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Aerosol delivery of spermine-based poly(amino ester)/Akt1 shRNA complexes for lung cancer gene therapy

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ABSTRACT

Polyethylenimine (PEI) has been commonly used as a cationic polymeric gene carrier due to high transfection efficiency, however, its cytotoxicity has hindered the practical application. In this study, we report the development of poly(amino ester) (PAE) based on glycerol propoxylate triacrylate (GPT) and spermine (SPE) as an alternative gene carrier for lung cancer therapy. GPT–SPE copolymer was prepared by Michael addition reaction between GPT and SPE, and the efficacy was evaluated using shAkt1 as a model therapeutic gene. The molecular weight and composition were characterized using gel permeability chromatography (GPC) and ^{1}H-nuclear magnetic resonance (^{1}H-NMR), respectively. The GPT–SPE could effectively condense DNA with about 163 nm size and protect the DNA from nucleases. GPT–SPE/DNA complexes showed excellent transfection with low toxicity both *in vitro* and *in vivo*. Furthermore, aerosol delivery of GPT–SPE/Akt1 shRNA complexes significantly suppressed lung tumorigenesis in K-*ras*^{LA1} lung cancer model mice. These results suggest that GPT–SPE can be used in shRNA-based lung cancer gene therapy.

1. Introduction

Gene therapy possesses tremendous potential for successful treatment of genetically based diseases, such as cancer (Merdan et al., 2002). Although gene therapy holds promise, progress in developing effective clinical protocols has been quite slow due to the lack of safe and efficient gene carriers (Gao et al., 2003; Jiang et al., 2007b). While most gene therapy protocols in present clinical trials employ recombinant viral vectors, safety concerns have led to the pursuit of non-viral alternatives (Jiang et al., 2007a). Non-viral vectors have attracted great interest, as they are simple to prepare, rather stable, easy to modify and relatively safe, compared to viral vectors (Lungwitz et al., 2005).

Polyethylenimine (PEI) is one of the widely used gene carriers which have become the gold standard of non-viral vector due to its superior transfection efficiency by high buffering capacity and consistency in transfection in many different types of cells (Boussif et al., 1995, 1996; Park et al., 2006). However, it has been reported that PEI is cytotoxic although the cytotoxicity of PEI is dependent on its molecular weight; lower molecular weight PEI has lower cytotoxicity (Fischer et al., 1999; Jiang et al., 2007a). On the other hand, the molecular weight of PEI must also be of at least 2 kDa, otherwise they are unable to form polyplexes of sufficient stability to protect the plasmid DNA from premature degradation by nucleases (Drake et al., 2010). Therefore, a number of different biodegradable bonds have been incorporated into low molecular weight PEI using various cross-linkers such as PEG diacrylate- (Arote et al., 2007; Park et al., 2005), glycerol dimethacylate- (Arote et al., 2008) glycerol triacrylate- (Arote et al., 2009), and disulfide-based reagents

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(Gosselin et al., 2001, 2002; Jere et al., 2009) to reduce cytotoxicity and increase transfection efficiency. However, these low molecular weight PEI-based polymers can be degraded into various new fragments, which may associate with additional safety issues. Furthermore, PEI is not easily biodegradable in cellular space and the presence of free PEI plays a significant role in disruption, permeability and modification of cell membrane as well as induction of mitochondria-mediated apoptotic program (Hunter, 2006; Moghimi et al., 2005). Therefore, biocompatible human endogenous amino compounds should be considered instead of PEI.

Spermine (SPE) is safe and naturally present in body tissues, is involved in cellular metabolism and present in all eukaryotic cells (Allen, 1983; Jiang et al., 2011). Also, SPE is a biogenic tetraamine with two primary and two secondary amino groups that allow the reaction with acrylate group by Michael addition reaction. Furthermore, polyspermine had high buffering capacity (Jere et al., 2009). Therefore, in this study, we prepared poly(amino ester) (PAE)-based on glycerol propoxylate triacrylate (GPT) and SPE as a biocompatible alternative gene carrier for lung cancer therapy. The GPT was selected as a cross-linking reagent because the acrylate group of GPT not only could react with SPE by Michael addition reaction but also the hydroxyl groups of GPT could stabilize the GPT–SPE/DNA complexes in *in vivo* situation. The physicochemical properties of GPT–SPE/shRNA complexes were analyzed, and their cellular uptake, transfection efficiency, and toxicity were characterized. Also, *in vivo* transfection efficiency and toxicity were also investigated after aerosol delivery. Furthermore, tumor-suppressing ability of GPT–SPE/Akt1 shRNA was also evaluated in K-*ras*^{LA1} lung cancer model mice.

2. Materials and methods

2.1. Materials

GPT (MW 428 Da), SPE (MW: 202.34 Da) and anhydrous ethanol were purchased from Sigma–Aldrich (St. Louis, MO, USA). The 5.3 kb pGL3 expression vector with driven by an SV40 promoter, and enhancer encoding firefly luciferase and Cell Titer 96® A_{queous} One Solution Reagent were purchased from Promega (Madison, WI, USA). Plasmids were propagated in *E. coli*, extracted by alkaline lysis, and purified with a QIAGEN kit (Chatsworth, CA, USA). The cassette of oligonucleotides encoding 19-mer hairpin sequences specific to target Akt1 mRNA was designed. The targeted Akt1 mRNA sequence was GAAGGAAGTCATCGTGGCC. A scrambled siRNA with same nucleotide composition as the siRNA but which lacks significant sequence homology to the genome was also designed. Antibody for Akt1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemicals were reagent grade or better.

2.2. Preparation of GPT–SPE

The GPT–SPE copolymer was synthesized according to modified Michael addition reaction method as reported previously (Arote et al., 2009). Briefly, SPE (2.5 M) and GPT (1.5 M) were separately dissolved in 2 ml of anhydrous ethanol at 4 °C, and GPT solution was slowly added to SPE solution with stirring at 4 °C for 2 h and at room temperature for overnight. The obtained polymer was dissolved in distilled water at 4 °C. After dialysis using Spectra/Por® membrane (MWCO = 3500) against distilled water at 4 °C for 24 h, the polymer was lyophilized and stored at −20 °C.

2.3. Characterizations of copolymer and GPT–SPE/shRNA complex

The composition of prepared GPT–SPE was estimated by measuring ^{1}H-nuclear magnetic resonance (^{1}H-NMR) using Avance™ 600 spectrometer (Bruker, Germany). For measurement of ^{1}H-NMR, polymers were dissolved in D_2O at concentration of 10 mg/ml. The molecular weight of the polymer was measured by gel permeation chromatography (GPC) with 690 nm laser wavelength (DAWN Eos, Wyatt, Santa Barbara, CA, USA).

All GPT–SPE/DNA complexes was freshly prepared before use and characterized by method of Jiang et al. (2007a). Complexes were prepared by adding DNA solution to equal volume of polymer solution, vortexing gently and incubating at room temperature for 30 min. The DNA condensation and protection abilities of the polymer were confirmed by electrophoresis. The morphology of GPT–SPE/DNA complexes was observed by energy-filtering transmission electron microscope (EF-TEM, LIBRA 120, Carl Zeiss, Germany). The size (scattering angle: 90°) and surface charge (scattering angle: 20°) of the GPT–SPE/DNA complexes were measured at 25 °C using an electrophoretic light scattering spectrophotometer (ELS 8000, Otsuka Electronics, Osaka, Japan).

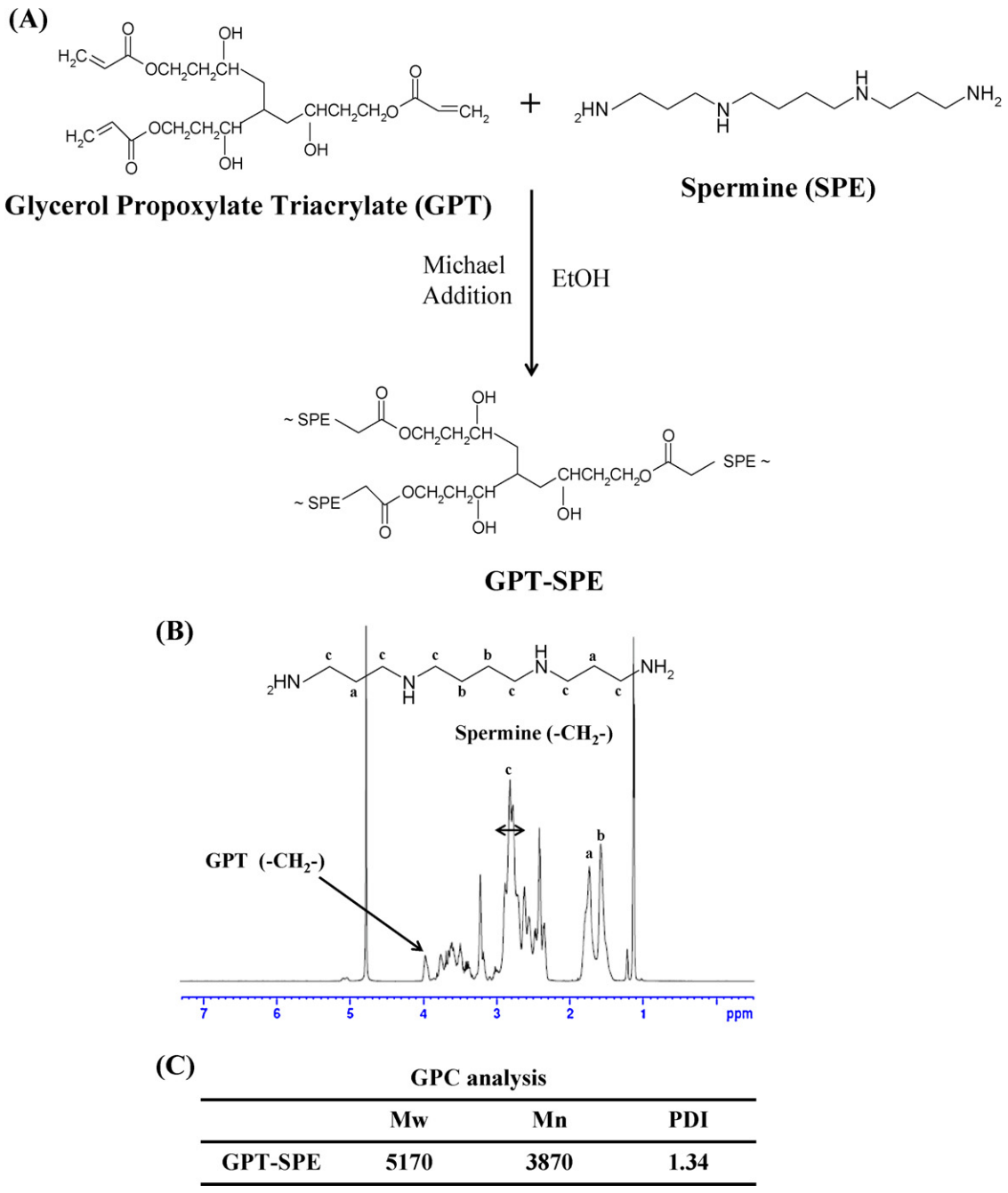
2.4. Cell viability and transfection efficiency

A549 (human lung carcinoma, ATCC, Manassas, VA, USA) cells were cultured in RPMI 1640 (Gibco-BRL Invitrogen, Grand Island, NY, USA) and HepG2 (human hepatoblastoma, ATCC), MCF-7 (human breast cancer, ATCC), and HeLa (human cervix epithelial carcinoma, ATCC) cells were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco-BRL Invitrogen), supplemented with 10% fetal bovine serum (FBS, HyClone, Logan, UT, USA), streptomycin at 100 μg/ml, and penicillin at 100 U/ml. All cells were incubated at 37 °C in humidified 5% CO_2 atmosphere. Cells were split, using trypsin/EDTA media when almost confluent.

In vitro cell viability and transfection efficiency studies were performed according to previously reported method (Jiang et al., 2007a). *In vitro* cytotoxicity tests were evaluated by Cell Titer 96® A_{queous} One Solution Cell Proliferation Assay (Promega, Madison, WI, USA). Briefly, cells were seeded in 96-well plates at an initial density of 1×10^4 cells/well in 0.2 ml of growth medium and incubated for 18 h prior to the addition of filtered polymers. Growth media was replaced by fresh, serum-free media, containing various amounts of polymers. After additional incubation for 24 h, the media were changed with growth media containing 20 μl of Cell Titer 96® A_{queous} One Solution Reagent. After further incubation for 3 h, the absorbance was measured at 570 nm, using an ELISA plate reader (GLR 1000, Genelabs Diagnostics, Singapore). *In vitro* transfection efficiency studies were evaluated by checking luciferase activity assay. Briefly, cells were seeded in 24-well plates at an initial density of 10×10^4 cells/well in 1 ml of growth medium. After incubation for 18 h, the media were replaced with serum-free or 10% serum-containing media with polymer/pGL3 (1 μg) complexes at various weight ratios and additionally incubated for 4 h. Then the media was exchanged by fresh media, containing serum, and allowed to incubate for 24 h at 37 °C. The luciferase activity assay was performed according to the manufacturer's protocol.

2.5. Aerosol delivery studies

Six-week-old female BALB/c mice (Breeding and Research Center, Seoul National University, Korea) were used following the policy and regulations for the care and use of laboratory animals (Laboratory Animal Center, Seoul National University , Korea). Animals were kept in the laboratory animal facility with temperature and relative humidity maintained at 23 ± 2 °C and 50 ± 20%, respectively, and 12 h light/dark cycle. For gene delivery, mice were placed in a nose-only exposure chamber and exposed to the aerosol based on the methods used previously (Jiang et al., 2009a). Four mice were randomly divided into three groups.



	Mw	Mn	PDI
GPT-SPE	5170	3870	1.34

Fig. 1. GPT–SPE copolymer synthesis and characterizations. (A) Proposed reaction scheme of GSC. (B) Representative ^{1}H-NMR spectrum of GSC in D_2O: δ = 1.6 (a) and δ = 1.8 ppm, (b) (–CH_2–, spermine), δ = 2.6–2.8 ppm, (c) (–NH–CH_2–, spermine), δ = 3.9–4.0 ppm (–CH_2–, GPT). (C) GPC analysis. Mw: weight average molecular weight, Mn: number average molecular weight, PDI (polydispersity index) = Mw/Mn, PDI indicates the distribution of individual molecular masses in a batch of polymers.

Treatment groups were exposed to aerosol containing GFP plasmid with or without GPT–SPE carrier, and one remaining group was used as an untreated control. Two days after exposure, mice were sacrificed and lungs were collected for the detection of GFP signal. Lung tissue was fixed at room temperature and embedded in Tissue-Tek OCT (Sakura, Torrance, CA, USA). Ten micrometers of tissue cryosections were cut with a microtome (Leica, Nussloch, Germany) and mounted on slides for analysis. Also, lung, liver, heart, spleen, kidney, brain were collected and fixed in 10% neutral buffered formalin for histopathological examination.

Six-week-old female K-*ras*^{LA1} mice, murine model of human non-small cell lung cancer, mice were obtained from Human Cancer Consortium-National Cancer Institute (Frederick, MD, USA) were divided into 3 groups (4 mice/group) and exposed to aerosol. The control group was untreated and the other groups were exposed to aerosol containing 8 mg of GPT–SPE and 0.8 mg of shRNA (scrambled or Akt1 shRNA) in distilled water. Mice were exposed to aerosol twice a week for 4 weeks. At the end of experiment, the mice were sacrificed and neoplastic lesions of lung surfaces were carefully counted under microscope. Simultaneously, the lungs were perfused with PBS, and a lobe of the left lung was collected and fixed in 10% neutral buffered formalin for histopathological examination. The remaining lungs were stored at −80 °C for further study. All methods used in this study were approved by the Animal Care

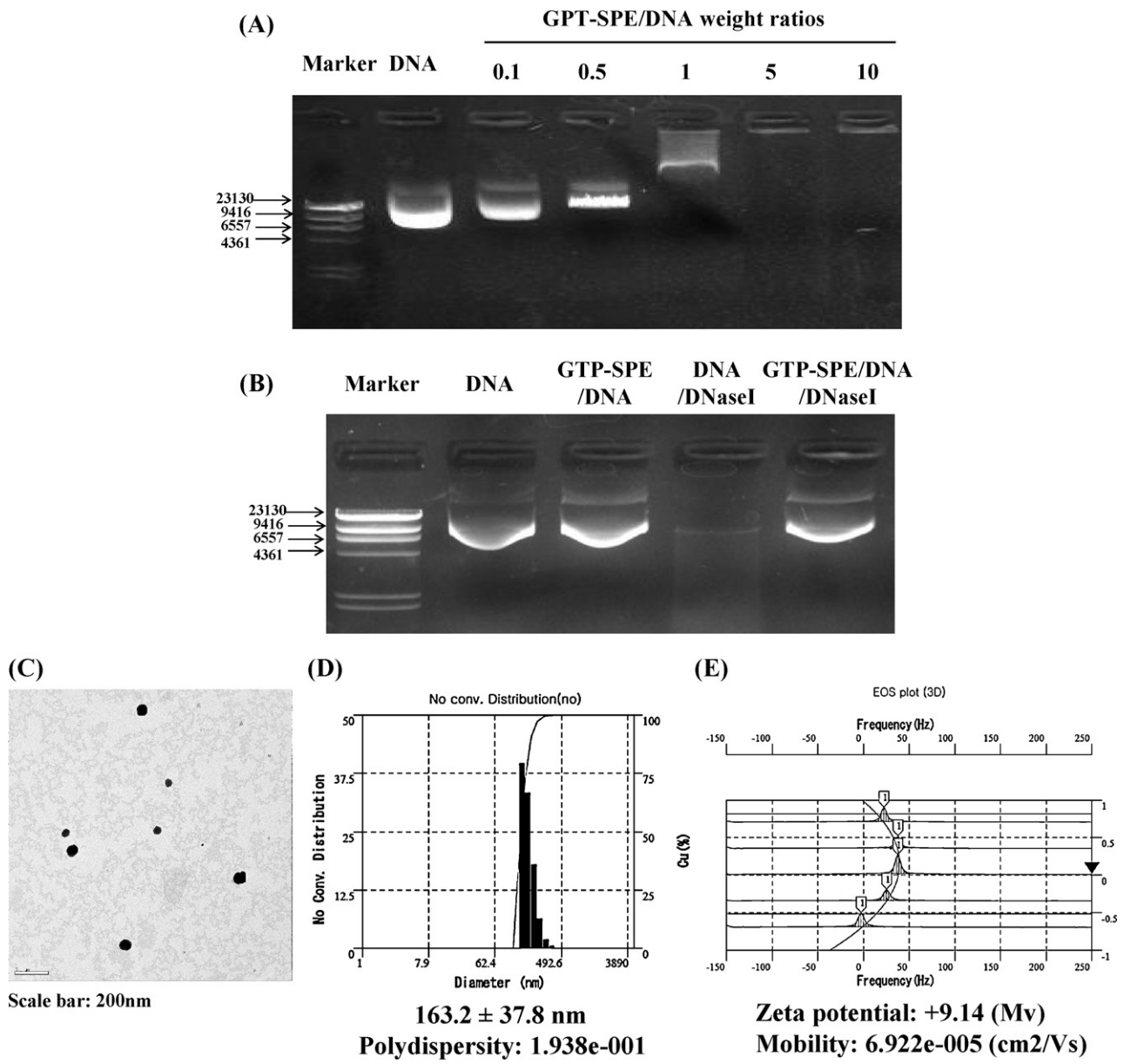


Fig. 2. Characterization of GPT–SPE/DNA complexes. (A) Agarose gel electrophoresis of GPT–SPE/DNA complexes at various weight ratios. The complexes were loaded onto 1% agarose gels with EtBr (0.1 μg/ml) and run with Tris–acetate (TAE) buffer at 100 V for 40 min. (B) DNA protection and release assay. Two units of DNase I or PBS in DNase/Mg^{2+} digestion buffer (50 mM Tris–Cl, pH 7.6 and 10 mM $MgCl_2$) was added to naked DNA or polymer/DNA complexes solution to check the protection ability of polymer. Electrophoresis was performed in 1% agarose gel with TAE running buffer for 1 h at 50 V. (C) EF-TEM images of GPT–SPE/DNA complexes at weight ratio 10. (D) Size distribution assay. (E) Particle surface charges of GPT–SPE/DNA complexes at weight ratio 10.

and Use Committee at Seoul National University (SNU-101211-2).

For Western blot analysis, lungs of three mice from the group of four mice were selected. Total protein was quantified by BCA protein assay kit (Promega). Thirty μg proteins were separated on SDS-PAGE, transferred onto nitrocellulose membrane, blocked and incubated overnight with Akt1 antibody. After washing, the membrane was incubated with secondary antibodies conjugated to horseradish peroxidase (HRP) for 3 h at room temperature. After washing, the bands-of-interest were analyzed by the luminescent image analyzer LAS-3000 (Fujifilm, Tokyo, Japan), and quantification analysis was done by using the Multi Gauge version 2.02 program (Fujifilm, Tokyo, Japan). All blood samples were collected by intracardiac puncture and analyzed for toxicological analysis.

2.6. Statistical analysis

All values are presented as mean values ± standard deviation. Statistical significance of differences among the groups was determined by an unpaired *t*-test. Probability values ($P < 0.05$) were considered as significant.

3. Results

3.1. Synthesis and characterization of GPT–SPE

The GPT, as a cross-linker, reacted with SPE by Michael addition to generate ester-based GPT–SPE [Fig. 1A]. The composition of the polymer was estimated by ^{1}H-NMR [Fig. 1B]. The chemical composition of the SPE in the polymer was determined to be

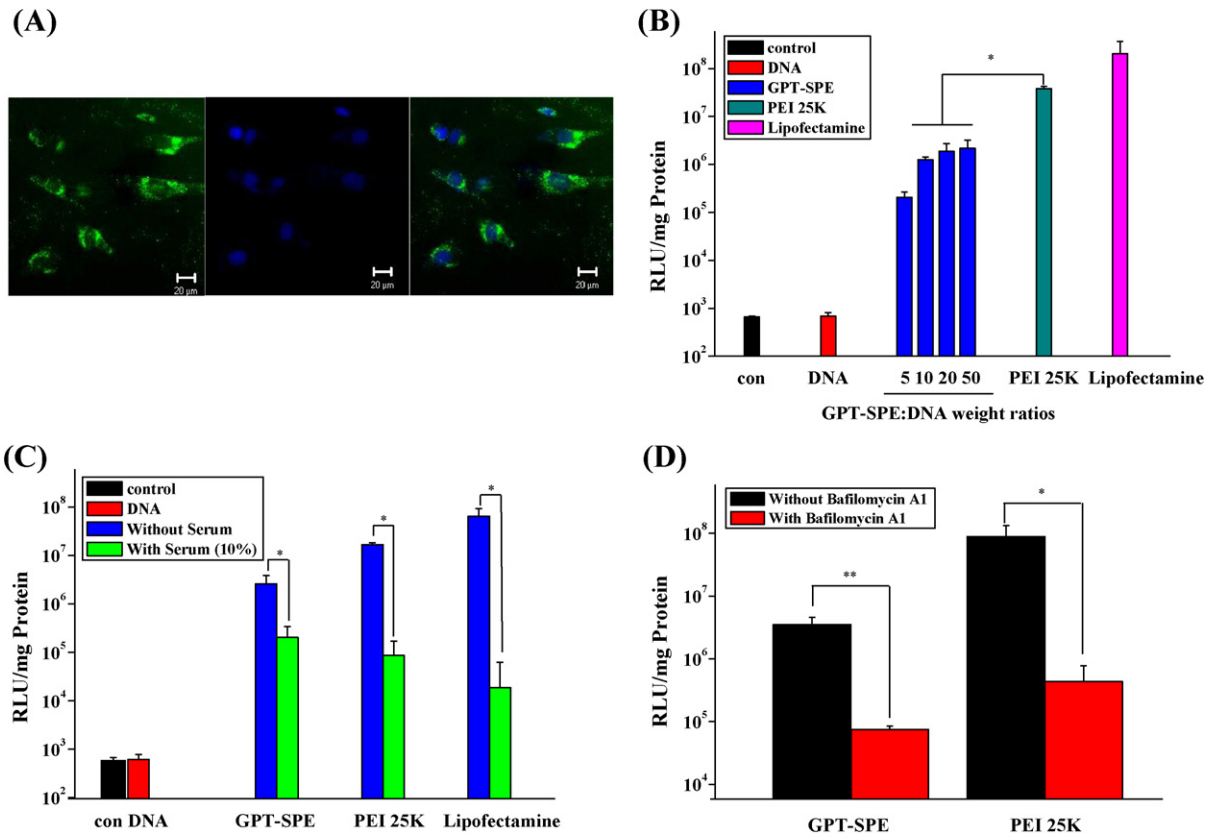


Fig. 3. Cellular uptake and transfection studies. (A) Cellular uptake study of GPT–SPE/DNA complexes in A549 cells. GPT–SPE was labeled with FITC, nucleus was stained with DAPI (scale bar = 20 μm). (B) Transfection efficiency study of A549 cells by GPT–SPE/pGL3 complexes at various weight ratios (mean ± SD, $n = 3$). (C) Effect of serum on gene transfection efficiency. A549 cells were incubated in the absence or presence of 10% serum with copolymer/pGL3 complexes (mean ± SD, $n = 3$). (D) Buffering capacity study of GPT–SPE/pGL3 complexes in A549 cells (mean ± SD, $n = 3$). Bafilomycin A1 (200 nM) diluted in DMSO were put into wells. After 10 min incubation period, transfection solutions were added into the wells for 4 h. Then the cells were incubated in the growth medium for 24 h.

64.07 mol% by assigning the protons of the methylene groups of SPE at $\delta = 1.6–1.8$ ppm and the protons of the methylene group of GPT at $\delta = 3.9–4.0$ ppm. GPC measurement was performed to measure the molecular weight of polymer. The molecular weight of polymer was 5.17 kDa with 1.34 polydispersity index [Fig. 1C].

3.2. Characterizations of GPT–SPE/DNA complexes

The condensation capability of GPT–SPE with DNA was evaluated using agarose gel electrophoresis. The migration of DNA was completely retarded when weight ratio of the GPT–SPE/DNA complexes was about five [Fig. 2A]. As shown in Fig. 2B, DNA in the complexes was protected from nucleases whereas naked plasmid DNA as a control was not protected. Fig. 2C showed representative morphologies of GPT–SPE/DNA complexes, which had well-formed, spherical shapes and compact structure. The average particle size of complex measured by dynamic light scattering was 163.2 nm (PDI: 1.938e−001) and homogenous size distribution of the complexes was unimodal [Fig. 2D]. The zeta potentials of GPT–SPE/DNA complexes were positive charged [Fig. 2E, +9.14 mV, mobility: 6.922e−005 (cm^2/Vs)].

3.3. In vitro cellular uptake and transfection studies

For confirmation of intracellular uptake of GPT–SPE/DNA complex, FITC-labeled-GPT–SPE/DNA complexes were delivered and visualized using confocal microscopy. As shown in Fig. 3A, significant fluorescence of labeled complexes demonstrated efficient intracellular uptake. Fig. 3B showed the transfection efficiency of GPT–SPE/pGL3 (encoding firefly luciferase) complex in A549 cells. GPT–SPE/pGL3 showed 1800-folds higher transfection efficiency compared to naked pGL3 in luciferase reporter gene expression due to effective gene binding ability. The transfection efficiency of GPT–SPE/pGL3 polyplex was lower than that of PEI 25K and Lipofectamine in serum free media. However, as shown in Fig. 3C, transfection efficiency of GPT–SPE/pGL3 in the presence of serum was good enough compared to PEI 25K/pGL3 and Lipofectsamine/pGL3. In fact, the transfection efficiency of GPT–SPE/pGL3 was slightly decreased (12.8-folds) whereas PEI 25K/pGL3 and Lipofectamine/pGL3 showed significant reduction (192.7- and 3468.4-folds, respectively) in the presence of 10% serum.

To further elucidate the mechanism of transfection, we analyzed the buffering capacity of the polymer. A549 cells were treated with bafilomycin A1 as an endosome proton pump inhibitor. The pGL3 expression was decreased 46.6-folds by bafilomycin A1 in GPT–SPE/pGL3 group [Fig. 3D], likewise PEI 25K, suggesting the involvement of proton sponge effect by spermine-mediated gene transfection.

3.4. Cytotoxicity of GPT–SPE

The polymer showed low cytotoxicity compared to PEI 25K in 4 different cell lines [Fig. 4A–D]. The GPT–SPE-treated cells exhibited good cell viability (62.48, 76.33, 85.05 and 79.00% in A549, HepG2, MCF7 and HeLa cell lines, respectively) even at high concentration (100 μg/ml), whereas the cell viability of PEI 25K-treated cells was

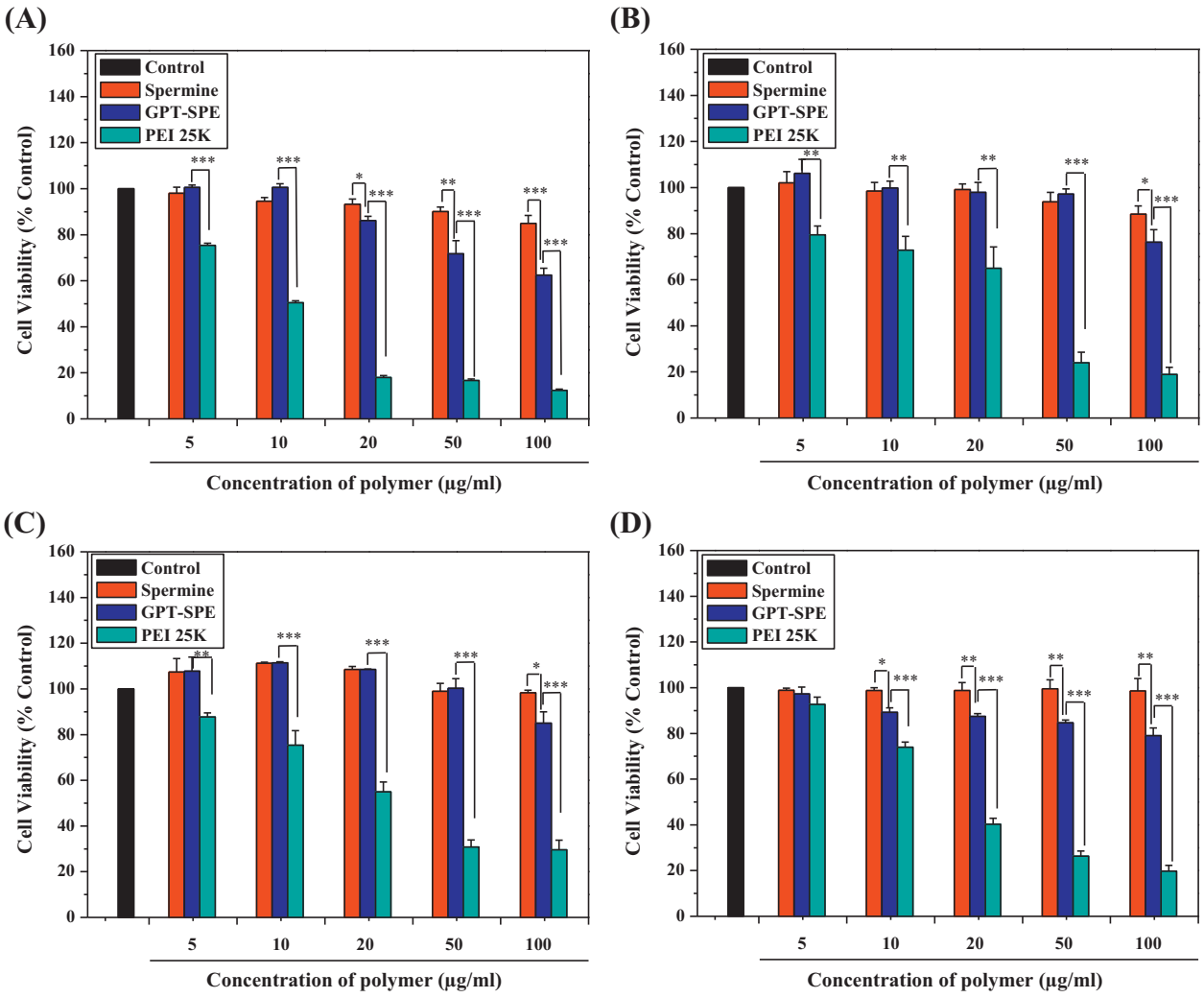


Fig. 4. Twenty four hours cytotoxicity of GPT–SPE copolymer at various concentrations in different cell lines. (A) A549, (B) HepG2, (C) MCF7, and (D) HeLa (mean ± SD, $n = 3$).

drastically decreased with increasing concentration (at 100 μg/ml: 12.32, 18.92, 29.58 and 19.71, respectively).

3.5. *In vivo* aerosol delivery of GPT–SPE

Based on *in vitro* results of increased cellular uptake, high transfection efficiency and low cytotoxicity, *in vivo* gene delivery efficiency of GPT–SPE was re-confirmed. GPT–SPE/GFP group showed strong fluorescence signals compared to control- and naked GFP-treated groups [Fig. 5A and supplementary data 1], without toxicity [Fig. 5B and supplementary data 2]. Also, aerosol delivery of Akt1 shRNA to the K-*ras*^{LA1} lung cancer model mice induced high transfection efficiency with significantly decreased tumor formation [Fig. 6A–D] through Akt signaling pathway [Fig. 6E] without toxicity [Table 1].

4. Discussion

An important step in the advancement of gene therapy is the development of a safe and efficient gene delivery system. As a cationic polymeric gene carrier, PEI has been widely used for non-viral transfection due to strong DNA condensation capacity as well as intrinsic endosomolytic activity. However, the progress toward clinical trials of PEI has been hampered by its toxicity (Lungwitz et al., 2005). Therefore, the present paper has described development of GPT–SPE using SPE and GPT cross-linker. The physicochemical and biological characterizations of GPT–SPE as a safe and efficient gene delivery carrier were evaluated.

We synthesized GPT–SPE with Michael addition reaction between acrylate groups of GPT and amine groups of SPE. Triacrylate linker reacted with SPE to generate ester-based GPT–SPE [Fig. 1A]. The obtained GPT–SPE was readily soluble in water due to its hydrophilic nature. As shown in Fig. 1B, the proton peaks of SPE ($-CH_2-$) appeared at 1.6–1.8 ppm, and the proton peaks of GPT ($-CH_2-$) clearly appeared at 3.9–4.0 ppm, indicating that GPT–SPE was prepared during the reaction. The molecular weight of GPT–SPE was 5.17 kDa with 1.34 polydispersity index [Fig. 1C].

High molecular weight is prerequisite for any polymer to give good transfection, theoretically, the maximum molecular weight should occur at stoichiometric equivalence (Jere et al., 2008). In addition, Anderson et al. (2005) also reported that amine terminated copolymers have better transfection efficiency than acrylate terminated when prepared with slight excess of amine monomer. Therefore, we selected a little increase in mole ratio of SPE (primary amine groups of SPE:acrylate groups of GPT = 10:9) than the ratio of stoichiometric equivalence (primary amine groups of SPE:acrylate groups of GPT = 2:3) with GPT. As expected, 64.07 mol% of SPE was introduced in the polymer.

Another prerequisite of polymeric gene carriers is high DNA binding affinity in order to form stable DNA complex. As shown

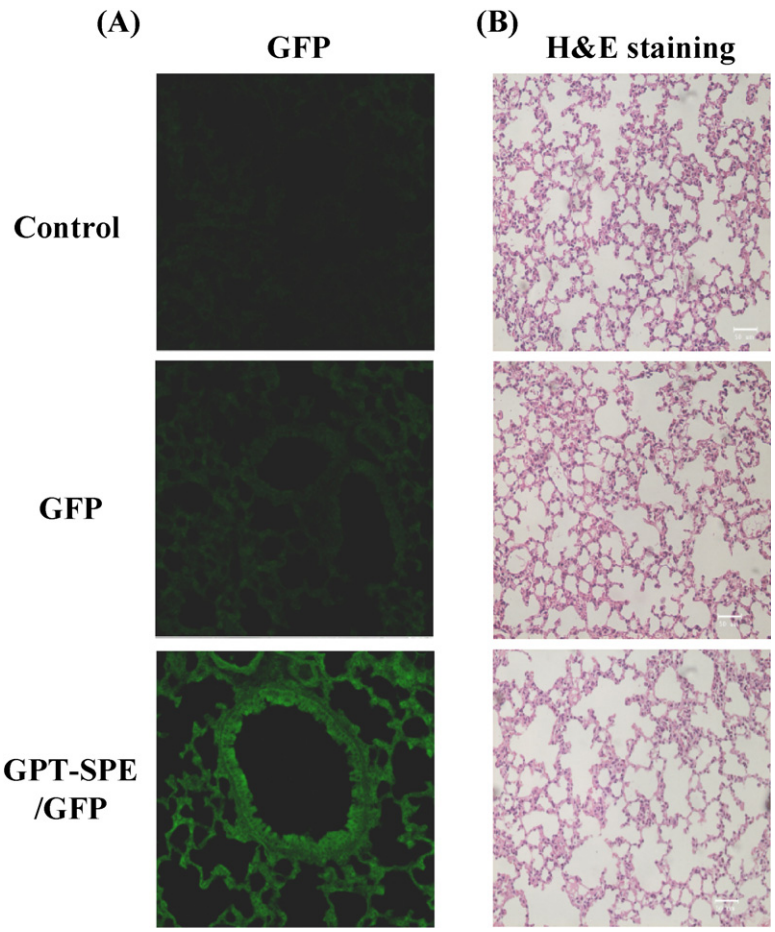


Fig. 5. *In vivo* analysis after aerosol administration to lungs. Two days after exposure, mice were sacrificed and lungs were collected for the detection of GFP signal and Hematoxylin & Eosin staining. (A) Transfection efficiency study: GFP expression analysis (magnification: 200×). (B) Lung histopathology study: Hematoxylin & Eosin staining (magnification: 200×, scale bar represents 50 μm).

in Fig. 2A, the polymer exhibited excellent DNA binding ability. At weight ratio 5, GPT–SPE showed strong DNA binding ability compared to naked DNA. The protection ability is also one of the important factors as a non-viral vector. For effective gene expression, the gene in the gene vehicle should be protected from enzyme degradation (Jiang et al., 2007a, 2009b; Park et al., 2005). As shown in Fig. 2B, DNA in the complexes was protected from nuclease attack whereas the naked DNA was degraded. This result suggests that intact DNA could be delivered into cells without degradation. GPT–SPE/DNA complexes had well-formed spherical shape and compact structure [Fig. 2C]. Successful gene carrier depends on its ability to condense negatively charged DNA into nanosized particles with positive charges so as to enter into the cells (Arote et al., 2009). The complex size and surface charge were measured by DLS as shown in Fig. 2D and E. All complexes were less than 200 nm which were suitable for intracellular delivery and had positive surface charges. Many lines of evidences demonstrate that positive surface charge is necessary for binding to anionic cell surfaces, which facilitates uptake by the cells (Jiang et al., 2008; Kunath et al., 2003). Therefore, it is reasonable to assume that GPT–SPE/DNA complexes are cationic enough for association with negatively charged cell membranes and subsequent uptake into cells.

For efficient transfection, GPT–SPE must deliver gene into cytosol in high concentration. For confirmation of post-transfectional DNA uptake by cells, FITC-labeled GPT–SPE/DNA complexes were delivered and visualized by confocal microscopy. As expected, GPT–SPE/DNA complexes induced high intracellular uptake as shown in Fig. 3A. Transfection efficiency is a key parameter for any gene delivery vector. Thus, transfection study was performed to check the gene expression ability of GPT–SPE/pGL3 complexes. Fig. 3B showed the transfection efficiency of GPT–SPE/pGL3 complexes in A549 cells. GPT–SPE/pGL3 complexes caused high transfection efficiency compared to naked pGL3 although transfection efficiency of the polyplexes was slightly lower than that of PEI 25K as well as Lipofectamine 2000. Interestingly, however, GPT–SPE/pGL3 complexes functioned well in the presence of 10% serum due to hydrophilic property of GPT moieties in GPT–SPE backbone whereas gene transfection capability of PEI 25K/pGL3 and Lipofectamine/pGL3 was decreased in the same condition [Fig. 3C]. These data demonstrate that GPT–SPE carrier would function properly under *in vivo* condition. Our findings are well matched with several lines of evidences, indicating that stability of Pluronic or poloxamer was increased in the presence of serum due to the property of PEO hydroxyl groups (Gebhart and Kabanov, 2001; Kabanov et al., 2005). To further elucidate the mechanism of transfection, we measured buffering capacity of GPT–SPE copolymer with bafilomycin A1, as a specific inhibitor of vacuolar type H^+ ATPase during transfection (Kichler et al., 2001). The transfection efficiency of GPT–SPE/pGL3 complexes was drastically decreased after bafilomycin A1 treatment, likewise PEI 25K [Fig. 3D]. The evidence of the reduction of transfection efficiency of GPT–SPE/pGL3 complexes in the presence of bafilomycin A1 strongly suggests that the GPT–SPE has a high buffering capacity.

Cytotoxicity with cationic polymeric gene carrier needs to be studied carefully for its successful clinical application. The

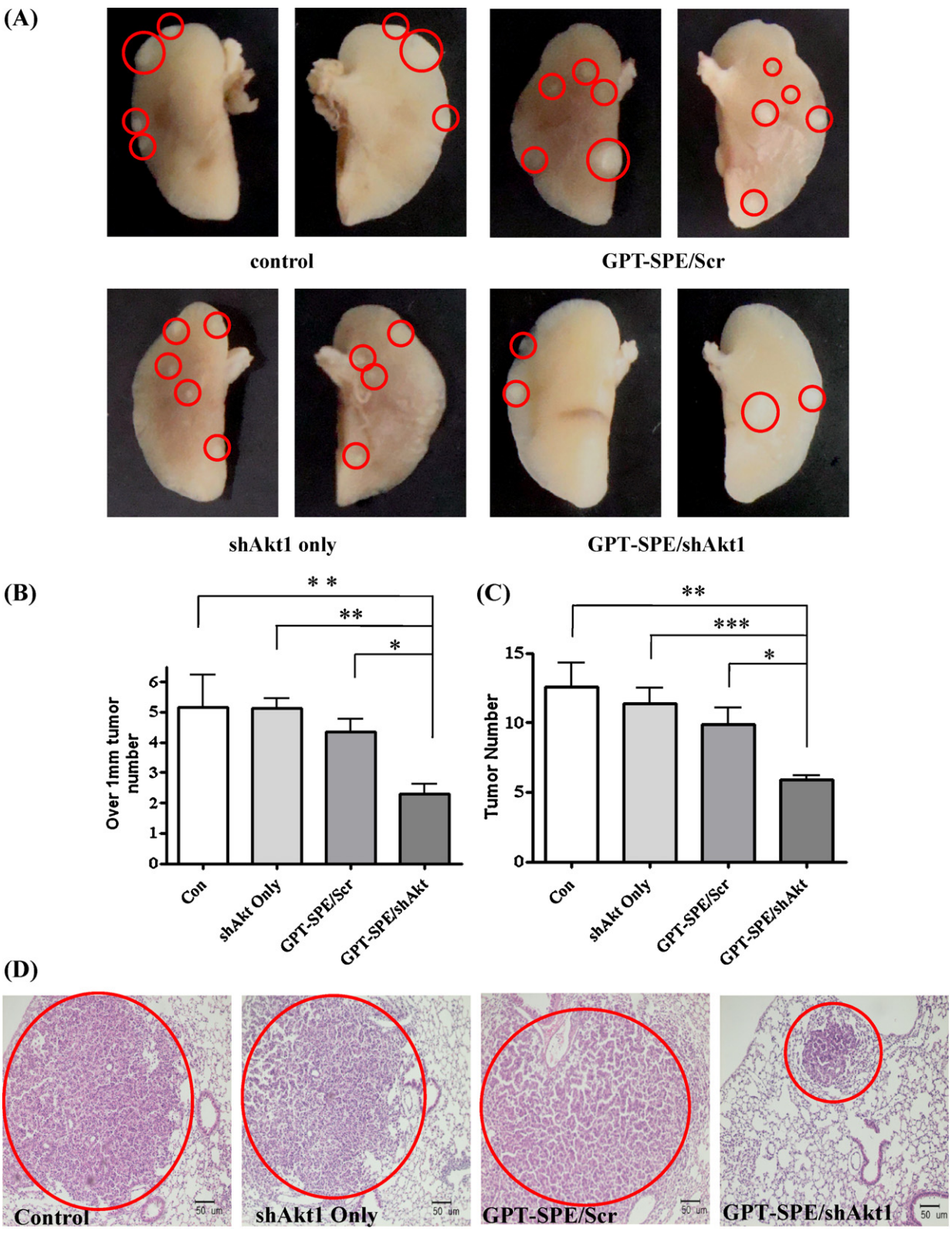


Fig. 6. Therapeutic efficiency of GPT–SPE as aerosol gene delivery carrier in lung tumor bearing K-*ras*^{LA1} mice. Aerosol delivery of GPT–SPE/Akt1 shRNA significantly inhibited lung tumor numbers: (A) Lungs showing numerous visible lesions (red circle represents tumor tissues). (B) Total tumor numbers ($n = 4$, $^{*}p < 0.05$, $^{**}p < 0.01$). (C) Tumor size over 1 mm tumor numbers ($n = 4$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Aerosol delivery of GPT–SPE/Akt1 shRNA significantly suppressed lung tumor progression through the Akt signaling pathway. (D) Histopathological characteristics. Red circle indicates the incidence in the lungs (magnification: 200×, scale bar represents 50 μm). (E) Western blot analysis of Akt1 protein expression in the lungs and bands-of-interest were further analyzed by densitometer ($n = 4$, $^{**}p < 0.01$, $^{***}p < 0.001$).

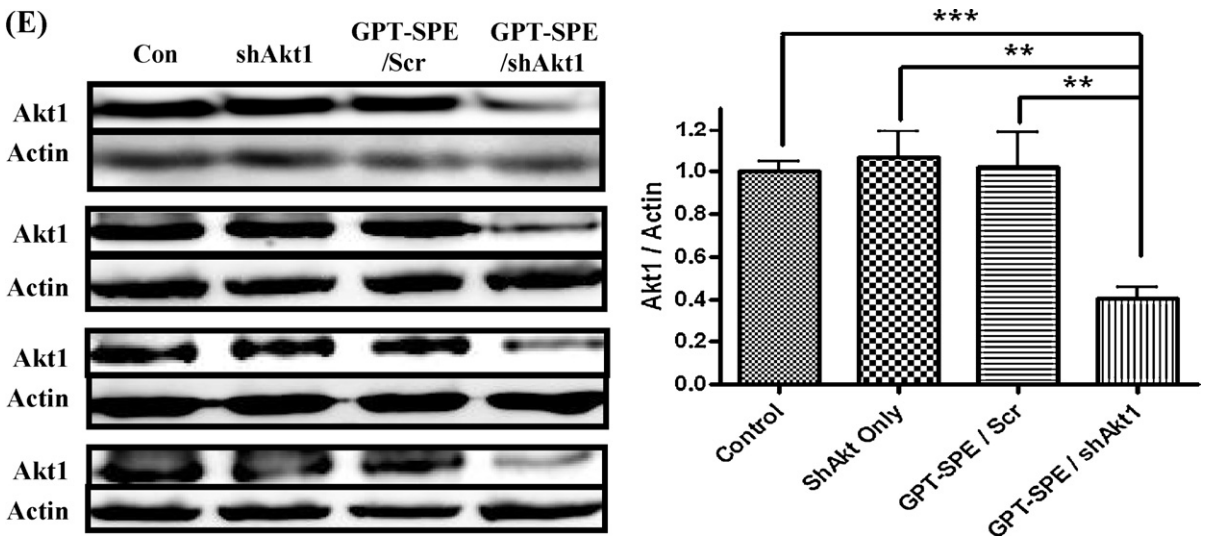


Fig. 6. (*Continued*)

cytotoxicity of cationic polymers is likely due to polymeric aggregation on cell surfaces, impairing important membrane functions. Also, cationic polymers may interfere with critical intracellular processes (Wong et al., 2006). Recent findings demonstrated that PEI and PEI/DNA complexes could induce mitochondria-mediated apoptosis (Hunter, 2006; Moghimi et al., 2005). Also, PEI is known to induce not only relatively weak apoptotic and strong necrotic effect, but also moderate genotoxic effect (Choi et al., 2010). On the other hand, among many attempts to reduce the cytotoxicity of gene carrier, the introduction of biodegradable linkage, i.e., ester linkage, has been the prominent method until today (Anderson et al., 2003; Arote et al., 2008, 2009; Lynn et al., 2001; Vandenbroucke et al., 2008). Degradation of these cationic polymers led to various new fragments, which may associate with unexpected additional safety issues, therefore, one of the reasonable strategy is to introduce human-oriented endogenous amine groups. In fact, SPE is safe and naturally present in body tissues (Jere et al., 2009). We found significantly higher cell viability of SPE- and GPT–SPE-treated cells compared with PEI 25K-treated cells in 4 different cell lines as shown in Fig. 4A–D. The viability of GPT–SPE-treated cells decreased slightly as a function of concentration, however, the viability was maintained more than 60% of non-treated control group even at high concentration. It has been reported that, although cationic polymers with high charge density had strong cell lytic and toxic properties, reduction of charge density resulted in less cell toxicity (Schipper et al., 1996). We observed low zeta potentials of GPT–SPE/DNA complexes (+9.14 mV) compared to PEI 25K/DNA complexes (+39.6 mV). Therefore, it is reasonable to assume that GPT–SPE has lower toxicity than PEI 25K due to the properties of biocompatible SPE and the shielding of the primary amines of SPE after Michael addition reaction with GPT.

Table 1
Toxicological analysis. Blood samples were collected for routine examination and to assess the potential toxicity of GTP–SPE.

	Con	GPT–SPE
(A) Routine CBC		
WBC ($\times 10^3$ cells/μL)	6.71 ± 2.06	5.41 ± 0.97
RBC ($\times 10^6$ cells/μL)	9.89 ± 0.66	10.26 ± 0.55
HGB (g/dL)	13.37 ± 1.30	13.47 ± 1.54
HCT (%)	46.50 ± 3.68	48.83 ± 2.63
MCV (fL)	47.43 ± 0.51	47.60 ± 0.26
MCH (pg)	13.50 ± 0.61	13.47 ± 0.38
MCHC (g/dL)	28.73 ± 0.64	28.40 ± 0.44
CHCM (g/dL)	27.80 ± 0.66	26.70 ± 0.66
RDW (%)	16.33 ± 1.37	16.43 ± 0.40
HDW (g/dL)	1.97 ± 0.31	1.87 ± 0.04
PLT ($\times 10^3$ cells/μL)	2084.00 ± 250.32	1778.33 ± 637.72
MPV (fL)	8.70 ± 0.30	8.60 ± 0.10
(B) Platelet parameters		
PDW (%)	60.27 ± 3.98	52.46 ± 0.36
PCT (%)	1.57 ± 0.30	1.39 ± 0.55
MPC (g/dL)	18.90 ± 0.36	16.36 ± 0.70
MPM (pg)	1.39 ± 0.02	1.20 ± 0.04
Large Pit ($\times 10^3$ cells/μL)	47.43 ± 0.51	41.63 ± 0.26

CBC, complete blood count; WBC, white blood cell; RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; MCV, mean cell volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; CHCM, mean cell hemoglobin concentration; RDW, red cell distribution width; PLT, platelets; MPV, mean platelet volume; PDW, platelet distribution width; PCT, plateletcrit; MPC, mean platelet component; MPM, mean platelet mass; Large Pit, large platelets.

The aerosol delivery of genes is a possible lung cancer gene therapy because the anatomical structure and location of the lungs make instant access and a non-invasive approach possible, with a high delivery efficiency that does not affect other organs (Gautam et al., 2003). Based on *in vitro* results, the *in vivo* gene delivery efficiency of GPT–SPE was re-confirmed using aerosol delivery. As shown in Fig. 5A and supplementary data 1, GFP signal was dominant in the lungs with GPT–SPE/GFP complexes-exposed group compared to the control and naked GFP-exposed groups. No necrosis, degeneration, metaplasia, anaplasia in pneumocytes, atelectasis, or emphysema were detected as shown in Fig. 5B and supplementary data 2. These results indicate that GPT–SPE functions safely and efficiently in aerosol delivery system. Of the K-*ras*, N-*ras*, and H-*ras* three members of the *ras* family, K-*ras* is found to be the most frequently mutated member in lung cancer (Pellegata et al., 1996). Moreover, K-*ras* gene mutation enhances motility of lung adenocarcinoma cells via Akt1 activation (Okudela et al., 2004), and radioresistance of K-*ras*–mutated human tumor is mediated through the epidermal growth factor receptor-dependent PI3K-Akt pathway (Toulany et al., 2005). Therefore, in this study, K-ras^{LA1} mice were used for *in vivo* effects of aerosol-delivered Akt1 shRNA in lung tumorigenesis. As expected, significant anticancer effects of GPT–SPE/Akt1 shRNA complexes in the lungs through aerosol inhalation were observed [Fig. 6A–E] without toxicity [Table 1]. Taken together, GPT–SPE could be a good shRNA carrier in aerosol-administered lung cancer gene therapy.

5. Conclusion

We successfully prepared and evaluated GPT–SPE as a new gene carrier for lung cancer therapy. The GPT–SPE contained high ability

to form complex with gene and had suitable physicochemical properties as a gene delivery system. This polymer had low cytotoxicity and exhibited enhanced gene transfer efficiency *in vitro* as well as *in vivo*. Therefore, GPT–SPE has the potential to be a safe and efficient gene carrier. To improve the cancer cell specificity, we are currently focusing on more comprehensive studies to develop cancer cell specific ligand conjugated GPT–SPE for lung cancer gene therapy via aerosol administration.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpharm.2011.08.045.

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