEIs-free media. We next evaluated the toxicity of M-PEIs/plasmid complexes. Although there was a slight reduction in cell viabilities at high concentration of M-PEIs ($20\,\mu g\,ml^{-1}$), no significant toxicity was detected ($P>0.05$). These results demonstrate that M-PEIs have negligible toxicity *in vitro*.

Toxicity after systemic application of M-PEIs nanogels

MDA, a biomarker of lipid peroxidation, would reflect the degree of cell damage. SOD is a scavenger of free radicals, which has important effects in the control of oxidation reactions in the body.[16] GSH is present in high concentrations in mammalian cells and play a major role in the protection against xenobiotics and oxidative event.[16] Previous studies have demonstrated that systemic administration of unshielded branched PEI (B-PEI800 kDa) had a lethal effect in animal models,[17] and injection of L-PEI/DNA was associated with the appearance of necrotic areas in the liver.[10,18]

The effects of intravenous injection of M-PEIs nanogels on the levels of SOD, MDA and GSH in the major organs from mice were examined (Figure 3b). No obvious changes in the activities of SOD, GSH and MDA from liver, kidney and heart were observed between M-PEIs-treated group and the control group. Moreover, no obvious changes were observed in the serum level of BUN, ALT and AST by M-PEIs treatment (Figure 3c). The present study showed that administration of repeated doses of M-PEIs to Kunming mice did not induce damages to liver, kidney and heart as revealed by an array of biochemical markers. All mice receiving M-PEIs nanogel survived throughout the duration of the experiment, and did not show any signs of acute toxicity after intravenous administration.

Many factors affect the cytotoxicity profile of PEI polyplexes such as molecular weight, degree of branching, ionic strength of the solution, zeta potential and particle size.[19] The low toxicity of M-PEIs was probably due to the $C=N$ bonds in its molecules which might help M-PEIs degrade into small molecules and excrete.[13] It might be also because the size of M-PEIs was small enough to be excreted by cells.

Gene delivery after systemic application

The studies of plasmid serum stability clearly demonstrated that the plasmids in the M-PEIs complexes were protected from serum degradation and were much more

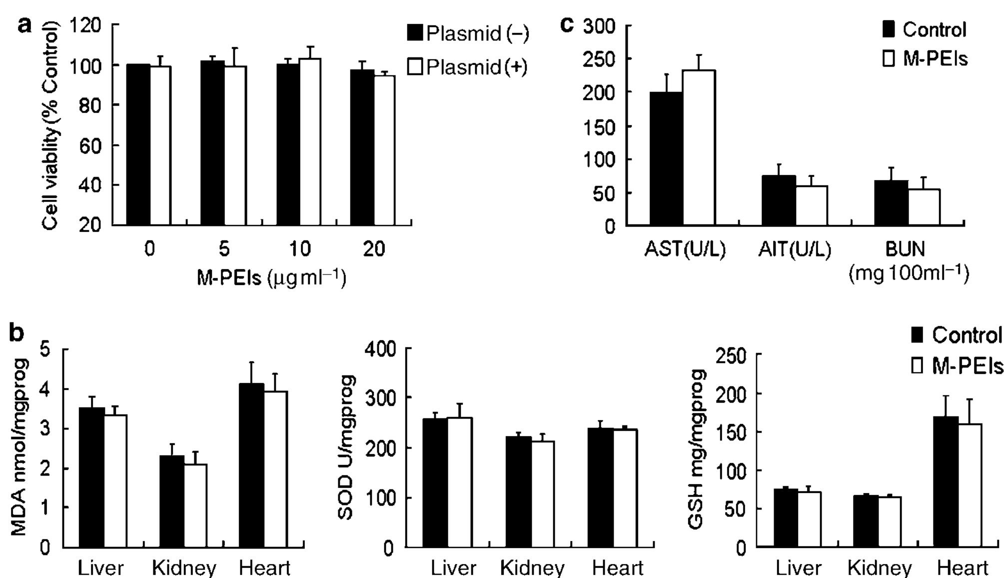

Figure 3 Cytotoxicity of polyethylenimine nanogels (M-PEIs) *in vitro* and *in vivo*. (**a**) Evaluation of the toxicity of M-PEIs containing plasmid (black bars) versus the equivalent dose of free M-PEIs (white bars). Relative cell viability was determined using an MTS assay where survival percentages were calculated as compared to an untreated control (100% survival). (**b**) Effects of systemic treatment with M-PEIs on malonaldehyde (MDA) and glutathione (GSH) level and superoxide dismutase (SOD) activities from liver, kidney and heart in control and treated mice. (**c**) The level of blood urea nitrogen (BUN), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the serum from control and treated mice. Data are expressed as means ± s.d. ($n=6$).

stable than naked plasmid. The enhanced stability of M-PEIs/plasmid complexes in the presence of serum implies that M-PEIs/plasmid complexes would have a great potential for *in vivo* applications.

To further investigate whether M-PEIs/plasmid complexes are suitable for transfection of tumors after systemic *in vivo* application, we implanted human HCC tumors (HCC-LM3) orthotropically into nude mice. Plasmids encoding a GFP reporter gene mixed with or without M-PEIs were injected intravenously into the tail vein. Six hours after injection, fluorescence was detected for GFP expression in the tumor tissues as well as in the major organs (Figure 4a). Intravenous administration of naked plasmid produced very little GFP fluorescence in the tumor, liver and lung. The lack of fluorescence and GFP expression from naked plasmid may be explained by its potentially metabolic instability resulting in quickly degradation because it was more labile in serum than that in the M-PEIs/plasmid complexes as shown in Figure 1. On the other hand, M-PEIs/plasmid complexes produced appreciable GFP fluorescence in the tumor tissues, but poor GFP fluorescence in the surrounding nontumor liver tissues and lung.

We further investigated the reporter gene expression (GFP) 48 h after injection by western blotting. As shown in Figure 4b, the GFP expression is mainly found in the tumor tissues and hardly detected in the surrounding nontumor liver tissues and lung, and undetectable in heart, kidney and spleen. The GFP expression profile showing highest expression in tumor tissues paralleled the tissue distribution observed with fluorescence microscopy (Figure 4a).

Discussion

Here, we describe the specific targeting of gene delivery to xenotransplants of human HCC by systemic administration of M-PEIs complexes. In our recent report elsewhere, we demonstrated that M-PEIs/DNA complexes had a low zeta potential.[13] The low surface charge of the complexes may contribute to the high tumor specificity observed. Cancer cells may promote internalization of PEI/DNA complexes which have marginally positive zeta potentials over normal cells.[7] Moreover, the low zeta potential may enable a prolonged circulation of PEI/DNA complexes by reducing nonspecific interactions with blood components and nontargeting cell.[9,20] Furthermore, the low positive zeta potential may promote the binding of PEI/DNA vector to the negatively charged glycoproteins on the tumor cell surface and favor better interaction with integrins that can be increased in proliferating tumor cells, leading to endocytosis and increased gene delivery.[7,21,22]

Transfection efficiency with nonviral vectors is strongly dependent on the breakdown of the nuclear membrane during cell division, which is rather low in normal tissues compared with highly proliferating tumor cells.[23,24] A similar DNA distribution with tumor was previous

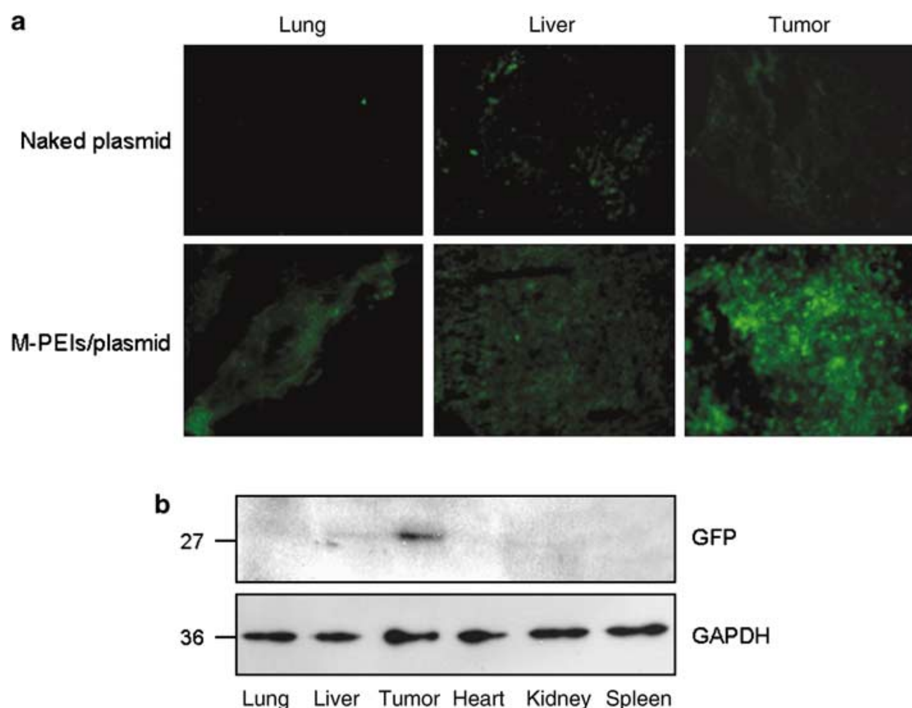

Figure 4 Green fluorescence protein (GFP) expression after systemic application of M-PEI/plasmid complexes in HCC-xenografted mice. (**a**) HCC-LM3 tumor-bearing mice received M-PEI/plasmid complexes by intravenous injection. The GFP expression was examined 6 h after injection on a fluorescence microscope as described in Materials and methods. (magnification, ×200). (**b**) GFP expression was measured by western Blotting with anti-GFP antibodies 48 h after application.

reported upon intravenous injection of DNA/PEI complexes[7] and polyethylene glycol-shielded DNA/PEI complexes.[20] The mechanisms for these results remain to be elucidated in further details.

In conclusion, our results indicate the potency of M-PEIs-mediated systemic gene transfer to tumor cells. The M-PEIs/plasmid complexes exhibited low toxicity both *in vitro* and *in vivo*. Importantly, intravenous injection of M-PEIs/plasmid complexes allows for highly specific expression of transgenes in tumors *in vivo*. M-PEIs could be potentially applied as a useful plasmid delivery system for cancer gene therapy.

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