

Full Length Research Paper

Synthesis and characterization of folate-poly(ethylene glycol) chitosan graft-polyethylenimine as a non-viral carrier for tumor-targeted gene delivery

Yi Zhou^{1#}, Jian-hai Chen^{1#}, He Wang², Cheng-xi Wang¹, Jian-ye Zhang³, Yi-wen Tao³, Guo-dong Zheng³, Hong-yan Xie³ and Yi Zhou^{3*}

¹Department of Pharmaceutical science, Nanfang Hospital, Southern Medical University 510515, China.

²Institute for Cancer Research, Southern Medical University, Guangzhou 510515, China.

³Department of Pharmaceutical science, Guangzhou Medical University, Guangzhou 510182, China.

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The use of chitosan and chitosan derivatives for gene delivery is limited due to the low transfection efficiency and difficulty in transfecting into a variety of cell types, including some cancer cells overexpressing folate receptor (FRs). In order to solve this problem, folate (FA) and poly(ethylene glycol) (PEG) was conjugated to chitosan-graft-polyethylenimine (CHI-g-PEI) to enhance water-solubility and the transfection efficiency. In the present study, a cell specific targeting molecule FA was linked on PEG and then grafted the FA-PEG onto CHI-g-PEI. The FA-PEG-grafted CHI-g-PEI (FA-PEG-CHI-g-PEI) effectively condensed the plasmid DNA (pDNA) into nanoparticles with positive surface charge under the suitable nitrogen/phosphorus (N/P) ratio. *In vitro*, transfection efficiency of the FA-PEG-CHI-g-PEI /pDNA complex in 293T cells and LoVo cells (FRs over-expressing cell lines) increased with increasing N/P ratio under N/P = 15 and was more than 50%, but no significant difference in human lung carcinoma cells (A549) cells (FRs deficient cell lines). Importantly, *in vivo* luciferase expression showed that the efficiency of FA-PEG-CHI-g-PEI-mediated transfection (50 µg luciferase plasmid (pLuc), N/P ratio = 15) was comparable to that of adenovirus-mediated luciferase transduction (1×10^9 pfu) in melanoma-bearing mice. It was concluded that FA-PEG-CHI-g-PEI, which has improved transfection efficiency and FRs specificity *in vitro* and *in vivo*, may be useful in gene therapy.

Key words: Folate poly(ethylene glycol)-chitosan-grafted-polyethylenimine (FA-PEG-CHI-g-PEI), gene transfection, non-virus vector, *in vitro*, *in vivo*.

INTRODUCTION

Gene therapy can be defined as the transfer of genetic

materials to specific cells in order to have a therapeutic effect. It is a promising approach to treat a wide range of diseases by producing bioactive agents or stopping abnormal functions in the cells (Huang, et al., 1999; Lieberman et al., 2002). Generally, two types of carriers such as viral and non-viral are used for the delivery of nucleic acids in gene therapy. Among the non-viral vectors, poly(ethylene imine) (PEI) was commonly applied to package plasmid DNA (pDNA) into nanoparticles for gene therapy. PEI offers the highest gene delivery efficiency among synthetic cationic polymers since it exhibits the unique "proton sponge effect" (Midoux et al., 2009). PEI has been shown to condense plasmids into colloidal particles which can effectively transfect a variety of cells, both *in vitro* and *in vivo*, due

*Corresponding author. E-mail: haifengteng88@163.com. Tel: +86 20 61642173. Fax: +86 20 61642173.

Abbreviations: FA, Folate; PEG, poly(ethylene glycol); FA-PEG-CHI-g-PEI, folate-poly(ethylene glycol)-chitosan-grafted-polyethylenimine; A549, human lung carcinoma cells; PBS, phosphate-buffered saline; DMSO, dimethylsulphoxide; MTT, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide; EDTA, ethylenediaminetetraacetic acid; N/P, nitrogen/phosphorus.

#These authors contributed equally to this work.

to its buffering capacity (Ooya et al., 2006). However, many studies have raised concerns regarding the toxicity of conventional PEI, which depends on its molecular weight; lower molecular weight PEI has a lower cytotoxicity (Kunath et al., 2003). Therefore, PEI needs structure modification for enhancing gene transfer efficiency and reducing cytotoxicity (Sun et al., 2010).

Chitosans, a family of linear binary polysaccharides containing β (1-4) linked 2-amino-2-deoxy- β -D-glucose (GlcN; D-unit) and the N-acetylated analogue (GlcNAc; A-unit) have been proposed as biocompatible alternative cationic polymers that are suitable for non-viral gene delivery (Koping-Hoggard et al., 2004). Wong et al. (2006) synthesized PEI-graft-chitosan by performing a cationic polymerization of aziridine to water soluble chitosan. The results indicated that PEI-g-chitosan had a lower cytotoxicity and showed higher transfection efficiency than PEI 25K both *in vitro* and *in vivo*. However, this system also has limited cell specificity and low water-solubility. Therefore, in this study, folate (FA) and poly(ethylene glycol) (PEG) modification was prepared to achieve better effect.

PEG modification (PEGylation), which could prolong the vector circulation in the bloodstream, becomes a well-established technique for drug delivery. The incorporation of the hydrophilic polymer PEG into polymeric gene carriers has showed to reduce the positive charge of self-assembling polymer/pDNA complexes and thus, improve the biocompatibility (Zeng et al., 2009). Furthermore, PEGylation could also provide a frame for other modifications, such as grafting a ligand for specific cell targeting. The cell-targeting ligands, including antibodies (Suh et al., 2001), growth factors (Toni et al., 2007), peptides (Shadidi et al., 2003) and folate (Hong et al., 2008), have been conjugated to the polymers. Among them, FA is a popular ligand for anti-cancer agents, because folate receptors (FRs) are often over-expressed in many cancer cells, but rarely found on the normal cell surface (Liang et al., 2008).

Therefore, in this study, we prepared FA-PEG-chitosan-graft-low molecular weight PEI (FA-PEG-CHI-g-PEI), which is not reported so far, to achieve better cancer cells specificity with FA receptor.

MATERIALS AND METHODS

Chitosan (Mw-255 kDa, viscosity: 200-800 cps in 1% acetic acid), FA, branched PEI (25 K and 1800 Da), N-hydroxysuccinimide (NHS), N,N-dicyclohexyl carbodiimide (DCC), (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and disuccinimidyl suberate (DSS) were purchased from Sigma-Aldrich (China). A hetero-bifunctional PEG derivative (NH₂-PEG-COOH, MW 4000) was purchased from NOF Corporation (Tokyo, Japan). Lipofectamine TM 2000 reagent was obtained from Invitrogen (China). Fetal bovine serum (FBS) was purchased from Gibco BRL (Grand Island, NY, USA). TransFast™ transfection reagent was obtained from Promega (Research Instruments, Singapore). Unless stated otherwise, all reagents and solvents were of biological grade

and were used without further purification. Green fluorescence protein expression was monitored by a fluorescence microscope (Olympus, IX71, Germany). Luciferase assay was performed by a microplate luminometer (LB 96V, EG&G, Berthold).

Animals

Normal nude mice (6 to 8 weeks) were obtained from animal experimental center of Southern Medical University. All experiments were in accordance with the recommendations of the European Union regarding animal experimentation (Directive of the European Council 86/609/EC).

Synthesis of FA-PEG-CHI-g-PEI

Folate-poly(ethylene glycol)-chitosan-grafted-polyethylenimine (FA-PEG-CHI-g-PEI) was synthesized by an amide formation reaction between activated carboxyl groups of folate-poly(ethylene glycol) (FA-PEG) and amine groups of chitosan-grafted-poly(ethylenimine) (CHI-g-PEI) in three steps. In the first step, the process of preparation of the NHS ester of folic acid is almost described by Singh et al. (2008). Meanwhile, the active ester of FA was prepared by a reported method (Figure 1a) (Lee et al., 1994). In the second step, CHI-g-PEI copolymer was synthesized in two steps following a modified method reported by Jiang et al. (2007). Then, the process of preparation of FA-PEG-CHI-g-PEI was followed: FA-PEG (0.1 mmol) dissolved in 10 ml of 2-(N-morpholino) ethanesulfonic acid (MES) buffer solution (0.1 M, pH 6.5) was activated with a mixture of NHS (1 mmol) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (1 mmol). After activating the carboxyl groups for 20 min, 0.001 mmol of CHI-g-PEI was added. The reaction was allowed to run for 12 h at 4°C followed by an additional 12 h at room temperature. The resulting product was purified using a dialysis tube (12,000, MWCO) against distilled water for 3 days. After dialysis, the copolymer was lyophilized. The reaction scheme was shown in Figure 1.

Characterization of polymers

The composition of the prepared FA-PEG-CHI-g-PEI copolymer was estimated by measuring ¹H nuclear magnetic resonance (¹H NMR) (AVANCE™ 600 FT-NMR, Bruker, Germany). The molecular weight of the FA-PEG-CHI-g-PEI copolymer was measured using a gel permeation chromatography (GPC) operating at a wavelength of a 365 nm (Dawn Eos, Wyatt, USA).

Gel retardation assay

In order to confirm the DNA condensation ability of the copolymers, electrophoresis was performed. Complex formation was induced at various N/P ratios from 1 to 10 in a final volume of 6 × agarose gel loading dye mixture (10 ul). Each mixture was vortexed and incubated for 30 min at room temperature and then analyzed on 1% agarose gel in 40 mM Tris-acetate, 1 mM EDTA (TAE) running buffer for 40 min at 80 V in a sub-cell system (Bio-Rad Laboratories, CA). The gel was stained with ethidium bromide (0.5 ug/ml) and the DNA bands were visualized and photographed by a UV transilluminator and BioDoc-It imaging system (UVP).

Preparation of plasmids

The plasmid pCAGLuc (a gift from Dr. He Wang, Institute for Cancer Research, Southern Medical University, Guangzhou, China)

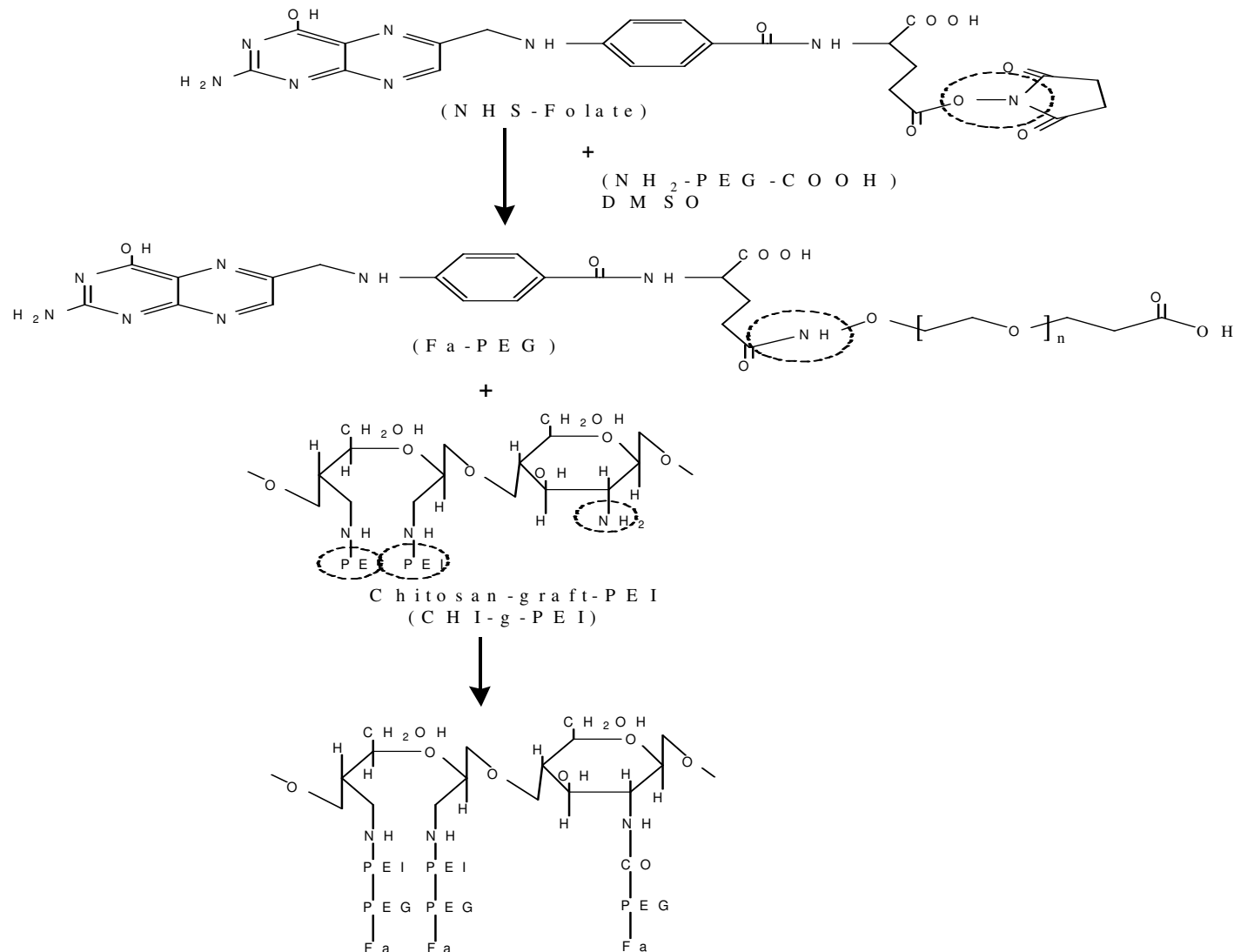


Figure 1. Proposed reaction scheme for synthesis of FA-PEG-CHI-g-PEI.

encoding firefly luciferase driven by a CAG promoter was amplified in *Escherichia coli* and purified using HiSpeed plasmid maxikit (Qiagen gmbH, Germany). The plasmid was quantified and qualified by PicoGreen assay (Molecular Probes, Inc., Eugene, OR) and by electrophoresis in 1% agarose gel, respectively.

Cell lines, culture and viability assays

Human embryonic kidney cell line (293T), human colonic cancer cell line (LoVo), human lung adenocarcinoma epithelial cell line (A549) and human cervical carcinoma cells (Hela) were cultured in Dulbecco's modified eagle medium (DMEM, Gibco BRL, Paris, France). All the media were supplemented with 10% fetal bovine serum (FBS, HyClone, Logan, Utah), streptomycin at 100 µg/ml and penicillin at 100 U/ml. Cells were incubated at 37°C in a humidified 5% CO₂ atmosphere. Cells were split using trypsin/ ethylenediaminetetraacetic acid (EDTA) medium prior to reaching confluency. *In vitro* cytotoxicity tests were performed with the cell titer 96[®] A Queous one solution cell proliferation assay (Promega, US). Cells were seeded in 96-well plates at an initial density of 1 ×

10⁴ cells/well in 0.2 ml of growth medium and incubated for 24 h to reach 80% confluency at treatment. Growth media were replaced by fresh, serum-free media containing various amounts of polymers or polymer/pDNA complexes at various N/P ratios (5, 10, 15, 20, 25 and 30). After an additional incubation for 24 h, the media were changed with growth media containing 20 µl of cell titer 96[®] aqueous one solution reagent. After further incubation for 3 h, the absorbance was measured at 570 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader (GLR 1000, Genelabs Diagnostics, Singapore) to obtain the metabolic activity of the cells.

Transfection protocol

The plasmid AAV-EGFP (1 µg) and the copolymer were mixed together with vortex. They were incubated for 30 min at room temperature. The original cell culture media were replaced with the complexes solution containing the complexes and an additional 200 µl serum-free DMEM for each well. They were incubated for 4 h at 37°C. Four hours later, the transfection media were changed with fresh and complete DMEM culture media. The cells were further

incubated for 20 h at 37°C and were observed under the fluorescence microscope (Nikon, Tokyo, Japan) equipped with the appropriate filter (Fluorescent filter slice). The fluorescent images were captured and recorded.

Toxicity assay

Sample cells were grown in 96-well plates at an initial density of 6000 cells/well. They were transfected with the same concentrations as that used for the 24-well plates. Transfection was performed using 150 ng of pDNA in 150 µl of serum-free growth medium. Each concentration was replicated in 3 wells. After incubation for 48 h, 150 µl of serum-free growth medium was replaced into the wells and 20 µl of 5 mg/ml MTT solution added. The cells were then incubated for about 4 h, before 100 µl of dimethylsulphoxide (DMSO) was added. After gentle agitation for 5 min, the absorbance at 570 nm of each well was recorded with the FLUOstar microplate UV spectrometer.

Reporter assay

The transfection efficiency of nanoparticles was evaluated by measuring the luciferase gene expression level (pGL3-luc plasmid). Cells were transfected with the complexes of various N/P ratios (from 5 to 30) at the same procedure in the 24-well plates in triplicate as previously described, while 1 µg pAAV-EGFP plasmid was replaced by 150 ng pGL3-luc plasmid in each well. After 48 h of incubation, the cells were washed with phosphate-buffered saline (PBS) and then 100 µl of lyses buffer was added to each well. The gene expression level in the lysates was evaluated using a luciferase assay kit (Promega, WI) with a luminometer (Lumat LB9501 instrument, Bad Wildbach, Germany). The data were expressed as relative light units (RLU) per well of total cell protein.

Flow cytometry

For flow cytometry, the plasmid pGL3-luc, was used to examine the GFP expression in HEK 293T, LoVo and A549 cells. All cells were respectively seeded in 6-well plates at density of 3×10^4 cells/well and incubated for being transfected. Two microgram (2 µg) of pGL3-luc-GFP plasmid in each well was performed to transfection and the N/P ratio of copolymers was 15. After 24 h, cells were washed with PBS and detached with 0.25% trypsin/EDTA. Subsequently, cells were resuspended in 500 µl of $1 \times$ Hank's balanced salt solution (HBSS, pH 7.4). Transfection efficiency was evaluated by scoring the percentage of cells expressing GFP using a FACS Aris™ system from Becton-Dickinson (San Joes, CA).

The transfection efficiency of FA-PEG-CHI-g-PEI in nudemice

Mouse melanoma LoVo cells in exponential growth phase were harvested and washed twice in PBS, resuspended in PBS at a density of 1×10^7 cells/ml and injected subcutaneously (0.1 ml injection volume; 1×10^6 cells) at the right dorsal flank of nudemice. When the tumor size reached 0.3 cm in diameter, mice were injected with FA-PEG-CHI-g-PEI / pLuc polyplexes (50 µg plasmid DNA, N/P ratio = 15), pLuc plasmid alone (50 µg) or rAdv-Luc (1×10^9 pfu) by intratumoral injection, respectively. Each treatment group was composed of five mice ($n = 5$). Mice were scarified by CO₂ inhalation and perfused by ice-cold PBS via cardiac puncture. Tumor tissues were then, extracted and homogenized in lysis buffer (Promega, Madison, WI) on ice. The homogenates were centrifuged and the luciferase activities in the supernatants were assayed by a luminometer. The following processes were the same as the

luciferase assay as described in the recommended protocol from the manufacturer (Technical bulletin No. 281, Promega). Briefly, cells were washed with TBS and lysed by the addition of 100 ml of cell culture lysis buffer (Promega, Madison, WI). The cell lysates from each well (20 ml) were used for luciferase activity assay with the Promega's luciferase reagent. Light units were measured with a microplate luminometer LB 96V (EG&G, Berthold). Protein assay kit (Bio-Rad, 500-0122) was used to determine the protein concentrations of cell lysates.

Luciferase activity (RLU/mg) = value of relative light unit (RLU)/ 20 ml total protein quantity (mg).

Statistical analysis

Statistical analysis was performed using Student's t-test (GraphPad Software, San Diego, CA). Data were expressed as mean \pm standard deviation (SD). Statistical significance was represented by * $P < 0.05$ and ** $P < 0.01$.

RESULTS AND DISCUSSION

Synthesis and characterization of the FA-PEG-CHI-g-PEI copolymer

Chitosan, is a biocompatible, biodegradable and low-toxicity material with high cationic potential, combined low-molecular-weight PEI and proved to be effective in the delivery of the genes into cells owing to the synergistic advantages of both PEI and cationic polymer (Kubota et al., 2000; Park et al., 2005). The molecular weight of CHI-g-PEI (24.99 kDa) in our results was higher than that of 25K PEI, which was used as a control (Remy et al., 1998). Due to commercial chitosan with low water-solubility, PEGylation of CHI-g-PEI can be effective to increase water-solubility and showed low-toxicity.

FA-PEG-CHI-g-PEI copolymer was successfully synthesized in our Laboratory as shown in Figure 1. The composition of the synthesized copolymer was analyzed by ¹H NMR (Figure 2). The copolymer showed signal at 3.8 ppm ((-CH₂CH₂O-)n) for PEG and signal at 1.8 (-OCCH₃) ppm for chitosan. FA-PEG-CHI-g-PEI show signals at 6.8, 7.7 and 8.7 ppm for FA. These results are consistent with the expected chemical structure of the copolymers. The proton peaks of PEI (-NHCH₂CH₂-) appeared at 3.3 to 2.5 ppm, indicating that PEI was grafted to the chitosan chain.

Complex formation between FA-PEG-CHI-g-PEI and CHI-g-PEI and plasmid DNA

As well as agarose gel electrophoresis, particle size and zeta potential demonstrated the ability of the cationic polymer to condense pDNA into particulate structures. The formation of polymer/pDNA complexes is an important prerequisite for gene delivery using cationic polymers. In this study, the complexation of cationic

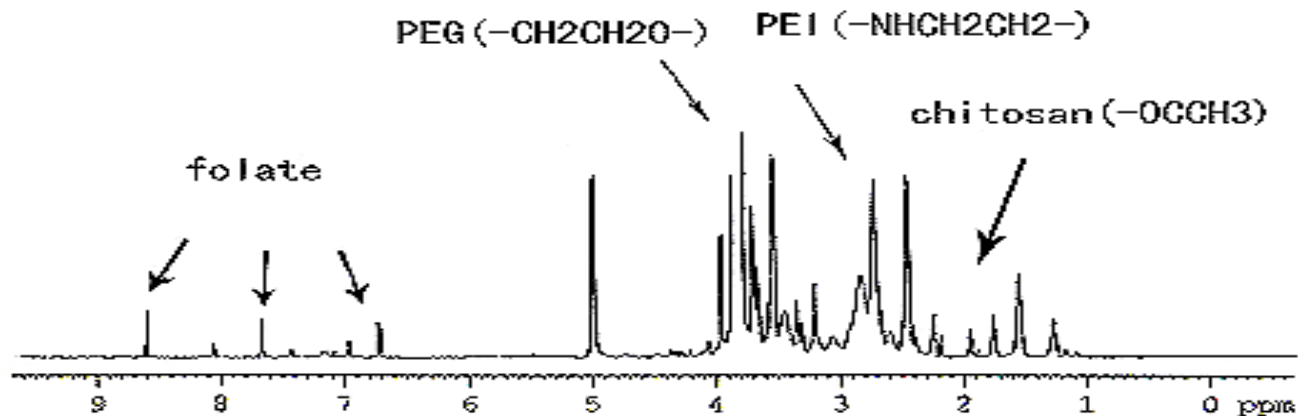


Figure 2. Representative ^1H NMR spectrum of FA-PEG-CHI-g-PEI in D_2O : $\delta=3.9-3.5$ ($-\text{CH}_2\text{CH}_2\text{O}-$, PEG ethylene), $3.3-2.5$ ($-\text{NHCH}_2\text{CH}_2-$, PEI ethylene) and 6.8 (d, $3'$, $5'$ -H, 2H), 7.7 (d, $2'$, $6'$ -H, 2H), 8.7 (s, C7-H, 1H) ppm for FA.

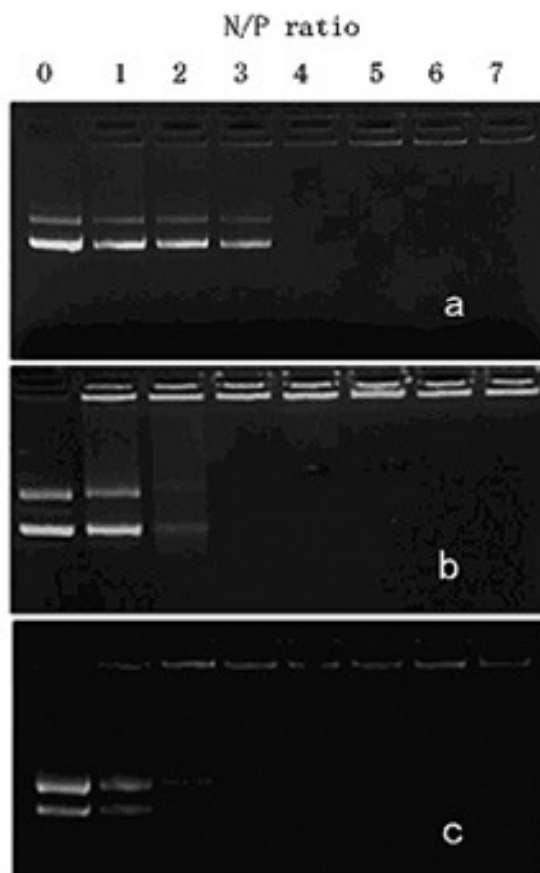


Figure 3. Electrophoretic mobility of plasmid DNA in the complexes between FA-PEG-CHI-g-PEI (a); CHI-g-PEI (b); pDNA in comparison with 25K PEI/pDNA (c) complex at various N/P ratios.

polymer with DNA was analyzed using agarose gel electrophoresis. Figure 3 showed the gel retardation results of cationic polymer/ pDNA complexes with

increasing N/P ratios in comparison with branched PEI-25 KDa/DNA complex. In comparison with PEI-25 KDa, FA-PEG-CHI-g-PEI and CHI-g-PEI could compact pDNA

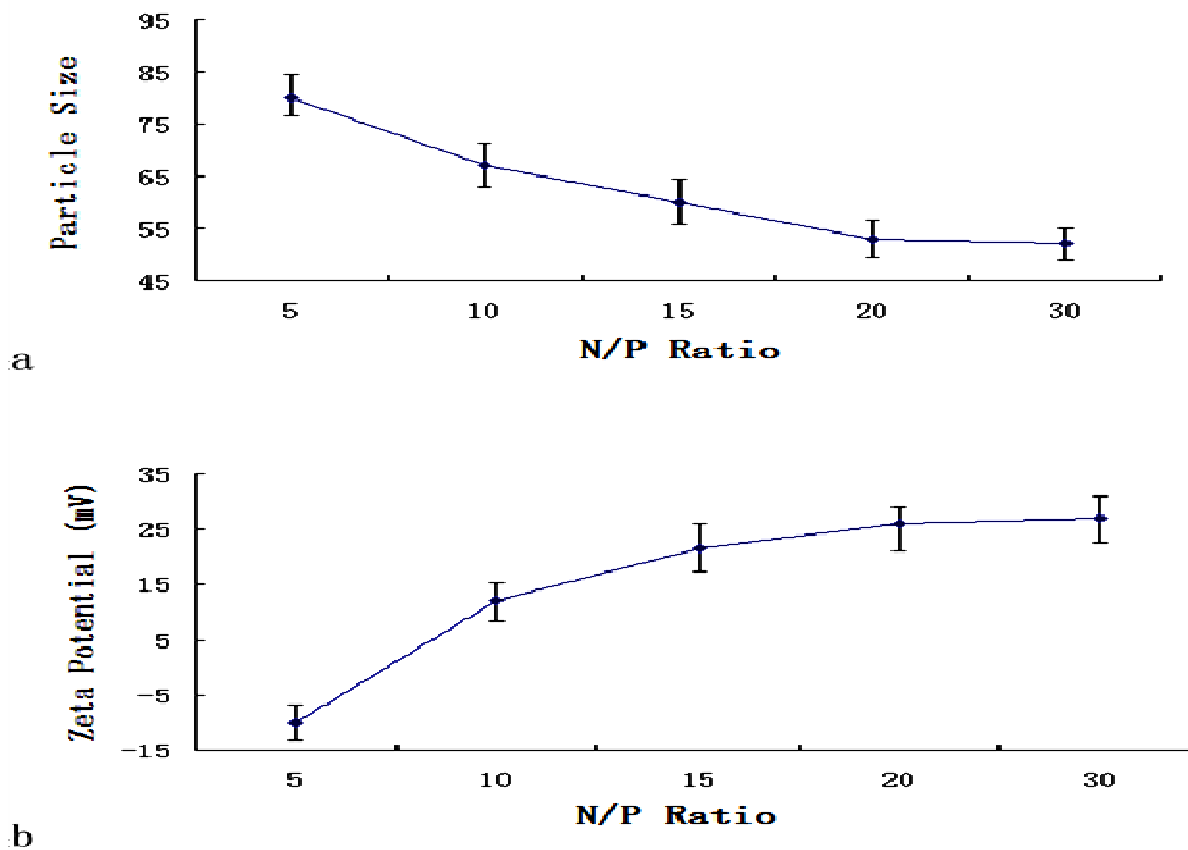


Figure 4. Particle size (a) and zeta potential (b) of FA-PEG-CHI-g-PEI/pDNA complex at various N/P ratios by dynamic light scattering.

entirely at the higher N/P ratio of 3 to 4, indicating that these polymers have slightly lower DNA condensation ability than PEI-25 kDa.

Figure 4 showed the effect of particle sizes and zeta-potential of FA-PEG-CHI-g-PEI/DNA complexes. In Figure 4a, FA-PEG-CHI-g-PEI could efficiently compact pDNA into small nanoparticles. The particle size of cationic copolymer/pDNA complexes decreased with an increase in the N/P ratios, that is all complexes were not more than 85 nm, on the contrary, zeta-potential of the complex were increased with the addition of N/P ratios. After the N/P ratio reached 15, the zeta potential of the complexes was positive and varies within the same range (20 to 30 mV), which results in a similar affinity for cell surface. The results show that the hydrophilic groups of the FA-PEG residue prevented aggregation of the complexes in Figure 4b.

Cytotoxicity of FA-PEG-CHI-g-PEI

For the concerns of efficient gene delivery and biocompatibility, copolymer should exhibit minimal cytotoxicity. To investigate the cytotoxicity of the FA-PEG-CHI-g-PEI

copolymer, HeLa cell line was tested at various concentrations of the copolymer (Figure 5a). Cells incubated with pure media without treatment were considered as controls and the cell viability values were set as 100%.

Cell viability decreased drastically with increasing concentration of PEI 25 KDa, whereas the FA-PEG-CHI-g-PEI and CHI-g-PEI did not exhibit cytotoxicity even at high concentration (130 $\mu\text{g/ml}$), which is highly toxic level for PEI in the texts (Jin et al., 2008; Davaran et al., 2008). Therefore, we assayed this novel copolymer of this concentration and the results showed no cytotoxicity in HeLa. The cell viabilities of FA-PEG-CHI-g-PEI/pDNA and CHI-g-PEI/pDNA complexes were also assayed at various N/P ratios (Figures 5b, c and d). When the N/P ratio was less than 20, FA-PEG-CHI-g-PEI and PEG-CHI-g-PEI copolymers showed significantly higher cell viability than 25 KDa PEI. Although there is difference of toxicity between FA-PEG-CHI-g-PEI and CHI-g-PEI in HeLa, it was not significant, because PEG which is known to help reduce the cytotoxicity of PEI amino groups (Jiang et al., 2007; Joung et al., 2008), resulted in lower cytotoxicity of FA-PEG-CHI-g-PEI.

The cytotoxicity of cationic polymers is likely due to

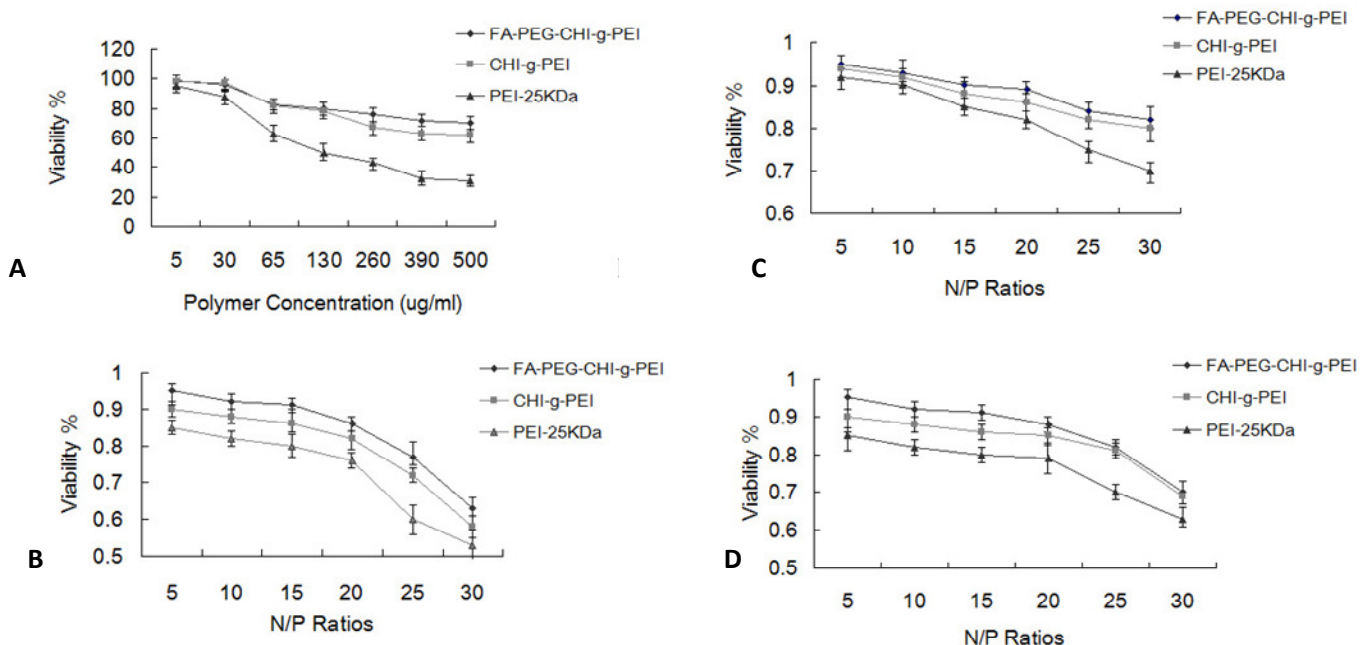


Figure 5. Cytotoxicity induced by the copolymer of a variety of concentrations in (a) HeLa cells (mean \pm SD, $n=3$). Cytotoxicity induced after transfection of CHI-g-PEI/pDNA, FA-PEG-CHI-g-PEI/DNA complexes into (b) 293T (c) LoVo, (d) A549 cells (mean \pm SD, $n=3$). X-axis indicates the polymer concentrations and the N/P ratios of the copolymers/pDNA complexes, while Y-axis shows the cell percentile viability.

polymer aggregation on cell surfaces, impairing important membrane function. Also, cationic polymers may interfere with critical intracellular processes: in particular, the primary amine was reported to disrupt PKC function through disturbance of protein kinase activity (Joung et al., 2008). So, PEI, especially, large molecular weight PEI, can exhibit higher cytotoxicity than other biodegradable materials. Modified vectors of which zeta potential is reduced can effectively decrease cytotoxicity. Concurrently, it was found that the toxicity of FA-PEG-CHI-g-PEI/pDNA and CHI-g-PEI/pDNA in HeLa was almost similar to other cell lines (293T, LoVo and A549) and also at various N/P ratio. Compared with the CHI-g-PEI and 25 KDa PEI copolymer, the over-expression of FA receptor on the surface of 293T and LoVo cells may significantly enhance the uptake of the FA-PEG-CHI-g-PEI copolymer via FA receptors mediated endocytosis and not result in higher cytotoxicity (Figures 5b, c and d).

Transfection efficiency

In vitro gene transfection efficiency and transgene expression level of complexes was visualized by observation of EGFP positive cells using a fluorescence microscope. The highest transfection efficiency and strongest fluorescent density were shown when cells were transfected with FA-PEG-CHI-g-PEI / pAAV-EGFP complexes while the lowest transfection efficiency and

the weakest fluorescent density were observed when cells were transfected with 25K PEI/ pAAV-EGFP copolymer complexes for 293T and LoVo cells (Figure 6). Moreover, transfection efficiency of FA-PEG-CHI-g-PEI/DNA complexes was obviously higher than the CHI-g-PEI/pDNA and 25 KDa PEI I/pDNA complexes in 293T, LoVo and A549, indicating the receptor-mediated endocytosis through FA moiety. But for A549 cells (FA-negative receptor), three complexes were taken up by A549 cells. These results indicated that folate receptor-mediated endocytosis may contribute to the high transfection efficiency mediated by FA-PEG-CHI-g-PEI. The mechanism of folate-mediated targeting of nanoparticles to FRs and the subsequent cellular uptake is perceived to be the same as that for the free folate, which is internalized into the cells via receptor-mediated endocytosis (Leamon and Reddy, 2004). The same reason can also be given to explain the discrepancy of the transfection efficiency of FA-PEG-CHI-g-PEI in various tumor cell lines.

The optimal N/P ratio of FA-PEG-CHI-g-PEI/pDNA complexes has contributed to transfection efficiency. Due to the higher transfection efficiency of FA-PEG-CHI-g-PEI/pDNA complexes, which is relative to higher amine content in the complex context of security, it similarly allows the complexes to escape the endosome easily due to a higher buffering capacity. In our experiment, A549 cells (FA-negative receptor) were transfected with complexes prepared at different N/P ratio (Figure 7a).

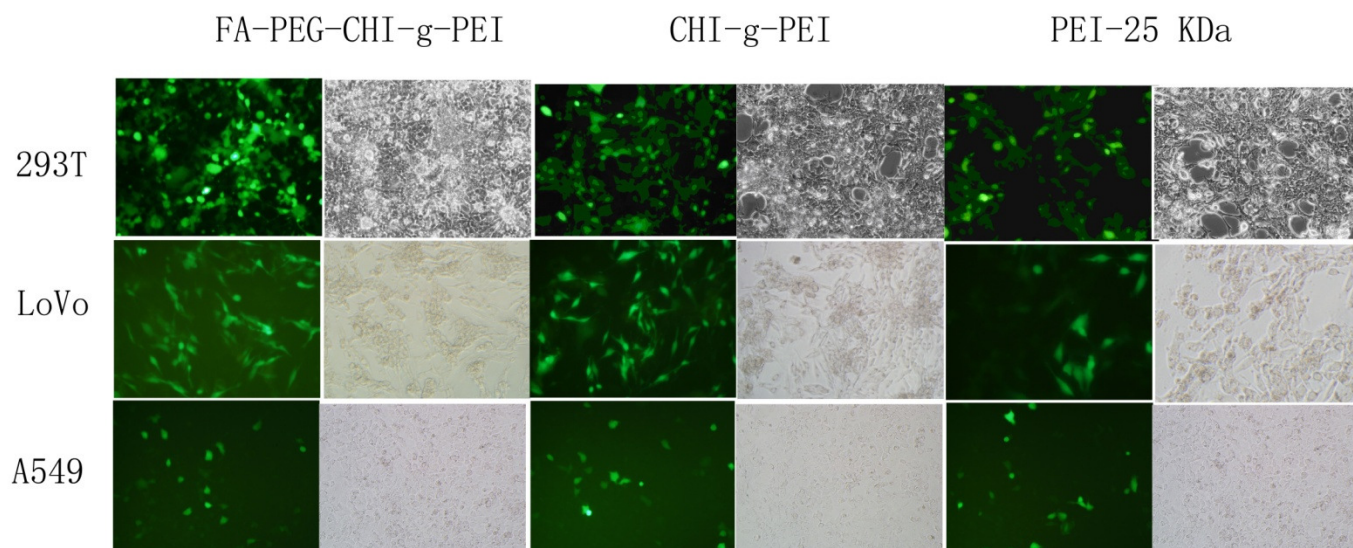


Figure 6. Fluorescent images of 293T cells, LoVo cells and A549 cells after transfection of FA-PEG-CHI-g-PEI/pEGFP, CHI-g-PEI/pEGFP, and PEI-25 KDa /pEGFP complexes at N/P = 15.

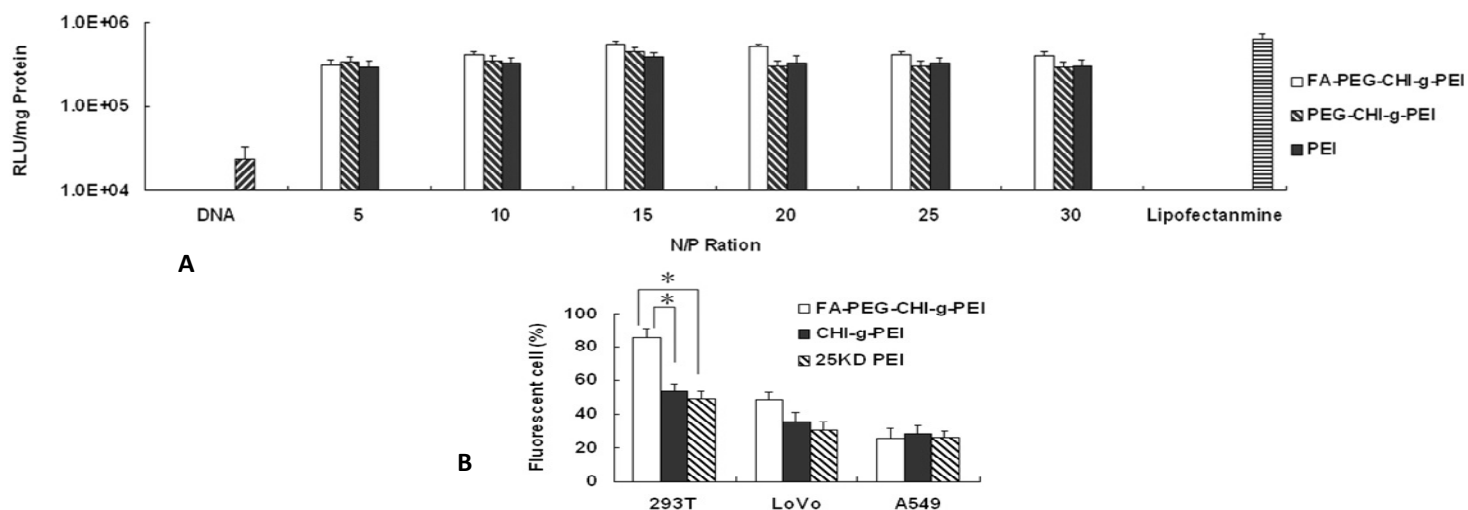


Figure 7. (A) Transfection efficiency of FA-PEG-CHI-g-PEI/pEGFP, CHI-g-PEI/ pEGFP and 25 KDa PEI/pEGFP complexes in A549 ($n=3$, error bars represent standard deviation); (B) transfection efficiency of FA-PEG-CHI-g-PEI/pEGFP, CHI-g-PEI/pEGFP and PEI-25KDs / pEGFP complexes by FACS analysis in 293T cells, LoVo cells and A549 cells at N/P=15. Compare with CHI-g-PEI and 25 KDa PEI, transfection efficiency of FA-PEG-CHI-g-PEI was significantly increased ($*P < 0.001$, $n = 3$, Student's *t*-test). Transfection was performed at a dose of 1 ug of DNA (mean \pm SD, $n = 3$). Y-axis shows the fluorescent cells.

The transfection efficiency of FA-PEG-CHI-g-PEI/pDNA and CHI-g-PEI/pDNA complexes increased with an increasing N/P ratio. The transfection efficiency of the three complexes was respectively reduced when N/P ratio overrides 15. In other words, the maximal transfection efficiency of FA-PEG-CHI-g-PEI/pDNA was shown at an N/P ratio of 15.

To further demonstrate transfection efficiency, flow cytometry assay was performed in the process of transfection of all complexes (Figure 7b). In our result, when

293T cells were respectively transfected with FA-PEG-CHI-g-PEI, PEG-CHI-g-PEI, 25 KDa PEI/pGFP complexes, the ratio of EGFP-positive cells is 85, 56 and 45%. Interestingly, compared with PEG-CHI-g-PEI and PEI-25 KDa, the transfection efficiency of FA-PEG-CHI-g-PEI significantly increased ($p < 0.001$) in 293T cells. Meanwhile, the transfection efficiency of FA-PEG-CHI-g-PEI, CHI-g-PEI, 25 KDa PEI/pGFP complexes in LoVo cells was 58, 45 and 35%, respectively. Consistent with previous results, no obviously difference in transfection

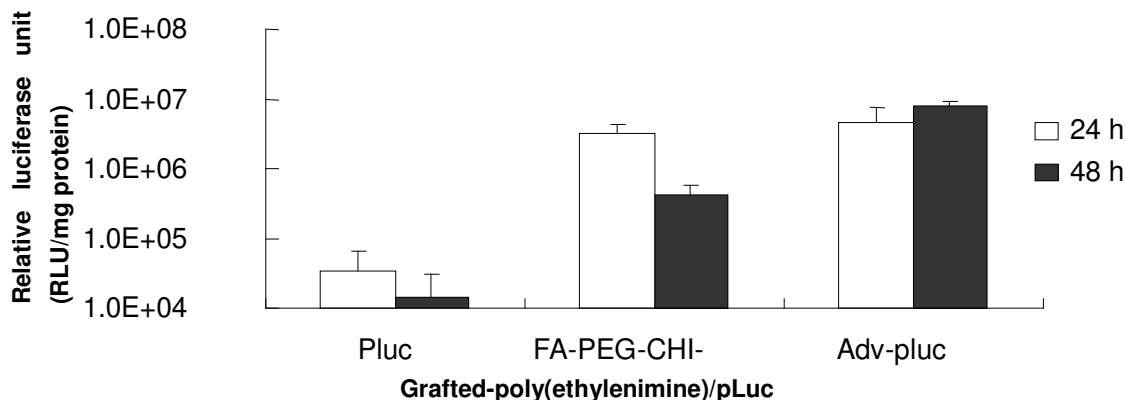


Figure 8. Determination of relative luciferase unit (RLU) of tumor tissue homogenates at 24 and 48 h after transfection by FA-PEG-CHI-g-PEI *in vivo*. The level of luciferase expression induced by FA-PEG-CHI-g-PEI/pLuc transfection was similar to that mediated by rAdv-Luc transduction at 24 h.

efficiency was discovered when A549 cells were transfected with the three copolymer/pDNA complexes.

The transfection efficiency and transgene expression level are the most important features of copolymers. The previous report demonstrated that the transfection efficiency of PEI-25 kDa is in a dose dependant manner, where as PEI reached optimum at N/P ratio of 15 and further decreased till N/P ratio of 30 because of the toxicity of PEI at high concentrations (Hima et al., 2007). Compared with other copolymers, the results of EGFP-emitted fluorescence by FA-PEG-CHI-g-PEI /pAAV-EGFP were obviously increased when the N/P ratio was 15; indicating FA-PEG-CHI-g-PEI /pAAV-EGFP possessed the highest transfection efficiency in the 293T and LoVo cells than other copolymers. The fluorescent spots were markedly reduced at a lower N/P ratio and as well as value over 15. Either the transfection efficiency of copolymers or the fluorescence density decreased with an increase in N/P ratios (data not shown). Furthermore, FA-PEG-CHI-g-PEI exhibited higher transfection efficiency than CHI-g-PEI in FRs over-expressing 293T and LoVo cells, but no difference in FRs-negative A549 cells. These results indicate that CHI-g-PEI and FA-PEG-CHI-g-PEI could be valuable gene delivery agents for cancer gene therapy.

The transfection efficiency and toxicity of FA-PEG-CHI-g-PEI *in vivo*

To evaluate the dynamic change of gene expression over time in transfected tumor cells *in vivo*, nude mice bearing LoVo tumors were injected intratumorally with pLuc (50 mg), FA-PEG-CHI-g-PEI /pLuc (DNA 50 mg, N/P ratio 15:1) or rAdv-Luc (1×10^9 pfu). Tumor tissue homogenates were prepared from pLuc, FA-PEG-CHI-g-PEI /pLuc and rAdv-Luc-injected tumor masses, respectively, at 24 and 48 h post injection. At 24 h, the luciferase

activity in FA-PEG-CHI-g-PEI /pLuc-injected tumor was almost 100-fold higher than that in pLuc-injected tumor and it was in the same order of magnitude compared with that in rAdv-luc-injected tumor (Figure 8). At 48 h post-injection, the luciferase activity in FA-PEG-CHI-g-PEI /pLuc-injected tumor was reduced 8-fold and that in rAdv-Luc-injected tumor was further increased by more than 17-fold.

Viral vectors with high transfection efficiency have been an important goal in gene delivery. Adenovirus can be used as a standard for comparing the efficiency of non-viral synthetic gene delivery vectors, because adenovirus has been one of the most commonly used vectors in gene therapy among various viral vectors. In this study, it was found that the luciferase activity of FA-PEG-CHI-g-PEI /pLuc-mediated transfection was 100 times higher than that of pLuc alone and was almost comparable to that of rAdv-Luc (1×10^9 pfu) in LoVo melanoma-bearing nude mice at 24 h postintra-tumoral injection. Similar results were observed in assaying the luciferase activity in the tissue homogenates prepared from the injected melanoma xenografts. Thus, it was concluded that the transfection efficiency mediated by a non-viral gene delivery vector can achieve a transgene expression level similar to that mediated by a recombinant adenoviral vector.

In this paper, it was shown that the PEG conjugated CHI-g-PEI copolymer has been designed for targeted delivery DNA for cancer treatment. FA-PEG conjugated CHI-g-PEI showed a narrow size distribution and lower zeta-potential after to condense the DNA in our results. Meanwhile, the copolymer showed low cytotoxicity and exhibited high FA-media receptor specificity *in vitro* when compared with 25 kDa PEI and CHI-g-PEI. Importantly, FA-PEG-CHI-g-PEI showed high transfection efficiency *in vivo*. It could, therefore, be concluded that the FA-PEG-CHI-g-PEI has a potential to be a safe and efficient gene carrier.

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