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Brush-shaped polycation with poly(ethylenimine)-*b*-poly(ethylene glycol) side chains as highly efficient gene delivery vector

Xi-Qiu Liu[a], Jin-Zhi Du[b], Chu-Pei Zhang[a], Fang Zhao[c], Xian-Zhu Yang[a], Jun Wang[a,*]

[a] Hefei National Laboratory for Physical Sciences at the Microscale and School of Life Sciences, University of Science and Technology of China, Hefei, Anhui 230027, PR China
[b] Department of Polymer Science and Engineering, University of Science and Technology of China, Hefei, Anhui 230026, PR China
[c] Department of Chemical Physics, University of Science and Technology of China, Hefei, Anhui 230026, PR China

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ABSTRACT

A brush-shaped polymer PHEMA-*g*-(PEI-*b*-PEG) with poly(2-hydroxyethyl methacrylate) (PHEMA) backbone and linear poly(ethylenimine)-*b*-poly(ethylene glycol) (PEI-*b*-PEG) side chains was synthesized and evaluated as a vector for potential cancer gene therapy. The characterizations by ^{1}H NMR and laser light scattering demonstrated the brush structure of the polymer. PHEMA-*g*-(PEI-*b*-PEG) was much less cytotoxic when compared with branched poly(ethylenimine) with M_w of 25 kDa. The capacity of plasmid DNA condensation by PHEMA-*g*-(PEI-*b*-PEG) was demonstrated by gel retardation assay, and they formed nanosized complexes with surface zeta potential around 20 mV at N/P ratios higher than 5:1. The complexes of PHEMA-*g*-(PEI-*b*-PEG) with plasmid DNA were more efficiently internalized by BT474 cells in comparison with the complexes of PEI25K, leading to higher gene transfection in cells. Further investigation using complexes of PHEMA-*g*-(PEI-*b*-PEG) with plasmid DNA encoding wild-type p53 gene showed its potential as a carrier for cancer gene therapy. The complexes of PHEMA-*g*-(PEI-*b*-PEG) successfully induced elevated wild-type p53 expression in BT474 cells and led to enhanced apoptosis of BT474 cells. Transfection of wild-type p53 using the complexes also significantly increased the sensitivity of BT474 cells to doxorubicin chemotherapy, suggesting the potential of this carrier in cancer gene therapy.

1. Introduction

The success in designing and preparing efficient gene vectors provides strong support for gene therapy. As a typical non-viral vector, cationic polyethyleneimine (PEI) is effective in gene delivery due to its proton sponge effect which can buffer the environment of endosome and cause the release of DNA to cytoplasm (Boussif et al., 1995; Akinc et al., 2005). It has been reported that the transfection efficiency and cytotoxicity of PEI are highly dependent on the molecular weight and structure (Dunlap et al., 1997; Godbey et al., 1999; Mintzer and Simanek, 2009). High molecular weight PEIs exhibit better transfection activity but also show higher cytotoxicity (Fischer et al., 1999; Godbey et al., 1999). On the contrast, PEI with molecular weight lower than 2 kDa is much less toxic but has almost no transfection activity (Baker et al., 1997; Fischer et al., 1999; Godbey et al., 1999). In view of this, great attention has been paid to develop gene vectors with high transfection efficiency and low cytotoxicity using low molecular weight PEI. A rational way is connecting low molecular weight PEI with biodegradable linkages (Gosselin et al., 2001; Petersen et al., 2002; Forrest et al., 2003; Kim et al., 2005; Peng et al., 2008). For example, Peng et al. synthesized a disulfide cross-linked PEI via thiolation of low molecular weight PEI and which was proved to be an efficient and non-toxic gene delivery system (Peng et al., 2008). An alternative strategy is to conjugate low molecular weight PEI to a polymeric backbone such as polysaccharide or polypeptide (Xiong et al., 2007; Lu et al., 2008; Sun et al., 2008; Wen et al., 2009). For example, Xiong et al. developed grafted PEI analogue to polyaspartate and demonstrated its low toxicity and high transfection efficiency for gene delivery (Xiong et al., 2007). The transfection efficiencies of those low molecular weight PEI-based carriers were generally comparable to that of branched PEI with M_w of 25 kDa (PEI25K) or even higher, while the cytotoxicity was significantly reduced (Tang et al., 2006; Wong et al., 2006; Jiang et al., 2007; Wen et al., 2009).

p53 is an important tumor suppressor, and in certain conditions such as DNA damage or deregulation of mitogenic oncogenes p53 can inhibit cell proliferation by leading to the induction of various cell checkpoints, apoptosis or cellular senescence (Prives, 1998; Sherr, 2004; Harris and Levine, 2005). p53 mutations increase cell survival and proliferation and can promote resistance to chemotherapies in some settings (Eastham et al., 2000; Giuliano et al., 2000; Harris and Levine, 2005; Bouchet et al., 2006). As loss of p53 function is a common feature of human cancer cells, it has been demonstrated that restoring p53 function with a functional

* Corresponding author Tel.: +86 551 3600335; fax: +86 551 3600402.
E-mail address: jwang699@ustc.edu.cn (J. Wang).

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wild-type copy can treat cancer (Fuster et al., 2007). It is also believed that restoring of p53 function in tumor cells may block tumor development and sensitize cells to cytotoxic therapeutics (Putzer et al., 1998).

We design a well-defined brush-shaped cationic polymer bearing low molecular weight PEI as a non-viral gene vector for gene therapy. The polymer consists of poly(2-hydroxylethyl methacrylate) (PHEMA) backbone and poly(ethylenimine)-*b*-poly(ethylene glycol) side chains, which is denoted as PHEMA-*g*-(PEI-*b*-PEG). PEI segment is expected to condense DNA while PEG is expected to increase the stability of complexes and potentially extends blood circulation in systemic administration (Woodle and Lasic, 1992; Torchilin, 2001). We investigate the *in vitro* cytotoxicity and transfection efficiency, and further use it to deliver wild-type p53 gene to cancer cells for restoring p53 activity. The combination of p53 gene therapy with doxorubicin chemotherapy for enhanced efficiency in inhibiting the growth of cancer cells is also studied.

2. Materials and methods

2.1. Materials

2-Hydroxyethyl methacrylate (HEMA, Acros Organics) was purified according to the literature (Beers et al., 1999). Ethyl 2-bromoisobutyrate (EBiB, Aldrich) was distilled just before use. Pentamethyldiethylenetriamine (PMDETA, Acros Organics), 2-methyl-2-oxazoline (MeOZO, Aldrich), acetonitrile, pyridine and ethanol were purified with CaH_2, followed by distillations. CuBr was purified in acetic acid, followed by washing with methanol and drying under vacuum. The macroinitiator α-methoxy-ω-4-toluene-sulfonate poly(ethylene glycol) (mPEG-OTs) was synthesized using monomethoxy poly(ethylene glycol) (M_n = 2000) according to the literature (Brissault et al., 2002; Zhong et al., 2005). Other reagents were used without further purification.

2.2. Synthesis of the polymer (Scheme 1)

2.2.1. Synthesis of the linear poly(ethylenimine)-b-poly(ethylene glycol) with hydroxyl end group (mPEG-b-PEI-OH)

mPEG-*b*-PEI-OH was synthesized by cationic polymerization of MeOZO under the initiation of mPEG-OTs, followed by the hydrolysis under acidic condition according to a reported procedure (Zhong et al., 2005). In a glove box under nitrogen atmosphere, a reaction vessel with mPEG-OTs (3.10 g, 1.44 mmol), MeOZO (6.11 g, 71.9 mmol), and CH_3CN (18 mL) was placed in an oil bath. The mixture was stirred for 48 h at 70 °C and then cooled to room temperature. To this mixture, was introduced 2 mL of methanolic KOH solution (0.1 N) to transfer the polymer chain end to hydroxyl group. The mixture was then passed through silica gel and concentrated under reduced pressure. The residue was precipitated in excess diethyl ether and mPEG-*b*-PMeOZO-OH was obtained after drying under vacuum. Yield: 5.40 g (58.7%). ^{1}H NMR ($CDCl_3$, ppm): 3.63 (–C***H***$_2$C***H***$_2$O–), 3.45 (–C***H***$_2$C***H***$_2$N–), 3.37 (–OC***H***$_3$), 2.07–2.13 (–C(O)C***H***$_3$).

mPEG-*b*-PMeOZO-OH (1.7 g) was dissolved in 12 mL of aqueous HCl solution (10 wt %) and the mixture was refluxed overnight under a nitrogen atmosphere. The pH of the reaction mixture was then adjusted to 10 with NaOH pellet, which led to precipitation of the resulted polymer. The precipitate was collected by centrifugation and it was washed twice with deionized water. mPEG-*b*-PEI-OH was obtained by subsequent lyophilization. Yield: 0.80 g (67.8%). ^{1}H NMR (DMSO-d_6, ppm): 3.50 (–C***H***$_2$C***H***$_2$O–), 2.57 (–C***H***$_2$C***H***$_2$NH–). The degree of polymerization (DP) of PMeOZO block was determined to be 40 according to ^{1}H NMR analysis.

2.2.2. Synthesis of PHEMA with side carboxyl groups (PHEMA-COOH)

A mixture of freshly purified HEMA (6.5 g, 0.05 mol), PMDETA (21 μL, 0.1 mmol) and ethanol (12.2 mL) in a dry glass tube was degassed via three freeze–thaw cycles, followed by addition of CuBr (14.4 mg, 0.1 mmol). The tube was further freezed for a moment and subsequently sealed under vacuum. The polymerization was carried out at 70 °C for 24 h. After cooling to room temperature, the tube was opened to air and stirred overnight. The crud product was diluted with ethanol and purified by passing the mixture through an Al_2O_3 column to remove copper catalyst. After concentration by a rotary evaporator under vacuum, the mixture was precipitated twice in water. The precipitate was collected and dried under vacuum to a constant weight to obtain PHEMA.

PHEMA (0.13 g, 1.0 mmol hydroxyl groups) was dissolved in anhydrous pyridine (5 mL) in a fresh dried round-bottomed flask and succinic anhydride (0.2 g, 2.0 mmol) was added. The mixture was stirred at 25 °C for 48 h. Thereafter, methanol (0.5 mL) was added to the flask to consume unreacted succinic anhydride. The resulted mixture was subjected to dialysis against water for 48 h to remove small molecule impurities, and PHEMA-COOH was obtained by lyophilization.

2.2.3. Synthesis of PHEMA-g-(PEI-b-PEG)

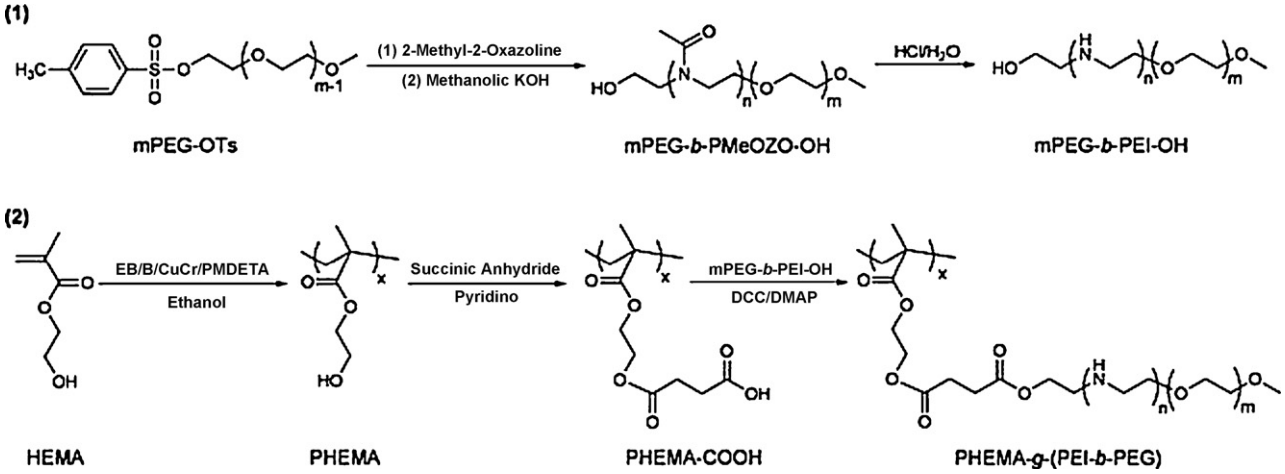
PHEMA-*g*-(PEI-*b*-PEG) was synthesized by the coupling reaction between PHEMA-COOH and mPEG-*b*-PEI-OH. PHEMA-COOH (8 mg, 3.45×10^{-2} mmol –COOH), mPEG-*b*-PEI-OH (0.13 g, 1 equiv to carboxyl groups), DCC (14.2 mg, 2 equiv) and DMAP (2.1 mg, 0.5 equiv) were dissolved in 20 mL of DMF/DMSO (50/50, v/v). The reaction was performed at room temperature for 48 h under N_2 atmosphere. Then, the solvents were evaporated under vacuum, followed by addition of 5 mL of deionized water. The solution was acidified with 1 N HCl. After centrifugation to remove the insoluble byproduct, the supernatant was dialyzed against deionized water using a membrane tubing (MWCO 15 000 Da) to remove unreacted mPEG-*b*-PEI-OH. The final product PHEMA-*g*-(PEI-*b*-PEG) was obtained by lyophilization.

2.3. Characterization of the polymer

^{1}H NMR spectra were recorded on a Bruker AV300 NMR spectrometer. Molecular weight of PHEMA-*g*-(PEI-*b*-PEG) was determined by laser light scattering (LLS) using a modified commercial LLS spectrometer (ALV/SP-125), which is equipped with an ALV-5000 multi-τ digital time correlator and a cylindrical 22 mW UNIPHASE He–Ne laser (λ_0 = 632 nm) as the light source. Detailed procedure for the measurement was following the previous report (Siu et al., 2002).

2.4. Cell lines and cell culture

HEK293 human embryonic kidney cells and BT474 breast cancer cells from ATCC were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, HyClone), streptomycin at 100 μg mL^{-1}, penicillin at 100 U mL^{-1}, and 4 mM L-glutamine at 37 °C in a humidified 5% CO_2-containing atmosphere. Mouse osteoblast cells were isolated from calvarias of 4-day-old neonatal Sprague–Dawley rats according to a standard procedure (Ishaug et al., 1994; Bakker and Klein-Nulend, 2003) and maintained in DMEM supplemented with 10% fetal bovine serum, penicillin (100 U mL^{-1}), streptomycin sulfate (100 U mL^{-1}), gentamycin (50 mg mL^{-1}), fungizone (1.25 mg mL^{-1}), ascorbate (100 mg mL^{-1}) at 37 °C in a humidified 5% CO_2-containing atmosphere.



Scheme 1. Synthesis of mPEG-*b*-PEI-OH (1) and PHEMA-*g*-(PEG-*b*-PEI) (2).

2.5. Amplification and purification of plasmid DNA (pDNA)

pGL3-Luc plasmid encoding firefly luciferase, pEGFP-N2 encoding enhanced green fluorescence protein and pcDNA3-p53 plasmid encoding wild-type p53 (a gift from Prof. M. Wu of USTC) were transformed in *Escherichia coli* DH5α and amplified in Terrific Broth media at 37 °C overnight with 250 rpm. The amplified pDNA was purified by a QIAGEN Giga plasmid purification kit according to the supplied protocol. Purified pDNA was dissolved in Tris–EDTA (TE) buffer, and its purity and concentration were determined by ultraviolet absorbance at 260 and 280 nm.

2.6. Cytotoxicity assay

The cytotoxicity of the brush-shaped polymer PHEMA-*g*-(PEI-*b*-PEG) to HEK293 cells was evaluated by MTT assay. HEK293 cells were seeded in a 96-well Plate 24 h before the assay at a density of 15 000 cells per well. The cells were then incubated for 24 h with 100 μL of complete DMEM containing PHEMA-*g*-(PEI-*b*-PEG) or PEI25K (Sigma, branched PEI with M_w 25 K) at different concentrations ranging from 0 to 125 μg mL^{-1}. MTT stock solution (25 μL, 5 mg mL^{-1} in PBS) was then added to each well and the cells were further incubated for 2 h. The extraction buffer (100 μL, 20% SDS in 50% DMF, pH 4.7, prepared at 37 °C) was added to the wells and incubated overnight at 37 °C. The absorbance of the solution was measured at 570 nm using a Bio-Rad 680 microplate reader and cell viability was normalized to that of HEK293 cells cultured in medium without polymer.

2.7. Preparation of complexes and DNA gel retardation assay

Complexes were prepared in 5% glucose by mixing different amount of PHEMA-*g*-(PEI-*b*-PEG) with pDNA to achieve various N/P ratios, and the resulting solution was vortexed for 30 s. PEI25K/pDNA complexes were prepared similarly. The complexes were incubated at room temperature for 30 min. Complexes with N/P ratios from 1:1 to 4:1 were analyzed by gel electrophoresis using 1% agarose gel, stained with ethidium bromide and visualized on a UV illuminator.

2.8. Particle size and zeta-potential measurements

Measurements of particle size and zeta potential of complexes were performed using a Zetasizer Nano ZS90 (Malvern Instruments, UK). Complexes were freshly prepared before measurements according to the procedure described above. The size measurement was performed at 25 °C at a 90° scattering angle. The mean hydrodynamic diameter was determined by cumulative analysis. The zeta-potential measurements were performed using an aqueous dip cell in an automatic mode.

2.9. Cellular uptake of complexes

Cellular uptake of complexes by BT474 cells was determined according to the literature (Remy-Kristensen et al., 2001). BT474 cells were seeded in a 24-well plate with 0.5 mL of complete DMEM medium and incubated for 24 h, yielding about 70% confluence. pDNA was fluorescently labeled by YOYO-1 at a ratio of one dye for 300 bases for 1 h at room temperature in the dark. Complexes were then prepared with YOYO-1 labeled pDNA and incubated at 37 °C with cells at 2.0 μg DNA/well for 4 h. Subsequently, cells were rinsed with phosphate buffered saline (PBS, 0.01 M, pH 7.4) to remove the cell surface-bound complexes. The cells were trypsinized, pelleted and resuspended in PBS. The residual fluorescence out of the cell membrane was quenched with 0.4% trypan blue for 2 min. Then cells were centrifugated and washed three times with PBS and subjected to flow cytometric analyses using a Becton Dickinson FACSCalibur flow cytometer.

2.10. In vitro transfection

In vitro transfection efficiency of complexes was evaluated in HEK293, BT474, and mouse osteoblast cells using both luciferase and EGFP as marker genes. Cells were seeded in 24-well plates at appropriate densities (7×10^4 for HEK293 cells, 1×10^5 for BT474 cells, and 5×10^4 for mouse osteoblast cells) with 1 mL of complete DMEM to achieve 70% confluence after 24 h incubation. At the time of transfection, the medium in each well was replaced with 400 μL of complete DMEM medium without antibiotics, followed by addition of various polymer/pDNA complexes containing 2 μg pEFGP-N2 or pGL3-Luc. Transfection with Lipofectamine 2000™ (Invitrogen) was performed by incubating the complexes with cells according to the manufacture's protocol. All of the transfections were performed in duplicate. After 48 h incubation, cells transfected with pEGFP-N2 were observed under a Nikon TE2000-U fluorescence microscope. Cells transfected with pGL3-Luc were washed with PBS and treated with 200 μL of cell lysis buffer (Promega). The luciferase activity in cell extracts was measured using a luciferase assay kit (Promega) on a luminometer (VERITAS microplate luminometer, Turner Biosystems) for 10 s. The relative

light units (RLU) were normalized against protein concentrations in the cell extracts, which were measured using a BCA protein assay kit (Pierce). Luciferase activity was expressed as relative light units (RLU/mg of protein in the cell lysate).

2.11. Western blotting

BT474 cells were transfected with complexes containing pcDNA3-p53 (10 μg per well in 6-well plates) for 48 h in a similar way as described above. Cells were lysed and proteins (50 μg) of whole cell lysate were treated with an electrophoresis loading buffer containing 5% (w/v) 2-mercaptoethanol, following analyses by SDS-PAGE. After electrophoresis, proteins were electrophoretically transferred to Immobilon™ transfer membrane (Millipore) and pre-incubated with a blocking reagent (5% skim milk) at room temperature for 1 h. Subsequently, the membrane was divided into two parts and incubated separately with primary antibodies for β-actin and p53 (1:5000 and 1:1000 dilution, respectively, Santa Cruz Biotechnology) overnight at 4 °C. They were labeled with horseradish peroxidase conjugated-antibody to mouse IgG (1:5000 dilution, Santa Cruz Biotechnology) at room temperature for 1 h. The chemiluminescence detection was then performed with SuperSignal West Pico Chemiluminescent Substrate (Pierce).

2.12. In situ cell death detection

BT474 cells (5×10^5) were seeded on the coverslip coated with poly-L-lysine (Sigma) in a 6-well tissue culture plate and transfected 24 h later with various complexes containing pcDNA3-p53 according to a procedure described above. The dose of pDNA was 10 μg per well. After 48 h, cells were fixed with 4% paraformaldehyde in PBS for 1 h at room temperature and rinsed with PBS. The cells were further incubated in 0.1% sodium citrate containing 0.1% Triton X-100 for 2 min on ice. After being rinsed twice with PBS, 50 μL of TUNEL reaction mixture (Roche) was added on the coverslips and the samples were incubated in a humidified atmosphere for 60 min at 37 °C in the dark. Samples were directly analyzed under a fluorescence microscope using an excitation wavelength in the range of 450–500 nm and detection in the range of 515–565 nm.

2.13. Cell viability assay of doxorubicin chemotherapy with wild-type p53 induction

For quantifying sensitization of the breast tumor cells to doxorubicin by wild-type p53 induction, BT474 cells were seeded at a density of 5×10^3 cells/well in a 96-well plate and after 24 h transfected with PHEMA-*g*-(PEI-*b*-PEG)/pcDNA3-p53 complexes (0.25 μg pDNA per well, N/P = 25). After 24 h incubation, the culture medium was replaced with 100 μL of fresh medium containing doxorubicin at various concentrations. After 24 h incubation, the cell viability was determined by MTT assay as described above.

To further confirm the combined effect of p53 gene therapy and doxorubicin chemotherapy, BT474 cells were treated with PHEMA-*g*-(PEI-*b*-PEG)/pcDNA3-p53 complexes (0.25 μg pDNA per well in 96-well plate, N/P = 25) with or without 1 μg mL^{-1} doxorubicin added. After incubation for 48 h, the cell viability was determined by MTT assay as described above.

2.14. Statistical analysis

All data were expressed as mean ± SD and compared between groups using the Student's *t*-test. Differences were considered statistically significant at $P < 0.05$.

3. Results and discussion

3.1. Synthesis and characterization of PHEMA-g-(PEI-b-PEG)

PHEMA-*g*-(PEI-*b*-PEG) was obtained by coupling mPEG-*b*-PEI-OH with PHEMA-COOH as shown in Scheme 1. In the first step, the hydroxyl-terminated mPEG-PMeOZO-OH was synthesized by cationic polymerization of 2-oxazoline monomer using mPEG-OTs as the initiator, followed by hydrolysis according to reported procedures after minor modification (Wang and Hsiue, 2003; Zhong et al., 2005). Characterization of mPEG-*b*-PMeOZO-OH by ^{1}H NMR showed resonances at 2.07–2.13, 3.45 and 3.63 ppm, assigned to methyl and ethylene protons of PMeOZO block and ethylene protons from poly(ethylene glycol) block. The degree of polymerization (DP) of PMeOZO block was determined to be 40, on the basis of the integral ratio of resonances at 2.07–2.13 and 3.63 ppm. mPEG-*b*-PMeOZO-OH was hydrolyzed in 12 wt% aqueous HCl solution and ^{1}H NMR of resulted polymer was analyzed in DMSO-d_6 to verify the structure. The proton resonance at 2.07-2.13 ppm of the acetyl group disappeared; meanwhile resonance assigned to ethylene protons of PEI backbone shifted to 2.57 ppm, indicating the successful deprotection.

On the other hand, PHEMA-COOH was synthesized by converting hydroxyl groups of PHEMA to carboxyl groups. PHEMA was firstly synthesized by atom transfer radical polymerization of HEMA in the presence of CuBr/PMDETA as the catalyst and EBiB as the initiator. The conversion of HEMA monomer was 38%, which was estimated by in situ ^{1}H NMR measurement. Therefore, the DP of HEMA was 152, based on the equation of $[HEMA]_0/[EBiB]_0 \times 38\%$, where $[HEMA]_0$ and $[EBiB]_0$ were the initial concentration of HEMA and EBiB, respectively. The ^{1}H NMR spectrum of PHEMA displayed in Fig. 1A demonstrated the correct structure. Conversion of hydroxyl groups of PHEMA to carboxyl groups was fulfilled by a reaction of PHEMA with excess succinic anhydride. The efficiency of such a method is very close to 100% according to the literature (Cai and Armes, 2005). ^{1}H NMR spectrum of PHEMA-COOH shown in Fig. 1B showed complete disappearance of hydroxyl protons at 4.80 ppm, and resonances of the methylene protons shifted to 4.05 and 4.20 ppm from 3.55 and 3.90 ppm, demonstrating the successful formation of ester linkages. It should be noted that the methylene protons of succinic anhydride segment were overlayed with the solvent signals at 2.50 ppm. This was confirmed when D_2O was used as solvent (data not shown).

The coupling of PHEMA-COOH and mPEG-*b*-PEI-OH was performed in the presence of DCC and DMAP. Unreacted mPEG-*b*-PEI-OH was removed by dialysis. The ^{1}H NMR spectrum of PHEMA-*g*-(PEI-*b*-mPEG) showed (Fig. 1C) clear signals at 2.73 and 3.50 ppm, assigned to methylene protons of PEI and PEG, respectively. Noteworthily, proton signals from PHEMA backbone were not detected due to unclear reason, which has also happened in other PHEMA-based brush polymers (Lee et al., 2006; Yuan et al., 2007).

The molecular weight of PHEMA-*g*-(PEI-*b*-PEG) was determined by light laser scattering in aqueous solution at pH 7.0, and the result indicated that the apparent M_w was 509 000 g/mol, whereas PEI-*b*-PEG diblock copolymer could not be detected under the same conditions due to the week scattering intensity. The calculated grafting efficiency of side chains was around 74%. Besides, R_g/R_h determined by LLS measurements indicated that the R_g/R_h was 1.1 and 1.7 at pH 7.0 and 5.0, respectively, implying that it adopted cylindrical brush topology at pH 5.0 (Storkle et al., 2007). This was not surprising due to the repulsion of the dense PEI-*b*-PEG side chains, which were stretched at acid condition owing to the protonation (Benns et al., 2000; Lee et al., 2006; Storkle et al., 2007).

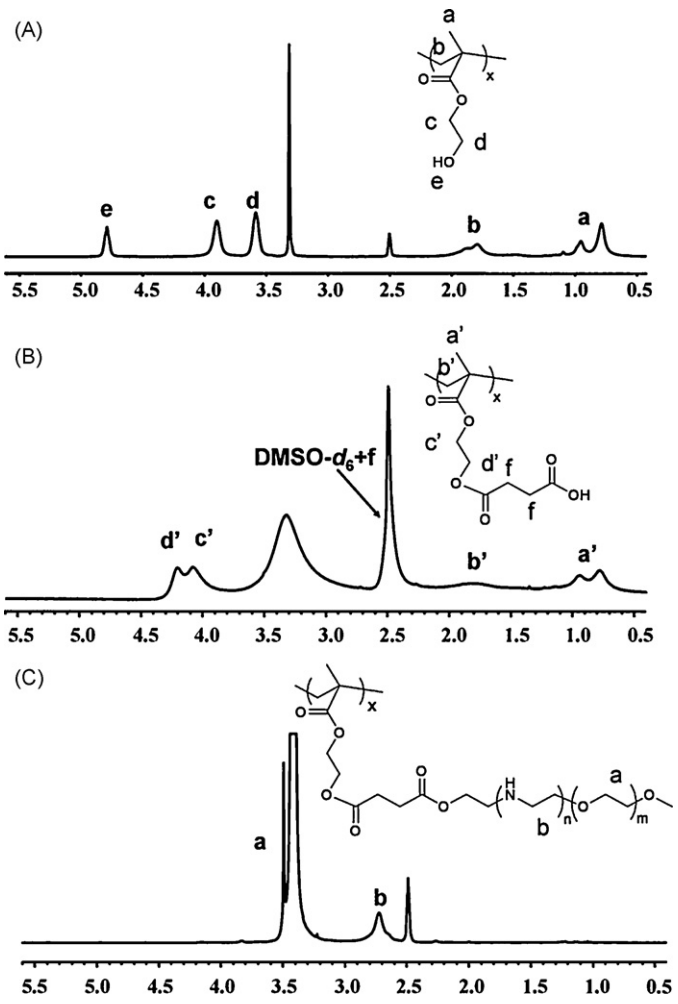


Fig. 1. ^{1}H NMR spectra of PHEMA (A), PHEMA-COOH (B), and PHEMA-*g*-(PEG-*b*-PEI) (C) in DMSO-d_6 (ppm).

3.2. Cytotoxicity of PHEMA-g-(PEI-b-PEG)

The cytotoxicity of PHEMA-*g*-(PEI-*b*-PEG) was assessed in a cell culture assay using HEK293 cells, which was performed in comparison with branched PEI25K. Cell viability was analyzed after the culture with polymer for 24 h using a standard MTT method. The results in Fig. 2 showed that PEI25K exhibited high toxicity to HEK293 cells with a LD_{50} (median lethal dose) below 20 μg mL^{-1}. However, PHEMA-*g*-(PEI-*b*-PEG) was tolerated by the cells up to a dose of 125 μg mL^{-1}. It has been demonstrated that PEI with lower molecular weights are less cytotoxic than that with higher molecular weights (Fischer et al., 2003), and linear PEI with low molecular weights exhibits even better cytocompatibility (Lee et al., 2007). The brush-shaped cationic polymer with grafted linear PEI-*b*-PEG side chains in this study led to a minimal toxicity to cells, which would be advantageous as a carrier for gene delivery.

3.3. Characterization of PHEMA-g-(PEI-b-PEG)/pDNA complexes

Gel retardation assay was performed to investigate whether the PHEMA-*g*-(PEI-*b*-PEG) could bind pDNA. As shown in Fig. 3A, pDNA was completely retarded by the brush-shaped polymer as the N/P ratio increased to 2:1, which was almost comparable to PEI25K.

Effective transfection efficiency of a gene carrier depends on its ability to condense negatively charged pDNA to nanosized particles so as to easily enter into the cells (Arote et al., 2007). To better understand the DNA condensation properties of the brush-shaped polymer, dynamic light scattering was used to measure the particle sizes of PHEMA-*g*-(PEI-*b*-PEG)/pDNA complexes at various N/P ratios. At the N/P ratio around 2.5:1, the average diameters of complexes suddenly increased from 139 to 432 nm (Fig. 3B) and it may be because particles began to aggregate while the zeta potential was close to 0 mV (Fig. 3C). At elevated N/P ratios above 10:1, PHEMA-*g*-(PEI-*b*-PEG) condensed pDNA into small particles in the range of 100–150 nm (Fig. 3B) with a positive zeta potential of 20 mV (Fig. 3C), which was suitable for endocytotic cellular uptake (Mislick and Baldeschwieler, 1996).

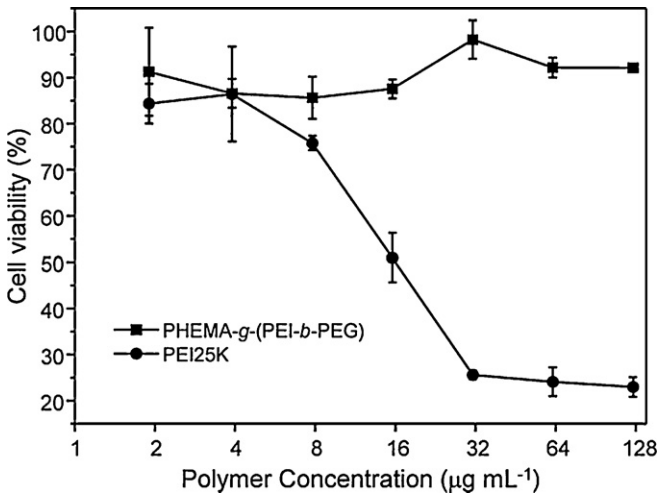


Fig. 2. Cytotoxicity of PHEMA-*g*-(PEI-*b*-PEG) and PEI25K to HEK293 cells.

3.4. Cell internalization of PHEMA-g-(PEI-b-PEG)/pDNA complexes

As a first step to investigate whether PHEMA-*g*-(PEI-*b*-PEG) could be an appropriate gene carrier, we analyzed cellular internalization of PHEMA-*g*-(PEI-*b*-PEG)/pDNA complexes by BT474 cells. Fig. 4 showed the cellular accumulation of fluorescence-labeled pDNA analyzed by flow cytometer. The cell fluorescence intensities

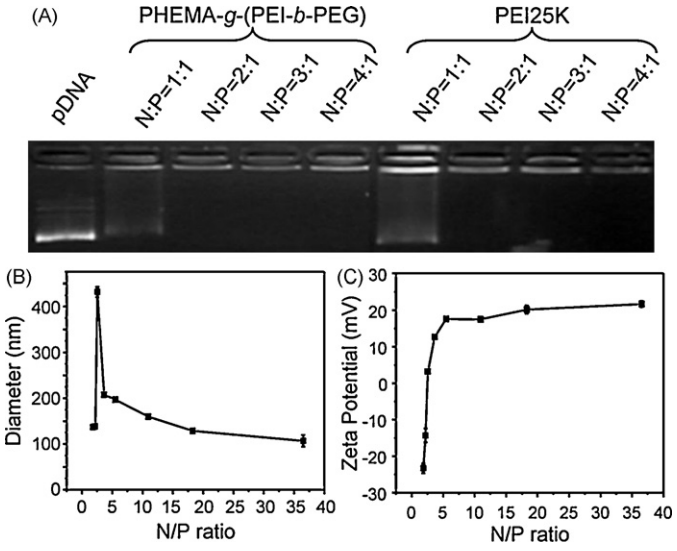


Fig. 3. Analyses of DNA binding properties of PHEMA-*g*-(PEI-*b*-PEG) and the complexes: (A) Agarose gel electrophoresis retardation assay; (B) particle size of PHEMA-*g*-(PEI-*b*-PEG)/pDNA complexes at various N/P ratios; (C) zeta potential of PHEMA-*g*-(PEI-*b*-PEG)/pDNA complexes at various N/P ratios.

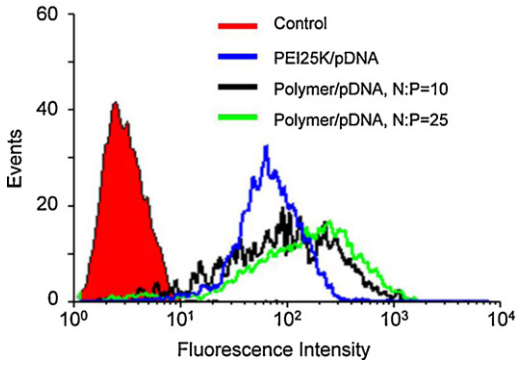


Fig. 4. Internalization of fluorescent labeled pDNA by BT474 cells: red, cells without any treatment; blue, cells incubated with PEI25K/pDNA complexes; black, cells incubated with PHEMA-*g*-(PEI-*b*-PEG)/pDNA complexes at N/P ratio of 10:1; green, cells incubated with PHEMA-*g*-(PEI-*b*-PEG)/pDNA complexes at N/P ratio of 25:1. Polymer stands for PHEMA-*g*-(PEI-*b*-PEG). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

after 4 h treatment with PHEMA-*g*-(PEI-*b*-PEG)/pDNA complexes at N/P ratios of 10:1 and 25:1 were significantly higher than those treated with PEI25K/pDNA complexes at N/P ratio of 10:1. This demonstrated that more PHEMA-*g*-(PEI-*b*-PEG)/pDNA complexes were accumulated in the cells, which implied that PHEMA-*g*-(PEI-*b*-PEG) might be able to induce higher transfection efficiency than PEI25K.

3.5. *In vitro transfection efficiency*

The transfection efficiency of PHEMA-*g*-(PEI-*b*-PEG)/pDNA complexes was then evaluated in HEK293 cells, BT474 cells, and mouse primary osteoblast cells in the presence of serum. Cells were incubated with 2 μg of pGL3-Luc complexed with PHEMA-*g*-(PEI-*b*-PEG) at N/P ratios from 10:1 to 50:1, while the concentrations of PHEMA-g-(PEI-b-PEG) were from 11.4 to 57 μg mL^{-1}.

The luciferase enzyme activity was measured and normalized to the total cellular protein. As shown in Fig. 5, when the N/P ratios were 25:1 and 50:1, luciferase expression level in HEK293 cells transfected with PHEMA-*g*-(PEI-*b*-PEG)/pDNA complexes was 5.5 to 7.0-fold higher than that transfected with PEI25K/pDNA ones. Meanwhile, luciferase expression in BT474 and mouse osteoblast cells after transfection with PHEMA-*g*-(PEI-*b*-PEG)/pDNA complexes was notably higher than that after transfection with PEI25K/pDNA ones, even when the N/P ratio was lowered to 10:1.

GFP expression in the transfected cells also revealed the remarkable transfection ability of PHEMA-*g*-(PEI-*b*-PEG) (Fig. 6). In HEK293 cells, there were comparable percentages of GFP-expressed cells in the treatments with PHEMA-*g*-(PEI-*b*-PEG) (at N/P ratio of 25:1), Lipofectamine 2000™ and PEI25K as carriers. In BT474 cells, remarkably higher transfection efficiency of PHEMA-*g*-(PEI-*b*-PEG) complexes was observed when compared with that of PEI25K complexes.

Combined the above results, it can be concluded that PHEMA-*g*-(PEI-*b*-PEG) is superior to PEI25K in various cell lines with much higher transfection efficiency and lower cytotoxicity. In agreement with the uptake result described above, the transfection efficiency increased from the enhanced ability of PHEMA-*g*-(PEI-*b*-PEG)/pDNA complexes to be internalized by cells.

3.6. *p53 gene therapy using PHEMA-g-(PEI-b-PEG) as the carrier*

Improved marker gene transfection ability of PHEMA-*g*-(PEI-*b*-PEG) over PEI25K indicated that it may be a good candidate for p53

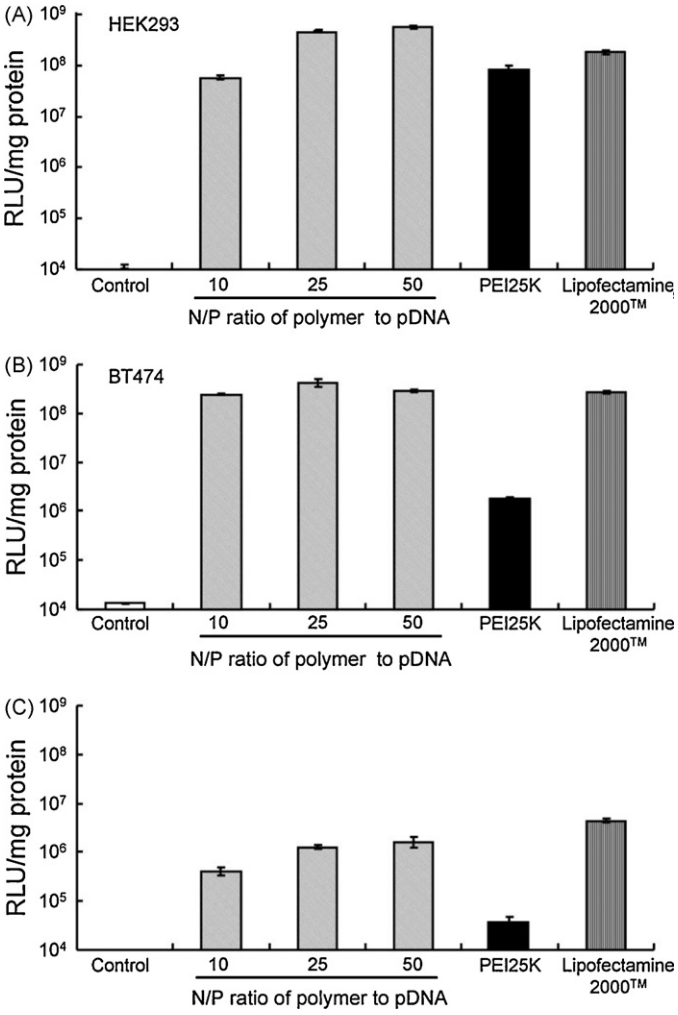


Fig. 5. *In vitro* transfection efficiency of PHEMA-*g*-(PEI-*b*-PEG)/pGL3-Luc complexes in HEK293 cells (A), BT474 cells (B) and mouse osteoblast cells (C) at various N/P ratios. Polymer stands for PHEMA-*g*-(PEI-*b*-PEG).

gene therapy. p53 is an important tumor suppressor that acts to restrict proliferation (Levine et al., 1991; Miyashita and Reed, 1995). Mutations of p53 increase cell proliferation and resistance to certain chemotherapies (Lowe et al., 2004), while loss of p53 function is a common feature of human cancers (Sherr, 2004). BT474 breast cancer cells carry the p53 gene in the mutant conformation at 37 °C (Muller et al., 2005).

The PHEMA-*g*-(PEI-*b*-PEG) polymer was used to form complexes with pcDNA3-p53 and incubated with BT474 cells using PEI25K and Lipofectamine 2000™ as controls. Western blotting analyses was used to evaluate the expression levels of wild-type p53 protein after transfection with different complexes. The results were displayed in Fig. 7A. Because the p53 antibody could detect both wild type protein and mutant one, there was mutant p53 protein in all of experimental groups which was carried by BT474 cells themselves. It could be seen that although PEI25K is one of the most powerful cationic polymers in gene delivery, there was low wild-type p53 expression in BT474 cells, which was in accordance with GFP and luciferase gene transfection results as described above. However, a remarkable expression of wild-type p53 was observed after transfection with PHEMA-*g*-(PEI-*b*-PEG)/pcDNA3-p53 complexes at N/P ratio of 25:1, and the band optical density ratio of wild type/mutant p53 was even slightly higher than that of Lipofectamine 2000™/pcDNA3-p53 complexes as shown in Fig. 7B.

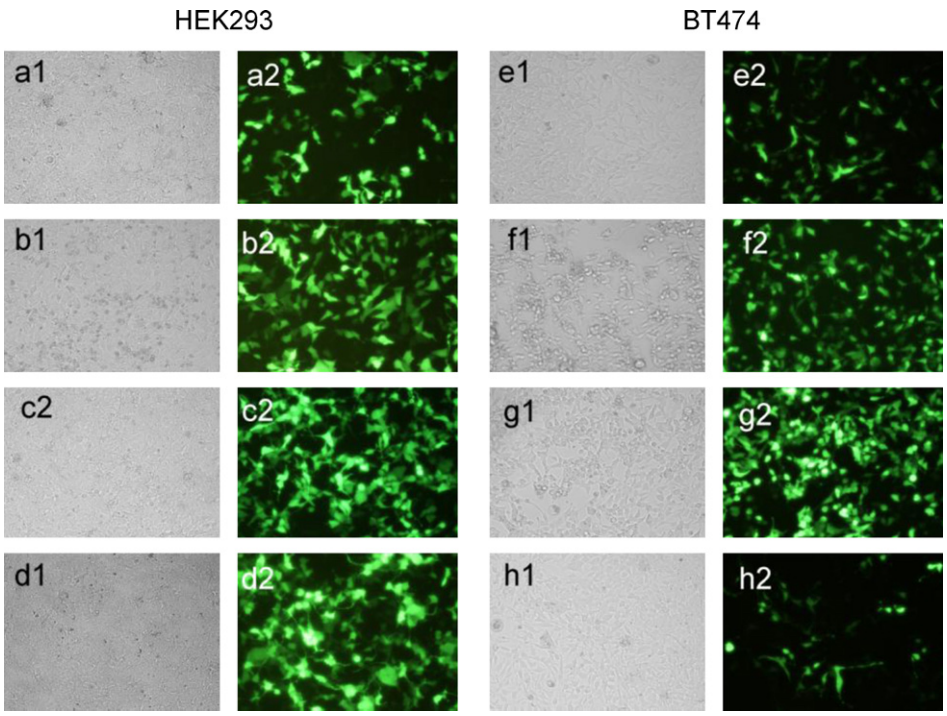


Fig. 6. Green fluorescent protein expression in HEK293 and BT474 cells after transfection with PHEMA-*g*-(PEI-*b*-PEG)/pEGFP-N2 complexes at N/P ratio of 10:1 (a and e) and 25:1 (b and f), Lipofectamine 2000™/pEGFP-N2 (c and g), PEI25K/pEGFP-N2 complexes at N/P ratio of 10:1 (d and h). (a1–h1) Differential interference contrast images; (a2–h2) fluorescence images. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

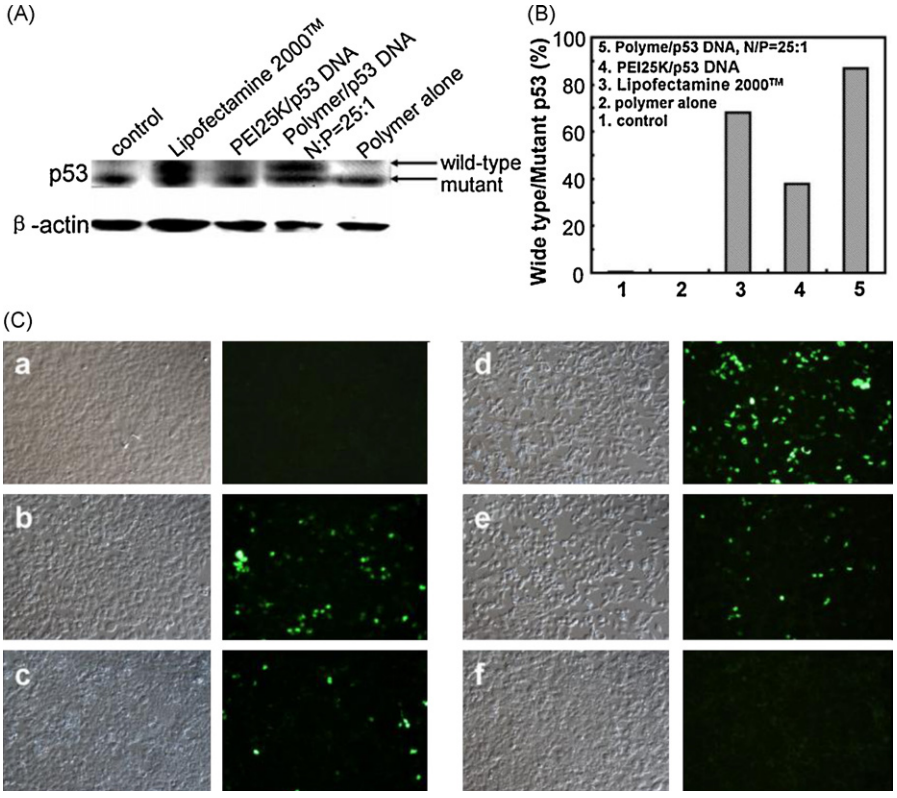


Fig. 7. (A and B) Western blotting determined p53 expression in BT474 cells after transfection with different complexes (A) and the band optical density ratio of wild-type p53/mutant type p53 (B); (C) TUNEL assay for apoptosis after transfection with different complexes in BT474 cells: (a) without any treatment; (b) transfected with Lipofectamine 2000™/pcDNA-p53; (c) transfected with PEI25K/pcDNA-p53; (d) transfected with PHEMA-*g*-(PEI-*b*-PEG)/pcDNA-p53, N/P = 25:1; (e) transfected with PHEMA-*g*-(PEI-*b*-PEG)/pcDNA-p53, N/P = 10:1; (f) incubated with PHEMA-*g*-(PEI-*b*-PEG). Polymer stands for PHEMA-*g*-(PEI-*b*-PEG).

Meanwhile, no wild-type p53 was detected in the cells treated with PHEMA-*g*-(PEI-*b*-PEG) alone, which demonstrated that the brush-shaped polymer itself would not disrupt original mutant p53 expression in BT474 cells.

The p53 protein plays a central role in eliciting cellular responses to a variety of stress signals and it results in the transcriptional regulation of genes that are involved in mediating key cellular processes, such as DNA repair, cell-cycle arrest, senescence and apoptosis (Vazquez et al., 2008). TUNEL assay was used for apoptosis detection in this study after wild-type p53 transfection. The nuclei of apoptotic cells were stained green by the incorporation of fluorescein-conjugated dUTP into the 3′OH ends of cleaved DNA. As shown in Fig. 7C, more TUNEL positive cells were observed after transfection with PHEMA-*g*-(PEI-*b*-PEG)/pcDNA3-p53. At N/P ratio of 25:1 the performance of PHEMA-*g*-(PEI-*b*-PEG)/pcDNA3-p53 complexes to induce apoptosis was even better than those of Lipofectamine 2000™. In contrast, few apoptotic cells was observed when cells were treated with PHEMA-*g*-(PEI-*b*-PEG) alone, which also indicated that the brush-shaped polymer itself would not induce cell apoptosis. This was coincided with the result of western blotting analyses. It should be mentioned that the negative control, obtained by omitting TdT from the TUNEL reaction, did not give TUNEL positive cell (data not shown).

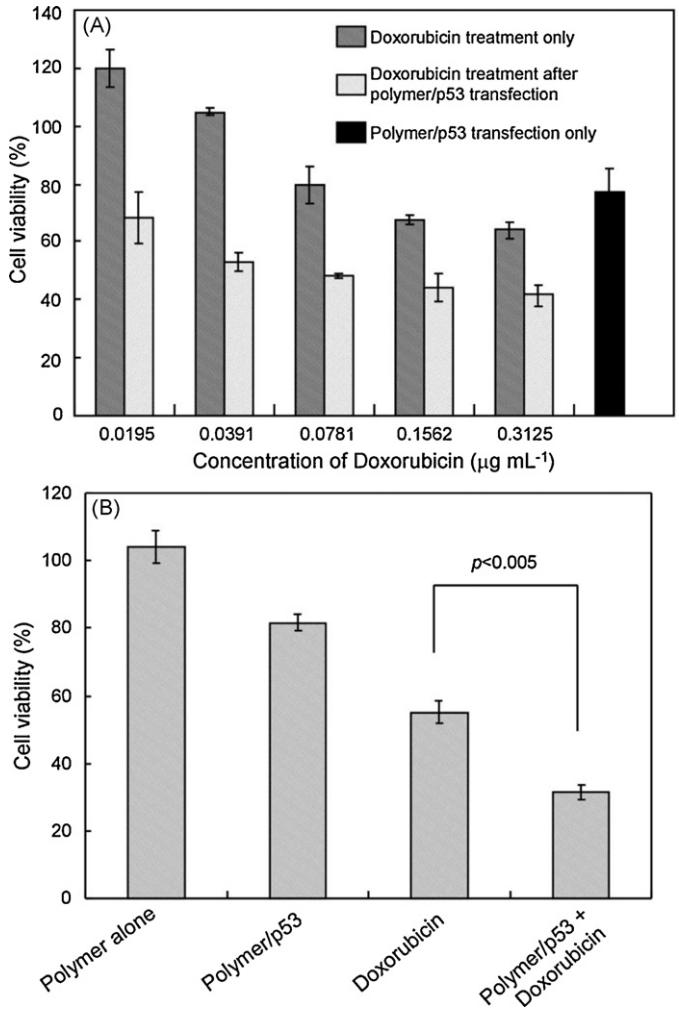


Fig. 8. (A) Sensitization of BT474 cells to doxorubicin after transfection with PHEMA-*g*-(PEI-*b*-PEG)/pcDNA-p53. (B) Cell viability after treatment with PHEMA-*g*-(PEI-*b*-PEG) alone, PHEMA-*g*-(PEI-*b*-PEG)/pcDNA-p53, doxorubicin, or combination of PHEMA-*g*-(PEI-*b*-PEG)/pcDNA-p53 and doxorubicin. Polymer stands for PHEMA-*g*-(PEI-*b*-PEG).

3.7. Sensitization of breast carcinoma cells to doxorubicin by PHEMA-g-(PEI-b-PEG)/pcDNA3-p53

Expression of p53 plays an important role in modulating drug sensitivity and p53 mutations have been proved to increase resistance of cancer cells to certain chemotherapies (Harris and Levine, 2005). In this study, we examined the effect of wild-type p53 gene expression in BT474 cells on the sensitivity to doxorubicin chemotherapy. The PHEMA-*g*-(PEI-*b*-PEG)/pcDNA3-p53 complexes at N/P ratio of 25:1 was selected in this study since optimized wild-type p53 expression was achieved under this condition as described above.

We first determined whether restoration of wild-type p53 level in BT474 cells could increase their sensitivity to doxorubicin. To address this issue, we first transfected BT474 cells with PHEMA-*g*-(PEI-*b*-PEG)/pcDNA3-p53 complexes and after 24 h incubation the cells were treated with different doses of doxorubicin. As shown in Fig. 8A, p53 transfection resulted in significantly enhanced sensitivity of BT474 cells to doxorubicin even at very low concentrations. For example, when the concentration of doxorubicin was 0.0391 $\mu g\,mL^{-1}$, doxorubicin alone did not inhibit the proliferation of BT474 cells. However, doxorubicin treatment after p53 transfection significantly increased the cell death of BT474 to 47%, while p53 transfection only induced about 23% cell death.

Then we compared the treatments of transfection with PHEMA-*g*-(PEI-*b*-PEG)/pcDNA3-p53 complexes, doxorubicin chemotherapy at the concentration of 1 $\mu g\,mL^{-1}$ and the combination therapy. After 48 h incubation, the cell viability determined by MTT assay was shown in Fig. 8B. The combination therapy resulted in about 31% cell viability while either p53 gene therapy or chemotherapy alone resulted in 55% or 80% cell viability. There was significant difference between the combination therapy and doxorubicin chemotherapy ($p < 0.005$). The observation also demonstrated that wild-type p53 restoration by PHEMA-*g*-(PEI-*b*-PEG) could sensitize BT474 cells to doxorubicin. Taken together, our results indicated that p53 gene transfer using PHEMA-*g*-(PEI-*b*-PEG) as vector could offer a novel powerful therapeutic approach to improve traditional chemotherapy.

4. Conclusion

We have constructed a novel brush-shaped hydrophilic polymer PHEMA-*g*-(PEI-*b*-PEG) gene carrier, offering potential for effective pDNA delivery. PHEMA-*g*-(PEI-*b*-PEG)/pDNA complexes possess nanoscaled particle sizes, and can be effectively internalized by cells. The brush polymer alone exhibits higher biocompatibility and better transfection efficiency than PEI25K in various cell lines. Furthermore, transfection of breast carcinoma cells with complexes of PHEMA-*g*-(PEI-*b*-PEG)/pcDNA3-p53 results in obvious apoptosis, and notably increased the chemo-sensitivity to DOX at low drug concentrations. These properties make it very attractive as a gene carrier for further therapeutic applications.

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