

Polylysine-modified Polyethylenimines as siRNA Carriers for Effective Tumor Treatment*

Jie Chen^a, Zi-xue Jiao^a, Lin Lin^a, Zhao-pei Guo^a, Cai-na Xu^a, Yan-hui Li^b,
Hua-yu Tian^{a**} and Xue-si Chen^a

^a Key Laboratory of Polymer Ecomaterials, Changchun Institute of Applied Chemistry, Chinese Academy of Science, Changchun 130022, China

^b School of Materials Science and Technology, Changchun University of Science and Technology, Changchun 130022, China

Abstract Polyethylenimine-poly(L-lysine) (PEI-PLL) copolymer was synthesized *via* ring-opening polymerization of L-lysine *N*-carboxyanhydride (Lys(Z)-NCA) initiated by PEI. The complexation of PEI-PLL with siRNA was studied by particle size and zeta potential measurements. The flow cytometric analysis and confocal imaging showed its excellent intracellular trafficking ability. PEI-PLL displayed higher gene silencing efficiency and lower cytotoxicity than commercial PEI-25k *in vitro*. In the antitumor study, PEI-PLL was further combined with siVEGF and showed obviously tumor inhibition effect for the treatment of CT26 tumor model. Therefore, PEI-PLL is a promising siRNA carrier candidate for further antitumor treatment *in vivo*.

Keywords: Gene carrier; Gene silencing; Polylysine; Tumor treatment; VEGF siRNA.

INTRODUCTION

RNA interference (RNAi) is a post-transcriptional mechanism of gene silencing to the target gene mediated by double strands RNA, which is a sequence specific process of mRNA degradation^[1]. In the target cells, the double strands RNA unwinds into an antisense strand and a sense strand with the assistance of adenosine triphosphate (ATP), then the antisense strand siRNA can form RNA induced silencing complex (RISC) by combining with endonuclease *et al*^[2]. Small interfering RNA (siRNA) with 21–23 nucleotide was first successful to mediate gene silencing in mammalian cells^[3]. Subsequently, researchers successfully proved that siRNA mediated targeted gene silencing technology was considered to be the most effective way to treat gene-related diseases^[4–6]. The application of siRNA-based gene therapeutic for tumors depended on the delivering of siRNA to the targeted tissues or cells *in vivo*. However, naked siRNA itself had low stability and could be rapidly cleared from the blood during systemic circulation^[6, 7]. Even more, siRNA itself could hardly penetrate cellular lipid membranes and entry into the targeted tissue or cells because of the negative charged surface of tumor cells^[8]. Therefore, siRNA had to be enclosed in carriers to improve the antitumor effect both *in vitro* and *in vivo*.

Recently, non-viral carriers had attracted our widespread attention because of their excellent performances,

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** Corresponding author: Hua-yu Tian (田华雨), E-mail: thy@ciac.ac.cn

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such as low immunogenicity, high transfection ability, repeatability and low cytotoxicity^[9–11]. Among them, polyethylenimine (PEI) played an important role as siRNA carriers in antitumor therapy. Hyperbranched PEI with a molecular weight of 25 kDa was considered as the "golden standard" for gene transfection^[12]. However, the application of PEI was limited due to the undesirable cytotoxicity effects caused by its high positive charge density and relative low transfection efficiency^[13]. Several strategies had been attempted to improve the transfection efficiency and reduce the cytotoxicity of PEI. For instance, the introduction of poly(ethylene glycol) (PEG)^[14, 15] and other biocompatible segments^[8, 16, 17] in PEI molecules, the crosslink of low molecular weight *via* disulfide bonds^[18, 19] and the conjugation of PEI with targeted ligands^[20, 21]. Based on our previous results, the transfection efficiency and cytotoxicity of PEI were obviously improved by introducing biocompatible polyamino acids^[22–25].

In our previous study, a series of polylysine modified PEI (PEI-PLL) were synthesized by ring-opening polymerization using ϵ -benzyloxycarbonyl-L-lysine *N*-carboxy anhydride (Lys(Z)-NCA) as the monomer and PEI-25k as a macro-initiator^[24]. PEI-PLL was developed as an efficient pDNA delivery system and exhibited significant tumor inhibition effect both *in vitro* and *in vivo*. In the present study, PEI-PLL was further explored as the siRNA delivery system and its therapeutic effectiveness was evaluated by targeting of vascular endothelial growth factor (VEGF) both *in vitro* and *in vivo*.

EXPERIMENTAL

Materials

Branched PEI with weight-average molecular weight of 25 kDa was purchased from Aldrich (St. Louis, MO, USA). L-Lysine was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Amresco (Solon, Ohio, USA). All other chemicals were purchased from Sigma-Aldrich (Munich, Germany). Cell culture media, antibiotics and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, USA). siRNA duplexes were synthesized in GenePharma Co. Ltd. (Shanghai, China) namely mouse VEGF siRNA (siVEGF; sense, 5'-AUGUGAAUG-CAGACCAAAGAAAdTdT-3' and antisense 5'-UUCUUUGGUCUGCA-UUCACAAdTdT-3'). Trizol reagent was purchased from Thermo Fisher Scientific Inc. Real-time PCR kit was purchased from TAKARA Biotechnology Co., Ltd (Dalian, China). Female BALB/C mice (5–6 weeks old) were purchased from Vital River Company (Beijing, China).

Synthesis of PEI-PLL

PEI-PLL copolymer was prepared according to our previous method^[24]. PEI-25k was used as a macroinitiator and PEI content in the final PEI-PLL was 60.9%.

Particle Size and Zeta Potential Analysis

The cationic carriers were utilized to form complexes with siRNA for particle size and zeta potential measurements. The carrier/siRNA complexes were formed at their optimal weight ratios according to our previous study^[24] and vortexed. After 20 min incubation at room temperature, the particle size and zeta potential of the complexes were measured by using a Zetasizer Nano ZS90 (Malvern Instruments, Malvern, UK).

Cell Culture

Murine colorectal adenocarcinoma cells (CT26 cells) were used in siRNA delivery experiments. CT26 cells were cultured in a DMEM medium containing 10% *V/V* heat-inactivated fetal bovine serum (FBS), penicillin (100 U·mL⁻¹) and streptomycin (100 mg·mL⁻¹) in a 5% CO₂ incubator at 37 °C.

Cytotoxicity Assay

The cytotoxicity of the PEI-PLL copolymer was assessed with MTT assay using CT26 cells. The cells were seeded in 96-well plates at a density of 1×10^4 cells per well and incubated at 37 °C in 5% CO₂ incubator for 24 h prior to the treatment of copolymer solutions with the concentration range from 1.56 $\mu\text{g}\cdot\text{mL}^{-1}$ to 25 $\mu\text{g}\cdot\text{mL}^{-1}$. PEI-25k was served as the control. After another 24 h, 20 μL of MTT stock solution (5 mg·mL⁻¹ in

PBS) was added to each well. 4 h later, the culture medium was carefully removed from each well, and 150 μ L DMSO was added to dissolve the MTT formazan crystals and shook for 10 min. The absorbance was analyzed using an ELISA microplate reader (Bio-Rad) according to the manufacturer's protocol. The cell viability was normalized to that of CT26 cells only cultured in the DMEM medium. Each experiment was performed as the average values of six runs and repeated for three times.

Quantification of VEGF Gene Expression by qRT-PCR

The *in vitro* gene silencing experiment was performed in CT26 cells using real-time PCR analysis. The cells were seeded in 6-well plates at 1.0×10^5 cells per well for 24 h prior the treatment of PEI-25k/siVEGF and PEI-PLL/siVEGF complexes. The weight ratios of PEI/siVEGF and PEI-PLL/siVEGF were selected as 2.5:1 and 5:1, respectively. After incubation for another 24 h, the total RNA was collected from the cells with Trizol reagent according to the manufacturer's protocol. The total RNA concentration and purity were evaluated using Nanodrop Plates (Tecan M200), and then the total RNA was reverse transcribed into cDNA as described of M-MLV manual. The primers for mouse VEGF were as follows: Forward, 5'-GGTGAGAGGTCTAGTTCCCGA-3'; Reverse: 5'-CCATGAACCTTCTGCTCTTC-3'. Then, the real-time PCR (Mx3005P, Strategene, USA) was performed using SYBR Green Mix Kit according to the manufacturer's protocol. The qRT-PCR amplification was performed as follows: initial heating at 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s, 56 °C for 60 s and 72 °C for 5 s.

Flow Cytometry Assay

To investigate the *in vitro* cellular uptake by CT26 cells, FAM-labeled siRNA was chosen as a tracker to formulate into the complex with PEI-25k or PEI-PLL copolymer. The cells were seeded in 6-well plates with 2.0×10^5 cells per well in 2 mL complete DMEM medium (90% DMEM, 10% FBS) and cultured at 37 °C in 5% CO₂ humidified atmosphere for 24 h. The medium was replaced with complete DMEM medium containing carrier/FAM-siRNA or FAM-siRNA. The final concentration of FAM-siRNA was 1 μ g·mL⁻¹. The cells were incubated at 37 °C for 4 h, and then the harvested cells were washed twice with cold PBS, detached with 0.25% trypsin and resuspended in 400 μ L of cold PBS (pH7.4). Finally, internalization efficiency was carried out using a flow cytometer (FACS Caliber, Becton-Dickinson, San Jose, CA, USA).

Confocal Laser Scanning Microscopy Assay

For confocal laser scanning microscope observation, CT26 cells were seeded on coverslips in a 6-well plate at a density of 2×10^5 cells per well and incubated for 24 h. The medium was replaced with complete DMEM medium containing PEI-25k/FAM-siRNA or PEI-PLL/FAM-siRNA complex. The final concentration of FAM-siRNA was 1 μ g·mL⁻¹. The cells were incubated for 4 h at 37 °C, then washed with cold PBS and fixed with 3.7% formaldehyde for 10 min at room temperature. The cell nucleus was stained with DAPI according to the manufacturer's protocol. The slides were observed by a confocal laser scanning microscope imaging system (LSM 780, Carl Zeiss Inc., Germany).

In vivo Tumor Treatment with Carriers/siVEGF Complexes

Female Balb/c mice (5–6 weeks old) were purchased from Vital River Company (Beijing, China), and all animal experiments were carried out under the guidance of the Animal Care and Use Committee of Northeast Normal University. In order to generate tumors, 50 μ L of 2×10^6 CT26 single-cell suspension in PBS medium was injected subcutaneously into the left foreleg of the mice using a syringe. Tumor size was measured across its longest (*a*, mm) and shortest (*b*, mm) diameters using a vernier caliper and the volume of tumor was calculated using the following equation: tumor volume (mm³) = $0.5ab^2$. After ten days, when the tumor size increased approximately 70 mm³, the mice were given an intratumoral injection of PEI-PLL/siVEGF complex, naked siVEGF and 5 wt% glucose solutions. All complexes were suspended in 50 μ L 5 wt% glucose solution, and a dose of 4.0 μ g siVEGF/head was injected intratumorally to mice four times during two weeks. The tumor volumes were measured three times a week.

RESULTS AND DISCUSSION

Synthesis of PEI-PLLs

PEI-PLL is a novel type copolymer of hyper-branched cationic polymer prepared by grafting polylysine segments onto the hyper-branched PEI-25k according to our previous study^[24]. It was used as a pDNA carrier and showed high gene transfection efficiency and antitumor effect both *in vitro* and *in vivo*^[24]. In the current work, the PEI-PLL copolymer was characterized as a siRNA carrier for efficient tumor treatment.

Formation of Complexes

Particle size and surface charge measurements could demonstrate the formation of carrier/siRNA complexes. Suitable size and surface charge were the essential process of efficient endocytosis and transfection for cationic carriers^[26]. For the current study, the particle sizes and zeta potentials of carrier/siRNA complexes were studied at their optimal transfection weight ratios. As shown in Fig. 1(a), the particle sizes of PEI-PLL/siRNA complex and PEI-25k/siRNA complex were about 110 nm and 120 nm, respectively. The results showed that the PEI-PLL copolymer had a similar property to that of PEI-25k in condensing siRNA into nanoparticles. Both PEI-PLL/siRNA and PEI-25k/siRNA complexes exhibited about 12 mV of zeta potential, which could further support the previous particle size results. (Fig. 1b)

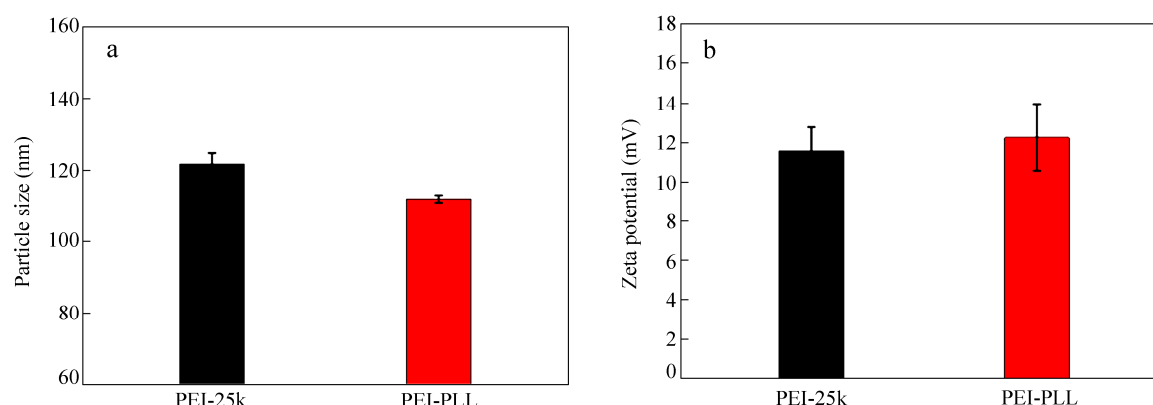


Fig. 1 Particle sizes (a) and zeta potential (b) of carrier/siRNA complexes at their optimum transfection weight ratios

Cytotoxicity of Copolymers

Cytotoxicity is a major obstacle for cationic carrier-mediated siRNA delivery both *in vitro* and *in vivo*. The cationic components can interact with negatively charged proteins on the cell membrane, therefore induce membrane destabilization and cell necrosis. The cytotoxicity of PEI-25k depends on the high density of positive charges on its surface. In order to improve the cytocompatibility of PEI-25k, the biocompatible polylysine moieties were introduced and shield the positive charge on the surface of PEI-25k. As shown in Fig. 2, PEI-PLL copolymer showed relatively lower cytotoxicity compared with the commercial PEI-25k, and exhibited the potential application *in vivo*.

VEGF Silencing with Carriers/siVEGF Complexes by qRT-PCR

To evaluate the activities of post-transcriptional gene silencing of the proposed siRNA delivery systems, the VEGF mRNA levels in CT26 cells were tested by qRT-PCR analysis after 24 h treatment with siVEGF, PEI-25k/siVEGF and PEI-PLL/siVEGF complexes. The VEGF mRNA level of untreated group was used as the control. The PEI-PLL/siVEGF complex showed a more dramatic decrease of about 60% in VEGF mRNA expression than that of PEI/siVEGF complex (about 30%), whereas the siVEGF group could not achieve any gene silencing efficiency (Fig. 3). These results clearly demonstrated that the PEI-PLL copolymer could achieve much more excellent performance compared with the commercial PEI-25k as siRNA delivery system.

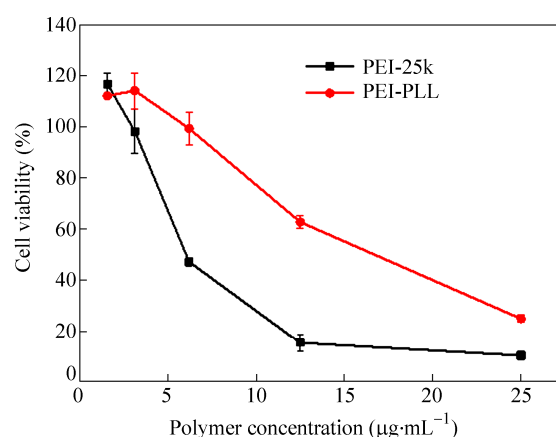


Fig. 2 Cytotoxicity of PEI-PLL copolymer to CT26 cells determined by MTT assay ($n = 4$)

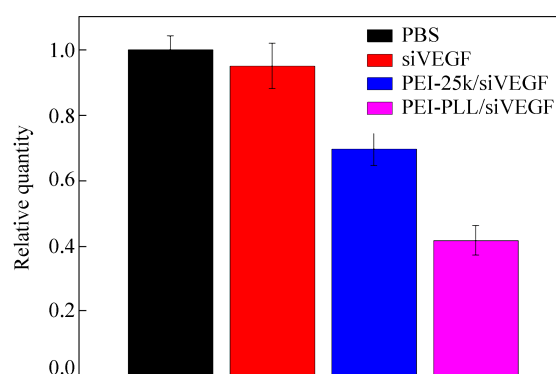


Fig. 3 Quantitative RT-PCR analysis for VEGF mRNA

CT26 cells without any treatment were used as controls. Relative quantification was performed using the GAPDH housekeeping gene as the internal control ($n = 3$).

Intracellular Trafficking of siRNA Complexes

The intracellular uptake of fluorescent-labeled siRNA (FAM-siRNA) formulations was monitored by confocal laser scanning microscopy (Fig. 4) and flow cytometry (Fig. 5) by using CT26 cells. All the tested carriers could internalize FAM-siRNA into cells, because the high levels of fluorescence were observed in the cytoplasm (Fig. 4). Both PEI-PLL and PEI-25k were highly efficient in siRNA intracellular trafficking, whereas no fluorescence spots were observed in the cells when treated with naked FAM-siRNA. However, more homogeneous green fluorescence spots were observed when treated by PEI-PLL/FAM-siRNA than PEI-25k/FAM-siRNA, which could be the reason that PEI-PLL achieved more gene silencing efficiency.

The internalization efficiency was further monitored in CT26 cells using flow cytometric analysis (Fig. 5). The fluorescence peaks for the cells treated with PEI-PLL/siRNA and PEI-25k were notably shifted to the right, whereas naked FAM-siRNA treated group showed no difference of fluorescence intensity compared with PBS treated group. Therefore, compared with the control groups, the internalization efficiency of siRNA was significantly improved by PEI-PLL or PEI-25k. However, PEI-PLL showed a much higher and uniform transportation capacity than PEI-25k, which further validated the previous CLSM observation.

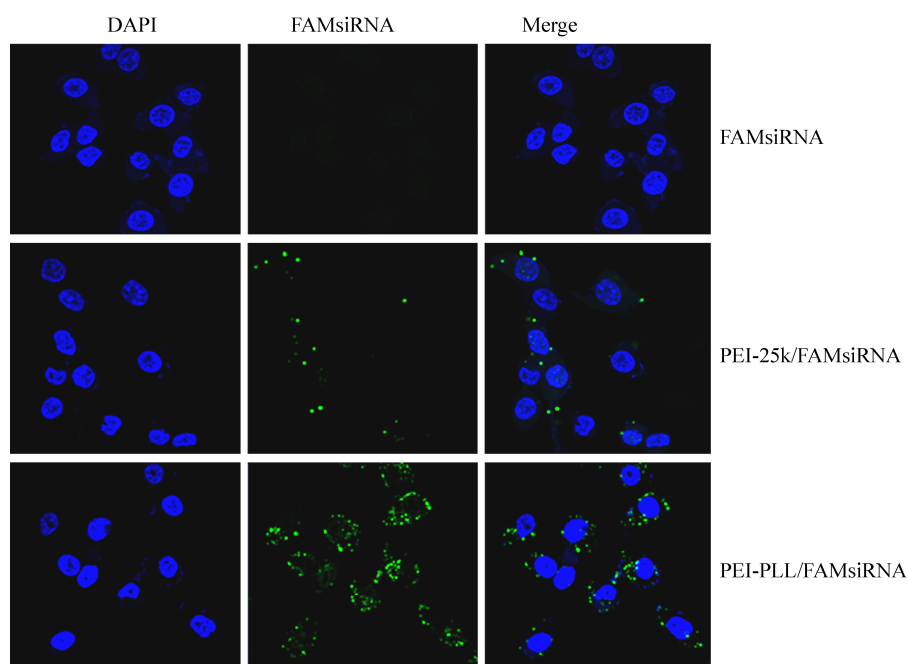


Fig. 4 Confocal laser scanning images of carrier/FAM-siRNA complexes in CT26 cells after 4 h of incubation

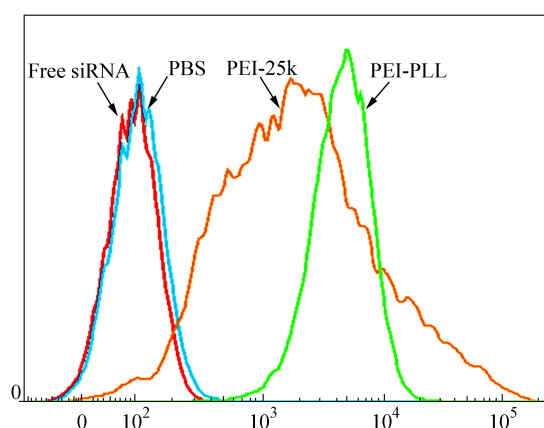


Fig. 5 Cellular uptake analysis of carrier/FAM-siRNA complexes in CT26 cells by flow cytometry after 4 h of incubation

In vivo Inhibition of Tumor Growth

To test the bioactivity of the inhibition of tumor angiogenesis by siRNA, a number of potential targets in cancer have been identified as positive regulators of tumors^[27–30]. Among them, VEGF has been thoroughly studied in antiangiogenic therapy by down-regulating the gene expression of vascular endothelial growth factor for tumor growth inhibition^[5, 31, 32]. In the current work, murine colorectal adenocarcinoma CT26 cells were collected and injected subcutaneously into the left foreleg of Balb/c mice for establishing the tumor models. When the tumor sizes reached approximately 70 mm³, the therapeutic procedures of PBS, siVEGF and PEI-PLL/siVEGF complex were carried out for four times therapy during two weeks. As shown in Fig. 6(a), the PEI-PLL/siVEGF complex achieved obvious tumor growth delay compared with the free siVEGF group. After four times injection of PEI-PLL/siVEGF complex, the tumor volume increased from 68.5 mm³ to 223.5 mm³, whereas the tumor volume increased from 67.8 mm³ to 693.4 mm³ in the siVEGF-treated group. The effect of tumor therapy was presented in Fig. 6(b), which further agreed with the inhibition curves in Fig. 6(a). These results confirmed that

the PEI-PLL/siVEGF complex could efficiently deliver siVEGF to tumor tissues and achieved successfully antiangiogenic effect for tumor treatment.

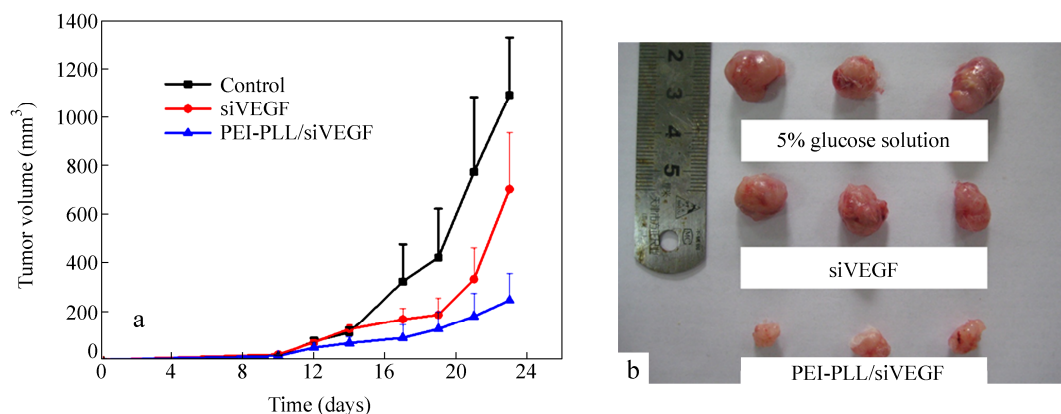


Fig. 6 Anti-tumor effect of PEI-PLL complex by intratumoral injection twice weekly at day 10, 14, 17 and 21 days (a) tumor inhibiting curves changed with increasing time and (b) efficacy of tumor inhibition

CONCLUSIONS

In this study, PEI-PLL copolymer was successfully applied as siRNA delivery system both *in vitro* and *in vivo*. The cytotoxicity of PEI-25k was significantly reduced after introducing the biocompatible polylysine segments. The siRNA condensation capability of PEI-PLL was characterized by particle size and zeta potential assay. The transfection ability of PEI-PLL was measured by qRT-PCR on the post-transcriptional level, and showed obviously enhanced gene silencing efficiency than PEI-25k. The higher transfection efficiency was mainly due to the excellent intracellular trafficking ability, which was obtained by confocal imaging and flow cytometric analysis. PEI-PLL was further combined with siVEGF and exhibited obviously therapeutic effects for the treatment of CT26 tumor model. Our results suggest that PEI-PLL will be a promising siRNA carrier for tumor gene therapy. Further investigation is underway on the targeted moieties and shielding systems for systematic delivery for tumor treatment *in vivo*.

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