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Folic acid conjugated mPEG-PEI600 as an efficient non-viral vector for targeted nucleic acid delivery

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ABSTRACT

In this study we describe a novel polymer, mPPS-FA, synthesized as a potential gene transfer vector. To complete mPPS-FA, folic acid was conjugated to a backbone (named mPPS) consisting of a copolymer of methyl PEG-2000, PEI-600, and sebacoyl chloride. ^{1}H NMR, FT-IR, and UV spectroscopy were used to characterize the structure of mPPS-FA. It was revealed that mPPS-FA holds the ability to bind plasmid DNA yielding positively charged particles (polyplexes). Dynamic light scattering (DLS) and TEM techniques were used to study the size and morphology of the formed mPPS-FA/DNA nanocomplexes. The mPPS-FA/DNA nanoparticles exhibited low cytotoxicity as transfection of B16-F0, U87MG, CHO-1, and Ho-8910 cells produced >80% viability indicating low cytotoxicity of the polymer. The ability of mPPS-FA to deliver EGFP plasmid to melanoma B16-F0, U87, CHO-1, Ho-8910, and A549 cells was investigated *in vitro* as compared to the lipid-based transfection agent Lipofectamine™2000 and Linear PEI 22kDa (L-PEI 22kDa). We found that mPPS-FA/DNA complexes yielded the highest GFP transfection efficiency in B16-F0, U87, CHO-1, and Ho-8910 cells, which all highly express folate receptors (FR), at an mPPS-FA/DNA ratio (w/w) of 15. Furthermore, the transfection of mPPS-FA/DNA complexes in CHO-1 cells could be competitively blocked by free folic acid molecules. In contrast, in low FR expressing A549 cells, mPPS-FA showed similar low transfection efficiency as mPPS. Taken together, mPPS-FA showed the highest efficiency *in vitro* and the potential to be developed as a nonviral gene carrier.

1. Introduction

Gene therapy is becoming one of the most rapidly advancing fields of biotechnology and holds great promise for treating a wide variety of diseases, mostly due to the outcome of the Human Genome Project and the continuous growing knowledge of gene functions (Anderson, 1992; Mulligan, 1993). Gene therapy can be used to compensate for dysfunctional or under-expressed genes by transferring exogenous nucleic acid into the cytoplasm or nucleus of pathogenic cells. However, current gene therapy seems far away from a successful clinical application largely due to the absence of an efficient and safe gene carrier for delivering the therapeutic genes to the desired targets (Somia and Verma, 2000; Verma and Somia, 1997). Virus-based vectors, e.g. the recombinant constructs from lentiviruses and adenoviruses, have been the most studied in laboratories and in clinical trials to date (Connolly, 2002; Mittereder et al., 1996; StGeorge, 2003; Thomas et al., 2003). Although viruses have adapted an exploitable means to deliver exogenous DNA into a host cell, viral gene delivery carries with it safety concerns which have hastened the development of the non-viral counterparts, particularly after the death of a patient in gene therapy trial using viral vectors (Glover et al., 2005; Marshall, 1999; Park et al., 2006; Thomas and Klibanov, 2003). Despite having lower gene transfer efficiency, non-viral vectors are easier to produce and easily amenable to chemical modifications which can increase transfection efficiency and target-specificity.

A great variety of synthetic and natural polymers, as well as off-the-shelf materials have been investigated as gene delivery, including poly(L-lysine), polyethylenimine (PEI), polyethylene glycol (PEG), dextrin, glatin, and dendrimers. Among these polymers, PEI has been widely used because of its superior transfection

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efficiency displayed over a wide range of cell types (Boussif et al., 1995; Neu et al., 2005). Importantly, the transfection efficiency and cytotoxicity of PEI were found to be correlated with both molecular weight and polymer structure. In general, PEI with higher molecular weight has higher transfection efficiency, but also has higher cytotoxicity resulting from interactions between its high positive surface charge and cellular components (Fischer et al., 1999; Kunath et al., 2003). Some strategies have been used to reduce cytotoxicity and to increase its circulation time *in vivo*, including polyethylene glycol (PEG) grafting and cross-linking low molecular weight PEI (Gosselin et al., 2001; Sung et al., 2003; Tang et al., 2003; Thomas et al., 2005). PEG has been considered an ideal biocompatible polymer with low toxicity, high water solubility, and negligible immunogenicity. Since these intrinsic properties of PEG can be transferred to another covalently bonded molecule, PEG-containing polymers are excellent candidates for gene delivery systems. Also, in contrast to viral vectors, non-viral vectors do not exhibit tropism for specific cell internalization. Conjugation of small molecules (e.g. peptides, carbohydrates and nutrient molecules) to a developed gene carrier is a common method to increase target specificity as well as to enhance transfection efficiency via receptor-mediated endocytosis.

In this work, we have synthesized a polymer (named mPPS-FA), by conjugating folic acid to the backbone (named mPPS) consisting of methyl PEG-2000, PEI-600, and sebacoyl chloride. It has been well established that the ability of polymeric delivery system to bind DNA and protect it from enzymatic degradation is one of the principle design criteria (Abdelhady et al., 2003; Bally et al., 1999). The electrostatic interaction between cations on polymer and phosphates must be strong enough to withstand competitive interaction from anionic species including endogenous proteins and nucleic acids (Mahato et al., 1997). We hypothesized that a sebacoyl chloride spacer can provide a torsional flexibility of the backbone to maximize cation and phosphate interactions while decreasing the positive charge density on its surface and hence reducing its cytotoxicity. In addition, we expected that PEG fragments in mPPS-FA can increase its biocompatibility and folic acid conjugation can confer a targeting ability specific to cancer cells that overexpress folate receptor (FR). To substantiate our expectations, we evaluated the transfection efficacy and toxicity of mPPS-FA a panel of cell lines and compared them with Lipofectamine^{TM}2000 and L-PEI 22kDa.

2. Materials and methods

2.1. Materials

Polyethylenimine (PEI-600, branched, Mn 600, Mw 800), sebacoyl chloride (97%, Mw 239.14), poly(ethylene glycol) methyl ether (mPEG, Mn 2000), polyethylene glycol (PEG), 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), terephthaloyl chloride and folic acid (FA, Mw 441.4) were purchased from Sigma, USA. Linear polyethyleneimine 22kDa (L-PEI 22kDa) was purchased from MBI Fermentas (St Leon-Rot, Germany). N-Hydroxy-succinimide (NHS) (98%, Mw 115.09) was purchased from Aldrich, USA. 1-ethy-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC HCl) (Mw 155.24) was obtained from GL Biochem (Shanghai) Ltd, China. Dimethyl sulfoxide (DMSO) of ACS grade was ordered from Labscan Asia Co. Chloroform of ACS grade was obtained from MERCK, Germany. Triethylamine was treated with toluene sulfonyl chloride and then distilled over sodium. Other chemicals and solvents were of analytical reagent grade and were used as received. Plasmid DNA encoding for EGFP driven by cytomegalovirus (CMV) promoter was obtained from Clontech, USA. DMSO, chloroform, and sebacoyl chloride were purified by refluxing distillation over sodium.

B16-F0, U87, and A549 cells were purchased from ATCC, and were maintained at 37 °C under 5% CO_2 and 90% relative humidity in Dulbecco's Modified Eagle's Medium (for b16-F0 cells), Eagle's Minimum Essential Medium (for U87 cells), and F-12K Medium (A549 cells). CHO-1 was obtained from Institute of Biochemistry and Cell Biology in Shanghai, Chinese Academy of Sciences, and was maintained in F-12K Medium. Ho-8910 was purchased from China General Microbiological Culture Collection, and was maintained in RPMI 1640 medium.

2.2. Synthesis of mPPS

mPEG-2000 (0.5 g, 0.25 μmol) and triethylamine (6.07 g, 0.06 mol) were dissolved in 40 mL of freshly dried chloroform in a 250 mL round-bottom flask cooled in dry ice/acetone bath. 20 mL of freshly dried chloroform containing 1.44 g sebacoyl chloride (1.287 mL, 0.06 mol) was slowly added to the flask for 1.5 h under stirring. PEI-600 (3.6 g, 6 μmol) in 10 mL chloroform was added into the flask. The reaction was then allowed to proceed at room temperature overnight. Solvent and excess reagents were removed by rotary evaporator followed by extensive dialysis (MWCO 3.5 kDa) in deionized water for 3 days. The crude product was filtered with 0.22 μm membrane and was further dialyzed against deionized water for 2 days. White semi-solid was obtained after lyophilization.

2.3. Synthesis of mPPS-FA

400 mg mPPS was dissolved in 50 mL DMF/DMSO (3:1, v/v). 20 mg (0.045 mmol) of folic acid (Mw = 441.4 g/mol) was reacted with 43 mg (0.225 mmol) EDC HCl (191.7 g/mol) in 40 mL DMF/DMSO solution (3:1, v/v) for 30 min. Subsequently, 5.2 mg (0.045 mmol) NHS was added to the mixture. After reacting for 15 min, folic acid solution was slowly added to mPPS solution and then stirred for 2 days. The reaction mixture was dialyzed against deionized water (MWCO 3.5 kDa) for 3 days to remove the excess folic acid and solvents. The product was then obtained by lyophilization.

2.4. ^{1}H NMR and size exclusion chromatography

^{1}H NMR spectroscopy was conducted on Bruker AVANCE 400 spectrometer (400 MHz). D_2O (99.9%, Cambride Isotope Laboratories, Inc., MA, USA) was used as the solvent at room temperature. Chemical shift was expressed in parts per million (δ) by using the proton peak of H_2O (set as 4.7pm) as an internal reference.

Size exclusion chromatography (SEC) was performed in 0.1 M NaNO3 at 25 °C with an elution rate of 0.8 mL/min on a Waters HPLC system with a G1310A pump and a G1362A refractive index (RI) detector, Ultrahydrogel 1000 (Waters) column was calibrated by polyethylene glycol standard (Polymer Source, Inc., Canada).

2.5. FT-IR

FT-IR spectroscopy was conducted on a FT-IR spectrometer (Spectrum 2000, PERKIN ELMER). The signals of 16 scans were averaged with a resolution of 2 cm^{-1} at room temperature. Samples were dispersed in anhydrous KBr and were compressed to form disks.

2.6. UV–Vis spectrum

UV–Visible spectrum was obtained in a 1 cm quartz cuvette using a Perkin Elmer Lamda bio40 spectrophotometer (Boston, MA) at room temperature. 0.2 mg mPPS and mPPS-FA was dissolved in 1% DMSO in water.

2.7. Agarose gel electrophoresis assay

The DNA-binding ability of mPPS-FA was determined by agarose gel electrophoresis. Complexes of mPPS-FA/DNA were prepared by mixing varying amounts of mPPS-FA with 0.5 μg of DNA in 5% glucose solution. Complexes with different N/P ratios were then diluted with distilled water to a final volume of 10 μL, and 6× DNA loading buffer (2 μL) was added to the solution. After 30 min incubation at room temperature, 10 μL of complexes were run on a 1% agarose gel containing 0.5% ethidium bromide in TAE buffer at 100 V for 60 min. DNA bands were visualized by UV illuminator.

2.8. Hydrodynamic radius and Zeta potential

1 mL mPPS-FA/DNA complex was freshly prepared at a DNA concentration of 0.1 mg/mL. Hydrodynamic radiuses of different N/P ratios were measured. The experiment was carried out by Zetasizer 3000HSA (Malvern Instruments, UK) with an angle of detection of 90° at room temperature. The mean sizes (±S.D.) were reported by a single run with 10 measurements.

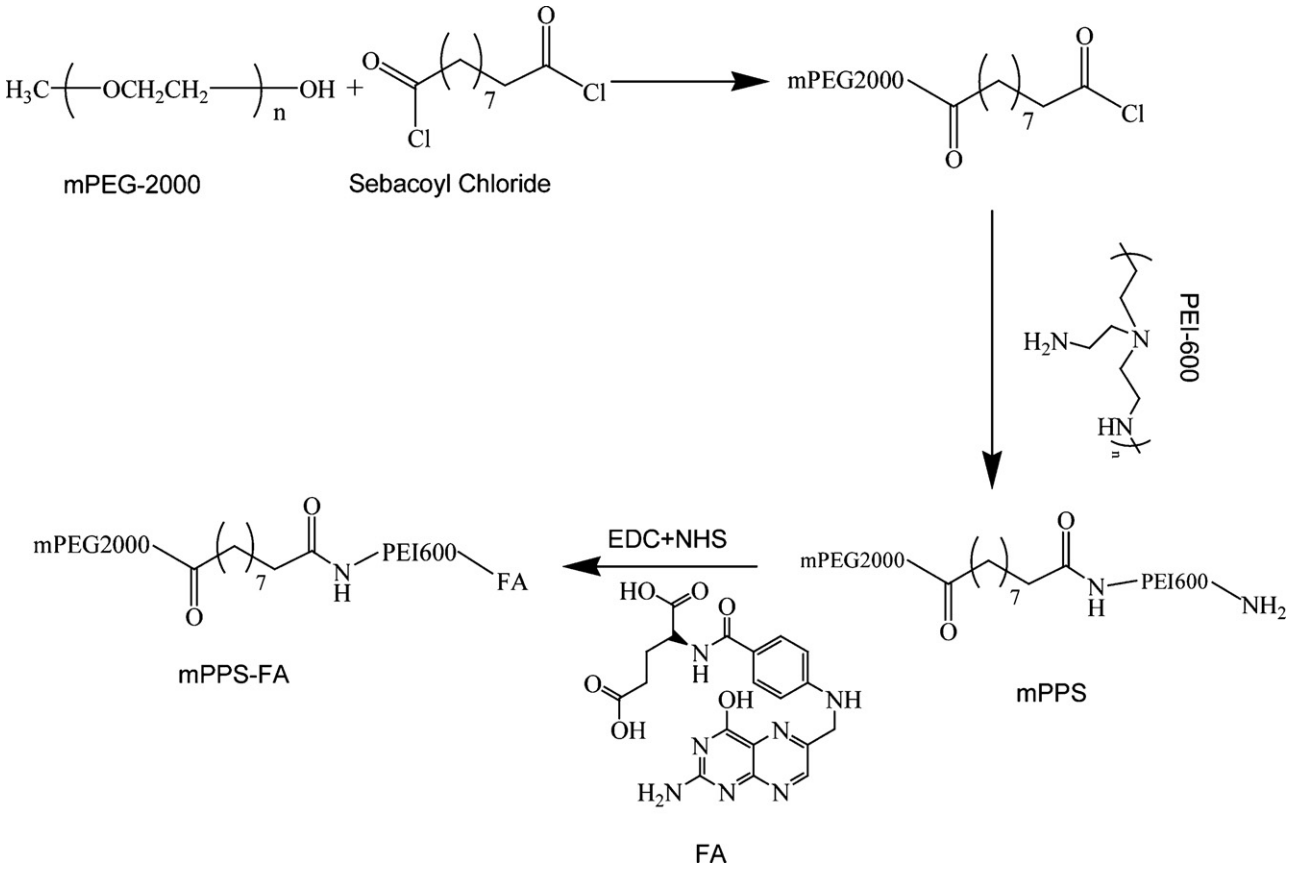
Complexes were formed by mixing 10 μg DNA in 150 μL 5% glucose with desired amount of mPPS-FA in 150 μL 5% glucose. After 30 min of incubation at room temperature, the complexes were diluted with MEM medium or PBS (pH = 7.0) to 10 mL. Zeta potentials were determined with an incident laser beam of 633 nm at a scattering angle of 90° using a Malvern ZetaSizer 3000 HSA. The charge of the complex particles and their mean values (±S.D.) were reported by a single run with 10 measurements.

2.9. Transmission electron microscopy

Transmission electron micrographs were taken with a JEOL-100 CX II transmission electron microscope (operated at 80 kV) in order to observe the shapes, morphologies, and sizes of the mPPS-FA/DNA complex. The samples were prepared by placing a drop of complex on a formvar/carbon-coated copper grid and allowing the solvent to evaporate.

2.10. Gene transfection assay

In vitro gene transfection assay was performed in B16-F0, U87, CHO-1, Ho-8910, and A549 cells using EGFP as reporter gene. Cells were seeded into a 24-well plate at a density of 7.5×10^4 cells/well in 500 μL of growth medium. After 24 h incubation, the culture media were replaced with 400 μL fresh growth media. Transfection complexes were prepared as follows: mPPS-FA with various concentrations and 1 μg reporter gene were diluted in 50 μL serum free media. After 5 min incubation, two solutions were mixed and incubated for additional 10 min before addition to the cells. Lipofectamine™2000 (procedure according to the supplier's protocol) and L-PEI 22kDa (at the optimized PEI/EGFP ratio of 40) were used as control to transfect the same panel of cell lines (Werth et al., 2006). After 6 h of transfection, the culture media was removed and replaced with 500 μL growth media. The EGFP expression was examined under an adverse fluorescent microscope (Olympus IX71, Japan) after an additional 18 h of incubation. EGFP positive cells and the total cells in three randomly selected sections were counted,



Scheme 1. Schematic illustration of synthetic process of mPPS-FA.

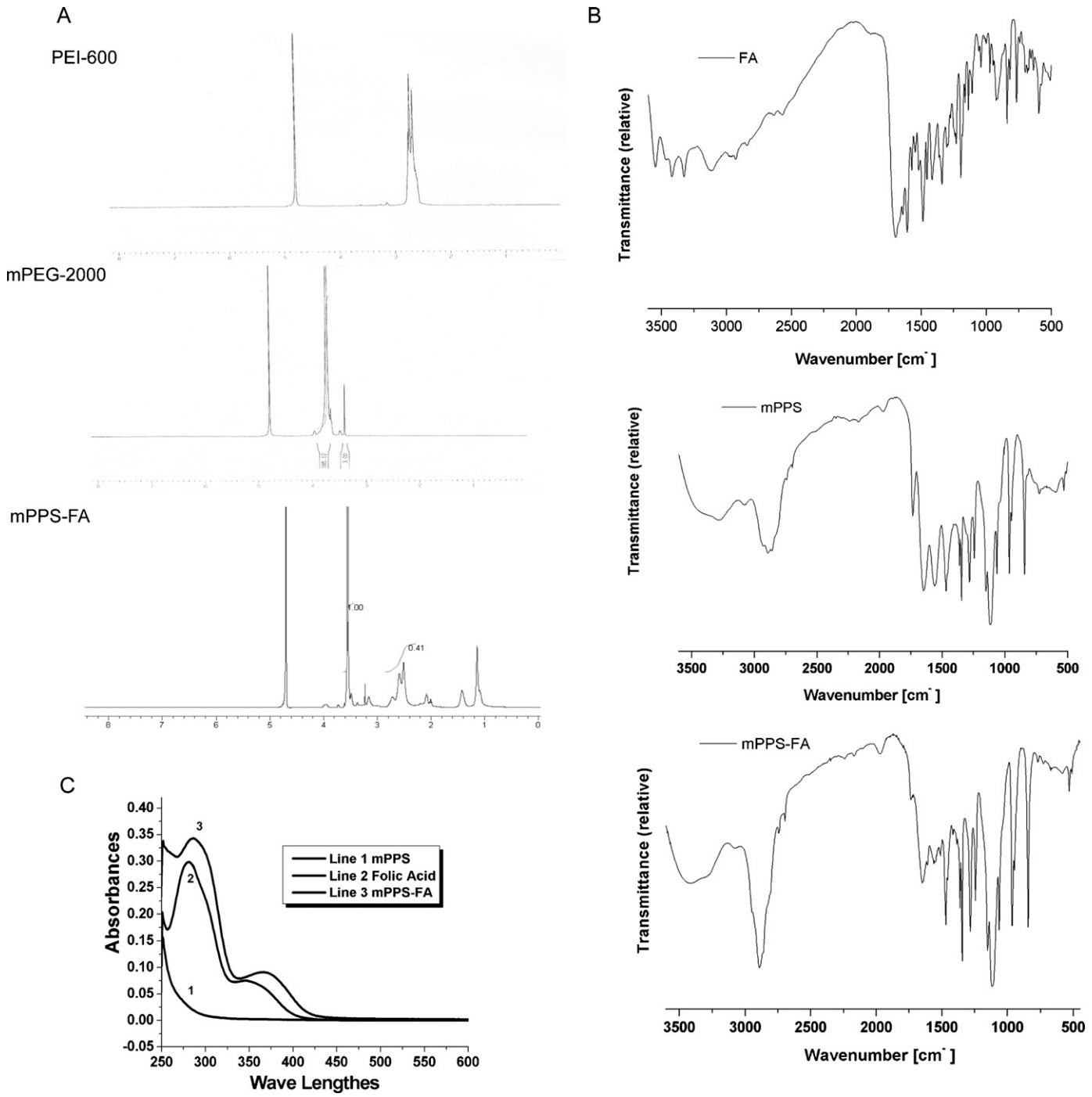


Fig. 1. (A) ^{1}H NMR spectra of PEI-600, mPEG-2000, and mPPS (3 mg/mL); (B) FT-IR spectra of folate acid, mPPS, and mPPS-FA; (C) UV–Vis spectra of the mPPS (0.2 mg/mL) (line 1), folic acid (0.005 mg/mL) (line 2), and mPPS-FA (0.2 mg/mL) (line 3).

and the transfection efficiency was calculated by the ratio between EGFP-positive cells and the total number of cells.

2.11. Cytotoxicity test

The cytotoxicity of mPPS and mPPS-FA was studied and compared with L-PEI 22kDa on B16-F0, U87, CHO-1, and Ho-8910 cell lines. Cells were maintained in the conditions previously described. The initial cell seeding density was 7500 cells in 96-well plate format. After overnight incubation, the culture media was replaced with 100 μL serum free fresh media. The complexes of mPPS-FA/EGFP and L-PEI 22kDa/EGFP with the same volume as used in gene transfection study were added to each well and the cells were incubated for 24 h. 10 μL of sterile filtered MTT (5 mg/mL) stock solution in PBS was added to each well reaching a final concentration of ~0.45 mg/mL. After 3 h of incubation, the media with un-reacted dye was removed. The reduced crystals were dissolved in 100 μL DMSO, and plate with cover was left in the dark for 2–4 h or overnight at room temperature. The absorbencies were then determined by a microplate reader (Thermo Electron

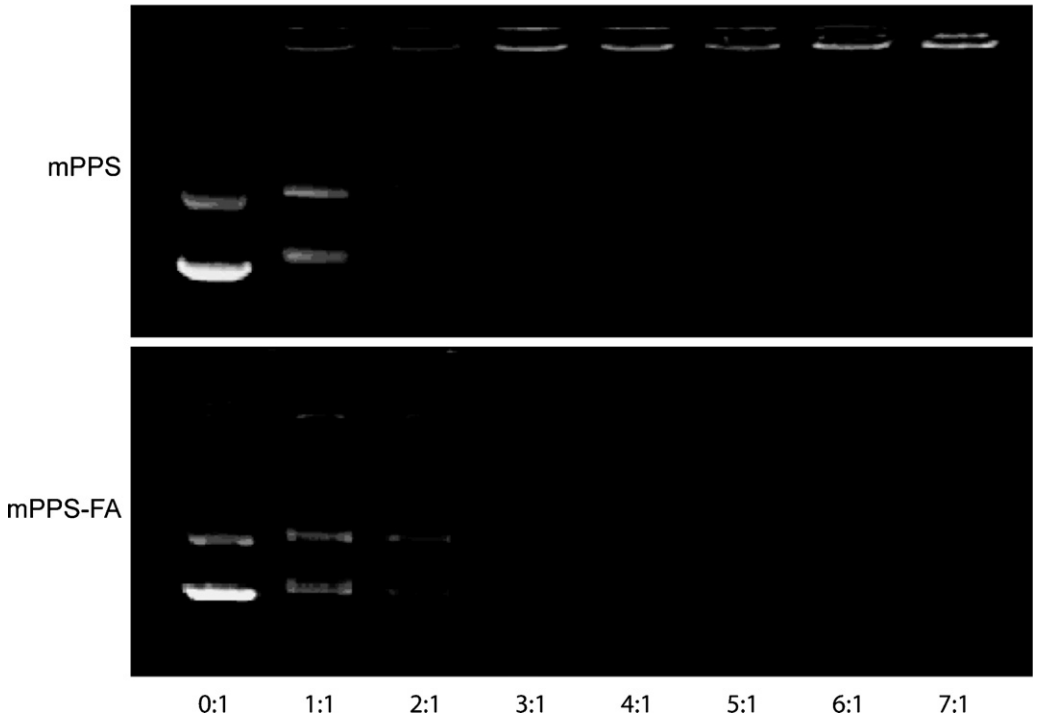


Fig. 2. Agarose gel electrophoresis of plasmid DNA in complexes with mPPS and mPPS-FA at different polymer/DNA weight ratios.

Corporation Multiskan Ex, USA) at detection wavelength 570 nm and with a reference wavelength 690 nm. The relative cell viabilities were calculated according to the equation:

$$\text{Cell viability}\,[\%] = \frac{\text{test Abs}_{570}}{\text{control Abs}_{570}} \times 100.$$

2.12. Live-cell confocal microscopy imaging

3.0×10^5 CHO-1 cells were seeded on sterile glass coverslips in glass-bottomed 35 mm tissue culture dish. After 24 h incubation, CHO-1 cells were mock transfected or transfected with PEI 22kDa/DNA or mPPS-FA/DNA for 48 h. Before image capture, 50 nM LysoTacker (LysoTracker Red DND-99, Invitrogen) was used to stain cells for 30 min. After washing with PBS three times, cells-adhered coverslips were mounted onto slides and immersed in CO_2 independent medium for imaging experiments. Fluorescence imaging was conducted on a Leica TCS SP5 equipped with Ti: sapphire pulse laser. Red fluorescence signals of LysoTracker were generated by 543 nm He–Ne laser and acquired by the PMT channel at 560–620 nm; and green signals of EGFP were excited by 488 nm Argon laser and received by the PMT channel at 500–540 nm.

3. Results and discussion

3.1. Synthesis of mPPS and mPPS-FA

As shown in Scheme 1, mPPS was prepared by the coupling of methyl PEG-2000, PEI-600, and sebacoyl chloride. Firstly, the main chain was obtained by condensation polymerization between PEG-2000 and sebacoyl chloride in the presence of excess triethylamine. Then low molecular weight PEI-600 was added into the reaction mixture. The introduction of PEG chains onto cationic main chain was aimed at increasing the hydrophilicity, polyplex stability,

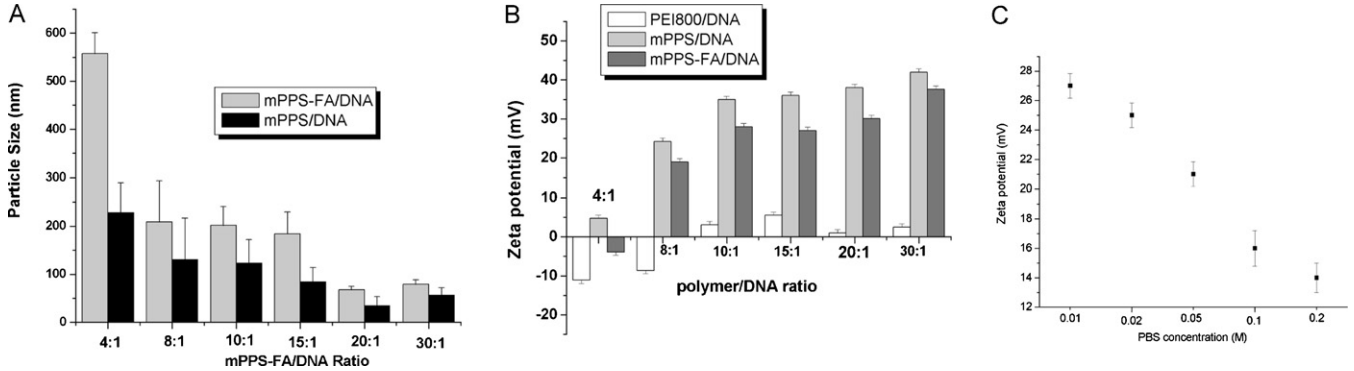


Fig. 3. Particle size and zeta potential of mPPS-FA/DNA complexes. (A) Effective diameter of mPPS/DNA and mPPS-FA/DNA complexes at N/P ratios specified. (B) Zeta potential of complexes of mPPS-FA/DNA. Complexes of mPPS-FA/DNA and PEI-600/DNA were measured as control. Mean ± S.D. (C) Zeta potential of mPPS-FA/DNA complexes in phosphate buffered saline (PBS) with different concentrations at pH 7.

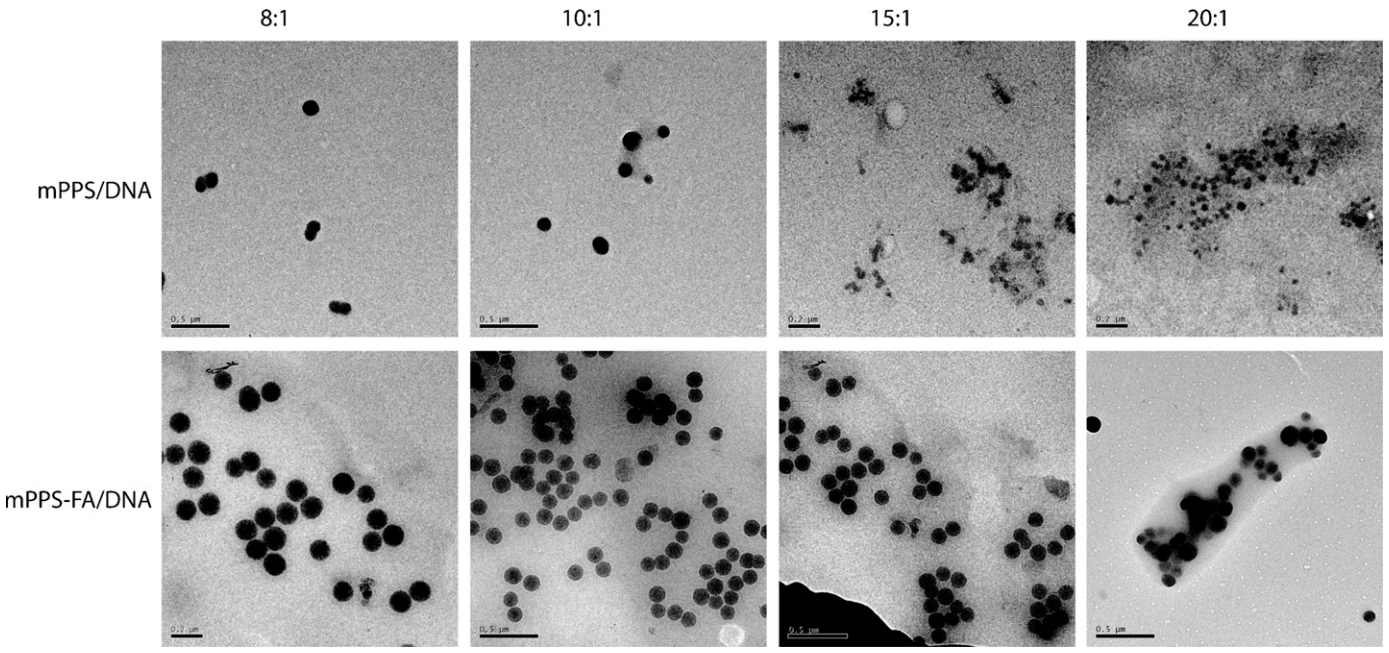


Fig. 4. TEM images of mPPS/DNA (up panel) and mPPS-FA/DNA complexes at N/P ratios specified.

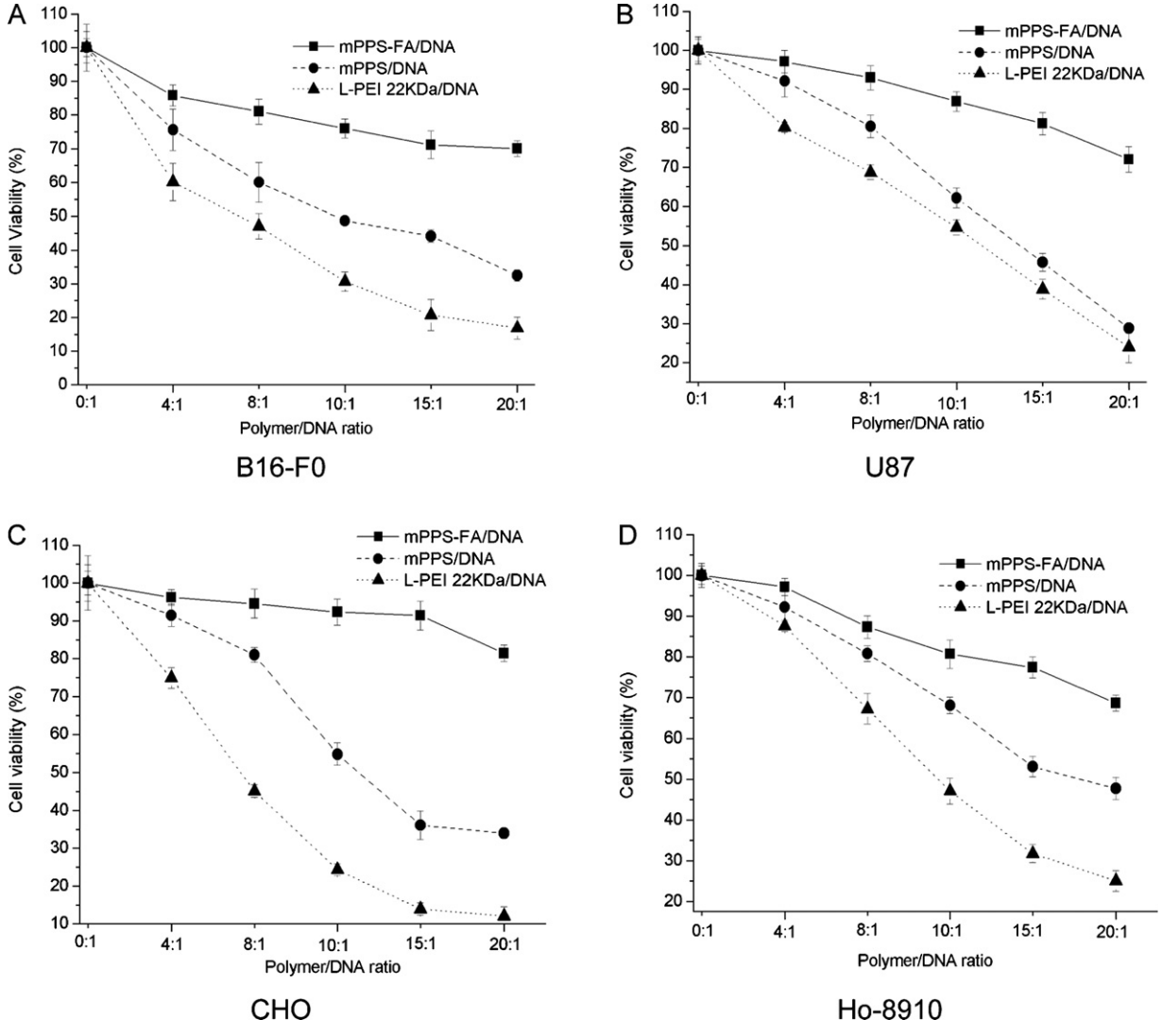


Fig. 5. Cytotoxicity of mPPS/DNA, mPPS/DNA, and PEI 22kDa/DNA in (A) U87 cells, (B) B16-F0 cells, (C) CHO-1 cells, and (D) Ho-8910 cells. Metabolic activity was measured by MTT assay following 24 h incubation of the cells with the polymer/DNA complexes (mean ± S.D., $n = 3$).

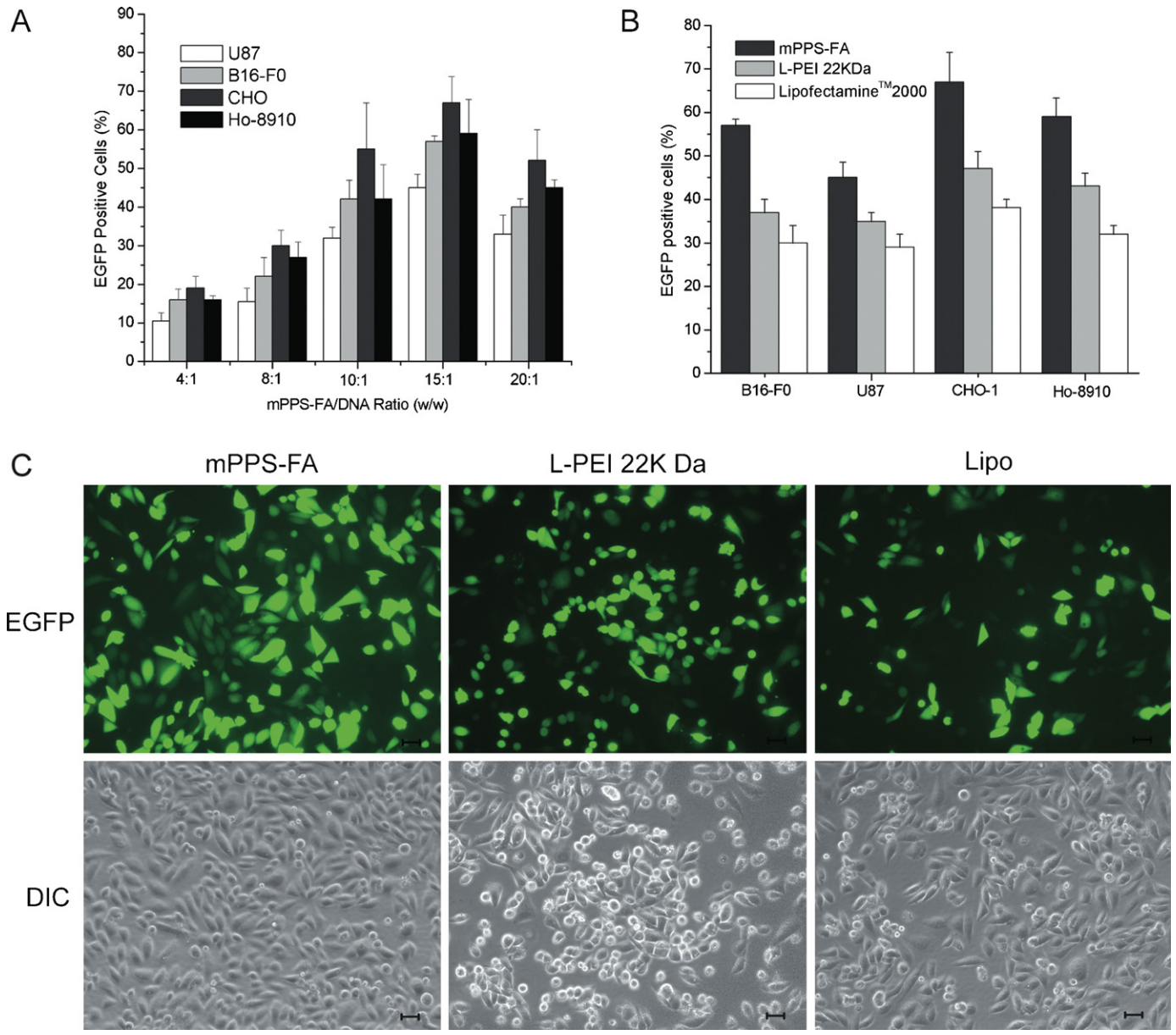


Fig. 6. Gene transfection efficiency of mPPS-FA. (A) Expression of EGFP in U87, B16-F0, CHO-1, and Ho-8910 cells at indicated ratios (w/w) after 24 h transfection (mean ± S.D., $n = 3$). (B) Comparison in EGFP transfection efficiency between mPPS-FA, L-PEI 22kDa and Lipofectamine ™2000 in B16-F0, U87, CHO-1, and Ho-8910 cells (mean ± S.D., $n = 3$). (D) Representative photos of EGFP expression in CHO-1 cells at mPPS-FA/DNA ratio of 15 after 24 h transfection (scale bar represents 5 μm).

and biocompatibility of mPPS while reducing protein adsorption and aggregation (Greenwald, 2001; Ogris et al., 1999). We further conjugated FA to mPPS using EDC.HCl and NHS as a coupling agent in hopes of achieving a level of cancer cell specificity. As it was not unequivocally determined which sites on the backbone were reacted with folic acid, the putative structure is not shown in Scheme 1.

We synthesized a series of mPPS-FA with different PEI-600, mPEG-2000, and sebacoyl chloride ratios, and tested their transfection efficiencies. The polymer that displayed the highest transfection efficiency was further characterized. The molecular structure of mPPS and mPPS-FA was characterized by ^{1}H NMR and FT-IR (Fig. 1A and B). Fig. 1A shows the ^{1}H NMR spectrum of PEI-600, mPEG-2000, and mPPS in D_2O. The ^{1}H NMR spectrum of sebacoyl chloride is not provided because it is not stable in D_2O. The peaks at δ 3.7 ppm were attributed to $-OCH_2-$ in PEG-2000. The peaks at δ 2.6–2.7 ppm were assigned to protons of $-CH_2CH_2-N$ in PEI-600. The ^{1}H NMR spectrum of sebacoyl chloride is not provided because it is not stable in D_2O. However, the peaks appearing at δ 1.18 and δ 1.46 ppm in mPPS-FA were assigned to $-CH_2-$ in sebacoyl chloride fragments. The stoichiometry of PEI-600, mPEG-2000, and sebacoyl chloride was approximated to be 5:2:1, calculated according to ^{1}H NMR. The conjugation of folic acid to mPPS was confirmed by FT-IR. MPPS-FA display IR absorbance peak at 1400 cm^{-1} corresponding *p*-amino benzoic acid of FA. The conjugation of folic acid to mPPS was also characterized by UV–Vis spectroscopy. The UV–Vis spectrum of mPPS-FA showed a maximum absorption at 280 nm and a broad shoulder peak at 370 nm, which are the characteristic peaks of folic acid (Fig. 1C).

Molecular weight and molecular weight distribution were measured by SEC with RI detector at 35 °C. The Mn and Mw of the mPPS were measured to be 10,155 g/mol and 5450 g/mol, respectively. The polydispersity index (Mw/Mn) is 1.86. For mPPS-FA, the Mn and Mw were 10,590 g/mol and 5975 g/mol with polydispersity of 1.77.

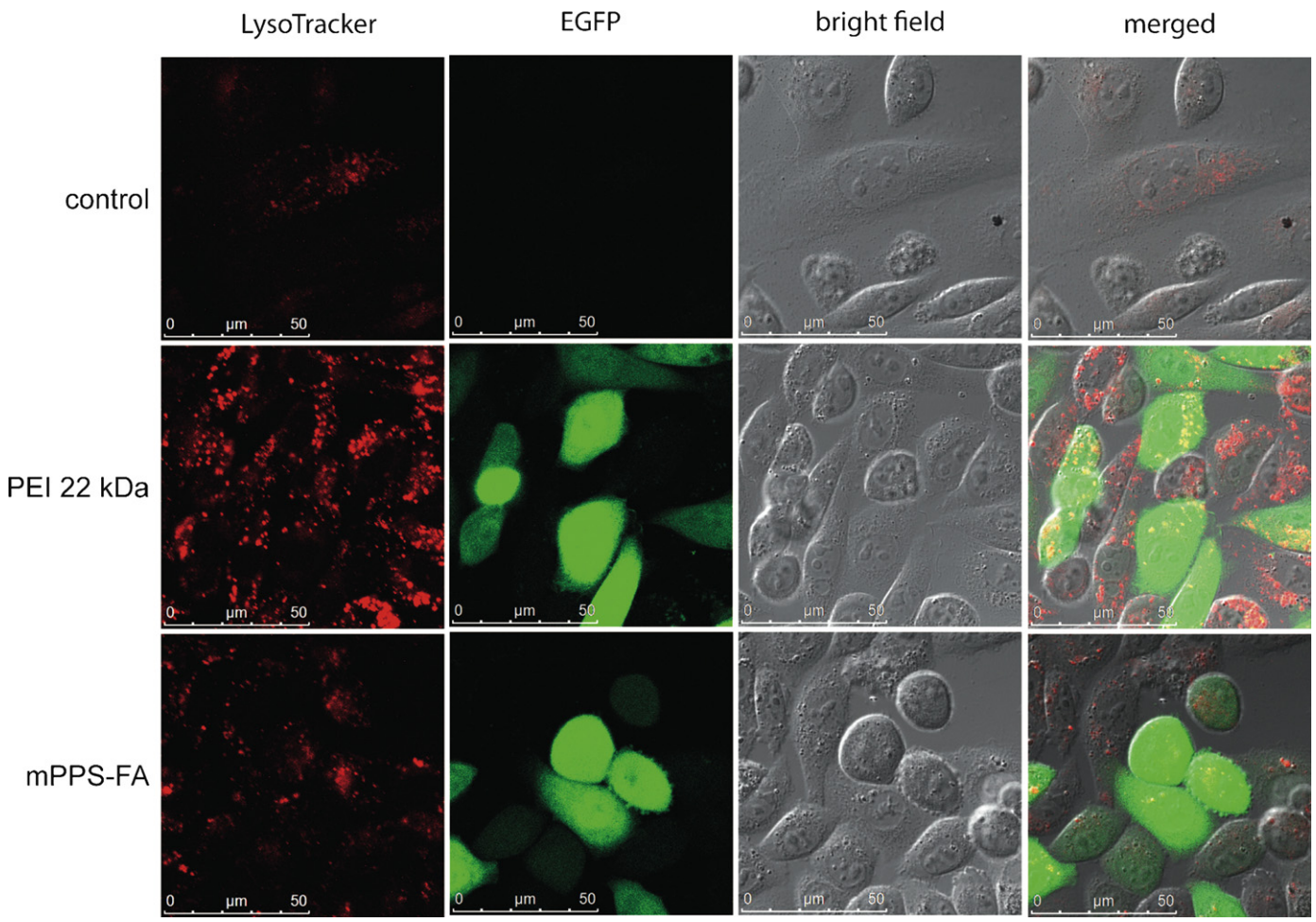


Fig. 7. Confocal fluorescence images (left, LysoTracker; left middle, EGFP), phase contrast (right middle), and merged images of CHO-1 cells (right). CHO-1 cells were mock transfected (top panel) or transfected with PEI 22kDa/DNA (middle panel) or mPPS-FA/DNA (bottom panel) for 48 h. Before image capture, 50 nM LysoTacker was used to stain cells for 30 min. After washing with PBS 3 times, red fluorescence signals of LysoTracker were generated by 543 nm He–Ne laser and acquired by the PMT channel at 560–620 nm and green signals of EGFP (right middle) were excited by 488 nm Argon laser and received by the PMT channel at 500–540 nm. All images were obtained on Leica SP5 with the same experimental conditions (40× oil objective, and 4× zoom factor; scale bar represents 50 μm). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

The polydispersity of mPPS and mPPS-FA is relatively high because a homobifunctional linker of sebacoyl chloride was used, thus making PEGylation of PEI-600 uncontrollable. New method of synthesis based on step-by-step would be applied to obtain more precisely controlled molecular-weight polymers rather than a broad distribution of molecular weights of polymers in the future.

3.2. Characterization of mPPS-FA/DNA complexes

The ability of mPPS-FA to bind DNA was demonstrated with an agarose gel shift assay. Fig. 2 depicts the migration of plasmid DNA (pEGFP) on an agarose gel (1%) in the presence of increasing concentrations of polymer mPPS-FA and mPPS. As shown in Fig. 2, retardation of DNA migration begins at mPPS-FA/DNA ratios as low as 1:1 (w/w) and migration is completely retarded at mPPS-FA/DNA ratio at 4:1 (w/w). The plasmid DNA was completely retarded at the ratio 3:1 (w/w) indicating that mPPS possesses a relatively greater DNA binding ability than that of mPPS-FA. This difference in DNA binding behavior between mPPS-FA and mPPS is probably caused by the partial substitution of positively charged primary amino groups by neutral folic acid, which results in lower surface charge and thus weaker DNA binding ability. The hydrophobicity of folic acid may also contribute to the reduction of mPPS-FA binding ability.

3.3. Particle size and zeta potential of mPPS-FA/DNA complexes

The mPPS-FA/DNA complexes were evaluated for particle size and zeta potential (Fig. 3A). Dynamic light scattering (DLS) studies using different mPPS-FA/DNA (w/w) ratios showed that the particle size of mPPS-FA/DNA complexes decreased with increasing mPPS-FA/DNA ratios (w/w). Particles with diameters less than 200 nm were obtained at ratios above 8:1 (w/w). After reaching a minimum of 70 nm at the ratio of 20:1 (w/w), the diameter increased slightly as weight ratio increased. To investigate the effect of conjugation of folic acid on the particle size, particle size of mPPS/DNA complexes was also measured. As shown in Fig. 3A, the size of mPPS/DNA was found to be smaller than mPPS-FA/DNA at each polymer/DNA ratio. This suggests that mPPS has higher surface positive charge density compared to mPPS-FA, and could form tighter complexes with DNA.

The relative surface charges of the resulting complexes were quantified with zeta potential measurements. Since the agarose gel shift assay showed that mPPS-FA completely complexes with DNA above the ratio of 4:1 (w/w), the zeta potentials were determined above that value. As shown in Fig. 3B, all the zeta potential values for mPPS-FA/DNA complexes were higher than +19 mV, indicating that an aqueous particle dispersion of complexes at a ratio above 4:1 (w/w) was stable. At the low mPPS-FA/DNA ratio of 4:1 (w/w) complexes tended to aggregate because there was not sufficient

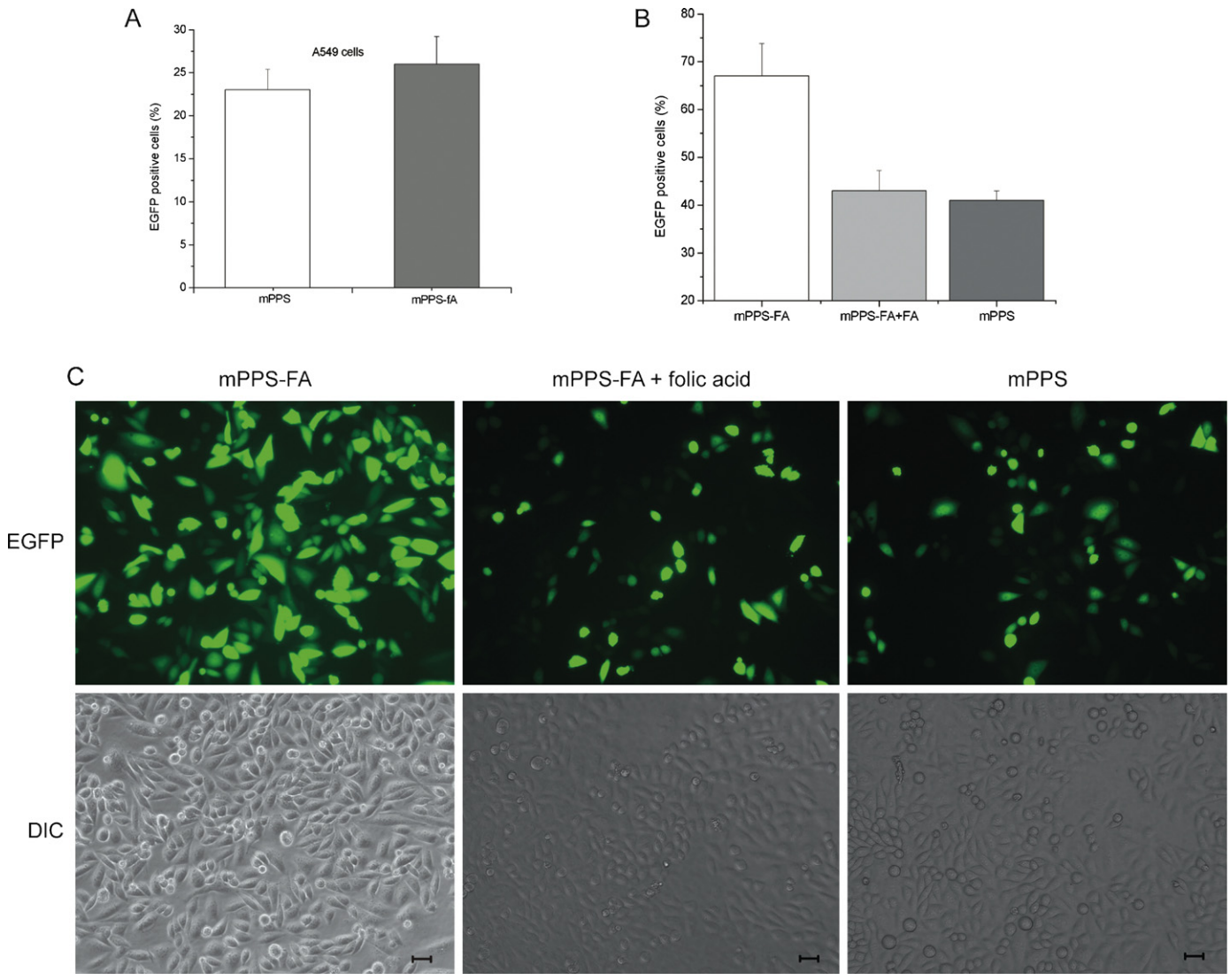


Fig. 8. Folic acid conjugation confers a targeting ability specific to cancer cells that overexpress FR. (A) Gene transfection efficiency of mPPS-FA and mPPS in A549 cells. (B) Gene transfection efficiency of mPPS-FA and mPPS in CHO-1 cells when 2 mM of folate was absent or present in the culture medium. (C) Representative photos of CHO-1 cells transfected by mPPS-FA and mPPS-FA at absence or presence of 2 mM of folic acid for 24 h (scale bar represents 5 μm).

positive charge to repel each other as was also confirmed by particle sizes measurements, which illustrated that the particle size at this ratio was larger than 550 nm. Comparatively, at the same polymer/DNA ratios, zeta potential values of mPPS/DNA complexes were higher.

The zeta potential under high salt conditions was measured to compare with the actual potential in biological buffer. Fig. 3C shows zeta potential of mPPS-FA/DNA complexes in phosphate buffered saline (PBS) with different concentrations at pH 7. The zeta potential decreases with increasing phosphate concentration, from +27 mV in 0.01 M PBS to +14 mV in 0.2 M PBS.

3.4. Visualization of mPPS/DNA and mPPS-FA/DNA complexes by TEM

The complexes of mPPS/DNA and mPPS-FA/DNA were characterized with TEM (Fig. 4). Representative TEM images of mPPS/DNA and mPPS-FA/DNA complexes are shown in Fig. 4. For mPPS/DNA complexes, condensation size decreases with increasing mPPS/DNA ratio, which can be attributed to the formation of tighter complexes between mPPS and DNA. Different from mPPS/DNA complexes, TEM revealed that well-defined spherical and mono-disperse complexes formed between mPPS-FA/DNA with estimated diameters around 160–180 nm across all polymer/DNA ratios from 8 to 20. Compare to mPPS/DNA complexes, the particle size of mPPS-FA/DNA complexes increased slightly most likely because of the formation of loosely packed polyplexes. However, at the mPPS-FA/DNA ratio of 20, the polyplexes were found not stable, and tended to form agglomerations, as seen in Fig. 4 (the lower right corner).

3.5. Cytotoxicity

A successful gene delivery vector must be capable of mediating sufficient levels of gene transfer and gene expression without compromising to the viability of the host cells. Toxicity of mPPS-FA was assessed in B16-F0, U87, CHO-1, and Ho-8910 cells by MTT assay, which exploits metabolic activity of cells as a measure of cell viability. Cytotoxicity was measured at polymer/DNA ratios (w/w) of 0:1, 4:1, 8:1, 10:1, 15:1, and 20:1. At all ratios, the four cell types treated with mPPS-FA/DNA complex had viability of >80%, which is much higher than that of cells treated with mPPS/DNA and PEI 22kDa/DNA complexes, indicating that mPPS-FA is less toxic than mPPS and L-PEI 22kDa (Fig. 5). As compared to mPPS/DNA

complexes, the reduced cytotoxicity of mPPS-FA/DNA complexes is believed to be a direct consequence of the reduced surface charge.

3.6. In vitro transfection efficiency of mPPS-FA

As shown in Fig. 6A, the gene transfection efficiency depends strongly on the cell type and the mPPS-FA/DNA ratio after 24 h transfection. The gene transfection efficiency increased with increased mPPS-FA/DNA ratio from 4:1 to a maximum of 15:1. At a mPPS-FA/DNA ratio of 4:1, the complexes with large size and neutral surface charge showed low gene expression, which is likely because the complexes do not possess favorable physicochemical characteristics required for cell adhesion, uptake, and lysosome escape (Moghimi et al., 2005; van de Wetering et al., 1997). At the mPPS-FA/DNA ratio of 20:1, gene expression levels drop, which is likely a result of higher cytotoxicity. B16-F0 cells, U87 cells, CHO-1 cells, and Ho-8910 cells were reported to overexpress FR (Joshi et al., 2010; Liu et al., 2007; Reimer et al., 1999; Xu et al., 2007), it is expected that mPPS-FA has a high gene transfection efficiency in these cells, at the polymer/DNA ratio of 15:1 (Fig. 6B). L-PEI 22kDa has proven to have a superior effect on transfection efficiency (Magin Lachmann et al., 2004; Wightman et al., 2001). Therefore, we compared the transfection efficiency of mPPS-FA with that of L-PEI 22kDa, as well as commercial transfection reagent Lipofectamine™2000. First, we determined their optimal conditions of L-PEI 22kDa and Lipofectamine™2000 when 1 μg of plasmid was used for each well in 24-well plate. The maximal transfection efficiency of L-PEI 22kDa was obtained at N/P ratio 40; while Lipofectamine™2000 displayed the highest transfection efficiency at the Lipofectamine™2000 (μL): DNA (μg) ratio of 2:1. As shown in Fig. 6B and C, transfection efficiency of mPPS-FA is statistically higher than that of L-PEI 22kDa and Lipofectamine™2000 in B16-F0, U87, CHO-1, and Ho-8910 cells. As the main chain contains PEI fractions possessing quaternary ammonium groups consisting of primary, secondary, and tertiary amines in a 1:2:1 ratio, mPPS-FA was believed to has strong lysosome buffering capacity thus facilitating endosomal escape (Kichler et al., 2001; Med, 2005). For comparison of the capacities of lysosomal buffering and endosomal escape of L-PEI 22kDa and mPPS-FA, CHO-1 cells were transfected with L-PEI 22kDa or mPPS-FA. After 48 h of transfection, lysosomes were stained with LysoTracker probes, followed by image acquisition by confocal microscopy. As shown in Fig. 7, lysosomes were found to be dispersive in the control cells, whereas lysosomes in L-PEI 22kDa-transfected cells appeared swollen probably due to trapping of large amounts of protonated amino groups of L-PEI 22kDa. Compared with L-PEI 22kDa-transfected cells, lysosomes in mPPS-FA-transfected cells also appeared swollen. However, number of swollen lysosomes in mPPS-FA-transfected cells was visibly smaller than that in the L-PEI 22kDa group, suggesting that some internalized mPPS-FA may have escaped from the lysosomes because of its lysosomal buffering capacity.

Another hypothesis is that a sebacoyl chloride spacer can provide some torsional flexibility of the backbone to maximize cation and phosphate interactions while decreasing the positive charge density on its surface. To test this, a control gene delivery vector (denoted as mPPT-FA) was synthesized in which the sebacoyl chloride spacer was replaced with a rigid linker terephthaloyl chloride (Fig. S1A and B). We found that the control polymer mPPT-FA could not form complexes with plasmid DNA even at the P/D ratio of 7 (Fig. S1C). It came as no surprise that the transfection efficiency of mPPT-FA was very low in CHO-1 cells (Fig. S1D).

To confirm our hypothesis that folic acid conjugation can confer a targeting specificity to cancer cells that highly express FR, we tested the transfection efficiency of mPPS-FA and mPPS in A549 cells that were reported to lowly express FR (Yoo and Park, 2004). We found that mPPS-FA has almost the same low transfection efficiency as that of mPPS (Fig. 8A). Whereas in high FR-expressing CHO-1 cells, mPPS-FA displayed much higher transfection efficiency than mPPS in CHO-1 cells. When 2 mM of folic acid was added into the culture medium, there was no difference in the transfection of mPPS-FA and mPPS, indicating that the presence of folic acid in the culture medium competitively inhibited the uptake of mPPS-FA by CHO-1cells (Fig. 8B and C).

4. Conclusion

A gene delivery vector, mPPS-FA, was successfully synthesized by conjugation of folic acid to a backbone produced by coupling methyl PEG-2000, PEI-600, and sebacoyl chloride. In this backbone, sebacoyl chloride is believed to provide a flexible distance between the PEI-600 fractions, thus reducing charge density of the polymer. This design is believed to provide torsionally flexible backbone which will maximize electrostatic interactions between the polymer and DNA and facilitate the formation of stable polyplexes. Methyl PEG-2000 fractions in mPPS-FA molecules were incorporated in order to improve its hydrophilicity while reducing its toxicity. In addition, the covalent attachment of folic acid to backbone can increase gene transfection efficiency in FA-bearing cells.

For our polymer, substantial cytotoxicity was observed only at concentrations well above the optimal transfection concentrations. *In vitro* transfections were also performed and it was found that EGFP expression was highest at polymer/DNA ratios of 15:1 in four high FR-expressing cell lines. Finally, EGFP reporter gene transfected with this polymer into a variety of cell lines that highly express FA gave better results than that of L-PEI 22kDa and Lipofectamine™2000. These results clearly show that mPPS-FA is an effective gene delivery system for *in vitro* transfection of plasmid DNA and its application *in vivo* warrants further investigation.

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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.2012.01.009.

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