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Low molecular weight PEI-based polycationic gene vectors via Michael addition polymerization with improved serum-tolerance

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ABSTRACT

A series of polycationic gene delivery vectors were synthesized via Michael addition from low molecular weight PEI and linking compounds with various heteroatom compositions. Agarose gel electrophoresis results reveal that these polymers can well condense plasmid DNA and can protect DNA from degradation by nuclease. The formed polyplexes, which are stable toward serum, have uniform spherical nanoparticles with appropriate sizes around 200–350 nm and zeta-potentials about +40 mV. *In vitro* experiments show that these polymers have lower cytotoxicity and higher transfection efficiency than 25 KDa PEI. Furthermore, the title materials exhibit excellent serum tolerance. With the present of 10% serum, up to 19 times higher transfection efficiency than PEI was obtained, and no obvious decrease of TE was observed even the serum concentration was raised to >40%. Flow cytometry and confocal microscopy studies also demonstrate the good serum tolerance of the materials.

1. Introduction

Gene therapy represents a promising option for the treatment of various diseases such as viral infections, inherited disorders and cancers [1–3]. The safe and effective delivery of genes with various types of vectors promises exceptional advancements in clinical disease treatment, next-generation vaccines, and tissue engineering [4–6]. Current gene delivery vectors can be divided into viral and non-viral. The use of non-viral vectors mitigates some issues generally associated with viral gene therapy, such as limited gene insertion size, immune response, mutagenesis and large scale production limitations [7–9]. These non-viral vectors consist of a diverse set of materials which are typically cationic, including natural and synthetic polymers, lipids, peptides, dendrimers and combinations of these structures [10,11]. Cationic polymers, which can efficiently complex with negatively charged DNA, thereby increasing DNA stability, were frequently studied. Among the polycations, poly(ethyleneimine) (PEI), a commercially available material, has been used as a gene delivery vector since 1995 and become one of the most promising and widely studied gene carriers [12,13] due in large part to efficient escape from the endocytic pathway through the proton-sponge mechanism. However, the gene transfection efficiency (TE) and cytotoxicity of PEI are heavily correlated with their chain length and topology [14,15]. High molecular weight (HMW) PEIs are effective in condensing nucleic acids but exhibit pronounced cytotoxicity and induce membrane damage in the initial stages of treatment [16] and mitochondrial-mediated apoptosis in the later stages. Meanwhile, low molecular weight (LMW) PEIs, bearing buffering capacity equivalent to their longer chain counterparts, are almost non-toxic but display poor TE owing to inefficient pDNA condensation and low cellular uptake through diminished charge-mediated interactions [17].

To achieve high TE as well as low toxicity, PEI might be modified by many strategies such as covalent grafting [18], cross linking [19] and electrostatic coating [20]. Crosslinked LMW PEIs have been examined over the past decade, and various types of hydrolytically or reductively degradable PEI polymers and networks have been designed for *in vitro* transfection. Zhong et al. reported the reversibly hydrophobilized 10 KDa PEIs based on rapidly acid-degradable acetal-containing hydrophobe for nontoxic and marked enhanced non-viral gene transfection [21]. Zhuo and co-

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workers described the disulfide cross-linked PEI via click reaction [22]. It was proved that these materials can maintain the TE of 25 KDa PEI with much less cytotoxicity.

For potential clinical applications, the interaction between electropositive polycation/pDNA complexes (polyplexes) and the negatively charged blood components cannot be ignored [23]. After intravenous injection, some unwanted effects would arise including the rapid clearance by the RES (Reticuloe Endothelin System) upon polyplexes aggregation, and structure destabilization as well as the premature DNA release and degradation [24]. Thereby it is highly indispensable to make models about the serum-conditioned transfection for the forecast evaluation on the *in vivo* TE of polycationic vectors. For example, zwitterionic betaine species were used for the functionalization of polymeric materials to enhance their serum-tolerance [25,26]. Zhuo et al. also put forward that branched PEI could be modified via the catalyst-free aminolysis reaction with 5-ethyl-5-(hydroxymethyl)-1,3-dioxan-2-oxo (EHDO) to promote the serum-tolerant capability [27]. Poly(ethylene glycol) (PEG) was also used to enhance the biocompatibility of the polymer vectors, and these modified PEIs maintained low cytotoxicity and showed enhanced transfection activity [28]. Meanwhile, they protected the polyplexes from undesired interactions with the negatively charged components in the bloodstream.

In the present study, we developed a series of polycations (**MP1**–**MP6**) via Michael addition from LMW PEI 600 Da and linking compounds (**LC1**–**LC6**). These materials showed good pH buffering capacity and DNA binding ability. Improved TEs were achieved compared to 25 KDa PEI, especially in the presence of serum.

2. Experimental details

2.1. Materials and methods

All chemicals and reagents were obtained commercially and were used as received. Anhydrous methanol and anhydrous chloroform were dried and purified under nitrogen by using standard methods and were distilled immediately before use. LMW PEI (branched, average molecular weight 600 Da, 99%) was purchased from Aladdin (Shanghai, China). 25 KDa PEI (branched, average molecular weight 25 KDa) was purchased from Sigma–Aldrich (St. Louis, MO, USA) and nucleic acid labeling kit Label IT® Cy5™ was obtained from Mirus Bio Corporation (USA). The plasmids used in the study were pGL-3 (Promega, Madison, WI, USA, coding for luciferase DNA) and pEGFP-N1 (Clontech, Palo Alto, CA, USA, coding for EGFP DNA). The Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were purchased from Invitrogen Corp. The MicroBCA protein assay kit was obtained from Pierce (Rockford, IL, USA). The luciferase assay kit was purchased from Promega (Madison, WI, USA). The endotoxin free plasmid purification kit was purchased from TIANGEN (Beijing, China).

^{1}H NMR spectra were obtained on a Bruker AV400 spectrometer. $CDCl_3$ or D_2O was used as the solvent and TMS as the internal reference. The molecular weight of polymers were determined by gel permeation chromatography (GPC) (Waters 515 pump, Waters 2410 Refractive Index Detector (25 °C, incorporating Shodex columns OHPAK KB-803). A filtered mixture of 0.5 mol L^{-1} HAc/NaAc buffer was used as the mobile phase with a flow rate of 0.5 mL min^{-1}. Molecular weights were calculated against poly(-ethylene glycol) standards of number average molecular weights ranging from 900 to 80,000 Da.

2.2. Synthesis and characterization linker *LC1*–*LC6*

Diols or diamine (0.048 mol) and triethylamine (4.81 g, 0.057 mol) were dissolved in anhydrous CH_2Cl_2 (50 mL). $(Boc)_2O$ (12.45 g, 0.057 mol) in anhydrous CH_2Cl_2 solution was added dropwise to the above stirred solution under the ice bath. The mixture was stirred overnight at room temperature, followed by evaporation of the organic solvents. The residue was purified with silica gel column chromatography (dichloromethane/methyl alcohol = 30: 1). The synthesis of naked two primary amine compound according to the literature [29]. Then, acryloyl chloride (6.95 g, 0.077 mol) in anhydrous dichloromethane (50 mL) was added dropwise to a stirred solution of diol or diamine (0.038 mol) and triethylamine (7.77 g, 0.077 mol) in anhydrous dichloromethane (50 mL) under the ice bath. The mixture was stirred overnight at room temperature and then filtered off generated salt, followed by evaporation of the volatile solvent. The residue was purified with silica gel column chromatography (PE: EA = 3: 1, v/v) to give **LC1**–**LC6**.

LC1 (Yield 41.2%): ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.44 (s, 9H, $(CH_3)_3CO$), 4.19–4.31 (m, 4H, $OCH_2CH(NH)CH_2O$), 4.84 (m, 1H, $OCH_2CH(NH)CH_2O$), 5.85–5.88 (d, *J* = 12 Hz, 2H, CH_2CHCO), 6.09–6.15 (t, *J* = 8 Hz, 2H, CH_2CHCO), 6.40–6.45 (d, *J* = 20 Hz, 2H, CH_2CHCO). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 165.92, 155.24, 131.80, 127.88, 80.24, 63.45, 48.61, 28.43. MALDI-HRMS: *m*/*z* 322.1265 ($[M+Na]^+$), $C_{14}H_{21}NO_6Na^+$, calc. 322.1267.

LC2 (Yield 55.3%): ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.42–1.71 (m, 6H, $CH_2(CH_2)_3CH_2$), 4.12–4.15 (m, 4H, $OCH_2(CH_2)_3CH_2O$), 5.77–5.80 (m, 2H, $(CH_2CHCO)_2$), 6.05–6.12 (m, 2H, $(CH_2CHCO)_2$), 6.34–6.39 (m, 2H, $(CH_2CHCO)_2$). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 166.27, 130.67, 128.54, 64.32, 28.28, 22.52. MALDI-HRMS: *m*/*z* 235.0947 ($[M+Na]^+$), $C_{14}H_{21}NO_6Na^+$, calc. 235.0946.

LC3 (Yield 52.6%): ^{1}H NMR (400 MHz, $CDCl_3$): δ = 3.71–3.73 (t, *J* = 4 Hz, 4H, $CH_2CH_2OCH_2CH_2$), 4.28–4.30 (t, *J* = 4 Hz, 4H, $CH_2CH_2OCH_2CH_2$), 5.80–5.83 (m, 2H, $(CH_2CHCO)_2$), 6.08–6.15 (m, 2H, $(CH_2CHCO)_2$), 6.37–6.42 (m, 2H, $(CH_2CHCO)_2$). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 166.13, 131.18, 128.22, 69.08, 63.58. MALDI-HRMS: *m*/*z* 237.0742 ($[M+Na]^+$), $C_{14}H_{21}NO_6Na^+$, calc. 237.0739.

LC4 (Yield 45.4%): ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.43 (s, 9H, $(CH_3)_3CO$), 3.50–3.54 (t, *J* = 16 Hz, 4H, $CH_2CH_2N(CO)CH_2CH_2$), 4.24–4.26 (t, *J* = 8 Hz, 4H, $OCH_2CH_2N(CO)CH_2CH_2O$), 5.83 (m, 2H, CH_2CHCO), 6.06–6.13 (t, *J* = 8 Hz, 2H, CH_2CHCO), 6.37–6.41 (d, *J* = 16 Hz, 2H, CH_2CHCO). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 166.02, 155.26, 131.36, 131.18, 128.26, 128.21, 80.43, 62.81, 62.68, 47.16, 46.93, 28.41. MALDI-HRMS: *m*/*z* 336.1426 ($[M+Na]^+$), $C_{15}H_{23}NO_6Na^+$, calc. 336.1423.

LC5 (Yield 33.2%): ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.44 (s, 18H, $\{(CH_3)_3CO\}_2$), 3.32–3.49 (t, *J* = 20 Hz, 8H, $CH_2CH_2N(CO)(CH_2)_2N(CO)CH_2CH_2$), 4.23–4.26 (t, *J* = 4 Hz, 4H, $OCH_2CH_2N(CO)(CH_2)_2N(CO)CH_2CH_2O$), 5.83–5.85 (d, *J* = 8 Hz, 2H, CH_2CHCO), 6.08–6.15 (t, *J* = 12 Hz, 2H, CH_2CHCO), 6.39–6.43 (d, *J* = 16 Hz, 2H, CH_2CHCO). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 166.04, 155.42, 155.21, 131.46, 131.26, 131.09, 128.35, 80.34, 80.14, 62.75, 62.61, 46.71, 46.19, 45.50, 28.46. MALDI-HRMS: *m*/*z* 479.2370 ($[M+Na]^+$), $C_{22}H_{36}N_2O_8Na^+$, calc. 479.2369.

LC6 (Yield 19.5%): ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.40 (s, 9H, $(CH_3)_3CO$), 3.43–3.53 (m, 8H, $NCH_2CH_2N(CO)CH_2CH_2N$), 5.63–5.66 (d, *J* = 12 Hz, 2H, CH_2CHCO), 6.07–6.34 (m, 4H, $\{CH_2CHCO\}_2$). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 169.04, 157.12, 132.11, 131.06, 128.41, 126.61, 80.81, 49.59, 47.96, 40.04, 39.03, 28.41. MALDI-HRMS: *m*/*z* 334.1740 ($[M+Na]^+$), $C_{15}H_{25}N_3O_4Na^+$, calc. 334.1743.

2.3. Synthesis and characterization of target cationic polymers *MP1*–*MP6*

Polymers were successfully synthesized following modified Michael addition reaction as reported previously. Briefly, PEI 600 (1.26 mmol) and linker **LC1**–**LC6** (1.26 mmol) were separately dissolved in 1.5 mL of anhydrous methanol and 1.5 mL anhydrous

chloroform, they were mixed in a flask, under a nitrogen atmosphere, the reaction mixtures were heated at 45 °C with constant stirring for 24 h in an oil bath. After the reaction, MeOH–HCl solution was added to Boc-containing polymers and the reaction mixture was stirred overnight at room temperature. The solvent was removed under reduced pressure, and the residue was dissolved in a little water, strong alkaline ion-exchange resin was added and then filtered off, the raw producs (**MP1, MP4–6**) were obtained by lyophilization. The mixture of the six crude product (**MP1–MP6**) was diluted with 3 mL of anhydrous methanol, and then precipitated by the addition of anhydrous diethyl ether. The precipitation was collected and dried in vacuum to get the product as colorless oil. The molecular weights of compounds **MP1–MP6** were measured by GPC.

MP1 (Yield 45.16%): ^{1}H NMR (400 MHz, D_2O): δ = 2.59–3.41 (m, 45H, (OCCH_2CH_2)$_2$, (CH_2CH_2NH)$_n$), 3.55–3.59 (m, 1H, $NH_2CH(CH_2)_2$), 3.67–3.83 (m, 4H, $NH_2CH(CH_2O)_2$).

MP2 (Yield 56.08%): ^{1}H NMR (400 MHz, D_2O): δ = 2.36–2.90 (m, 80H, (OCCH_2CH_2)$_2$, $CH_2(CH_2)_3CH_2$, (CH_2CH_2NH)$_n$), 3.13–3.21 (m, 2H, (OCCH$_2CH_2$)$_2$), 3.32 (m, 4H, (OCCH$_2CH_2$)$_2$, $CH_2(CH_2)_3CH_2$), 3.47 (m, 2H, $CH_2(CH_2)_3CH_2$).

MP3 (Yield 57.21%): ^{1}H NMR (400 MHz, D_2O): δ = 2.36–2.99 (m, 79H, (COCH_2CH_2)$_2$, (CH_2CH_2NH)$_n$), 3.13–3.19 (m, 2H, $CH_2CH_2OCH_2CH_2$), 3.32 (m, 4H, (OCOCH_2CH_2)$_2$, $CH_2CH_2OCH_2CH_2$), 3.47 (m, 2H, (OCOCH_2CH_2)$_2$).

MP4 (Yield 42.37%): ^{1}H NMR (400 MHz, D_2O): δ = 2.43–2.87 (m, 72H, (COCH_2CH_2)$_2$, (CH_2CH_2NH)$_n$), 3.11–3.17 (m, 2H, (OCH$_2CH_2$)$_2$NH), 3.30–3.32 (m, 4H, (OCH$_2CH_2$)$_2$NH, (COCH$_2CH_2$)$_2$), 3.50–3.59 (m, 2H, (COCH$_2CH_2$)$_2$), 3.68–3.71 (m, 4H, (OCH_2CH_2)$_2$NH).

MP5 (Yield 40.32%): ^{1}H NMR (400 MHz, D_2O): δ = 2.37–2.96 (m, 68H, NHCH_2CH_2NH, (OCCH_2CH_2)$_2$, (CH_2CH_2NH)$_n$), 3.08–3.25 (m, 2H, (OCH$_2CH_2$NH)$_2$), 3.34–3.51 (m, 4H, (OCH$_2CH_2$NH)$_2$, (OCCH$_2CH_2$)$_2$), 3.52–3.67 (m, 2H, (OCCH$_2CH_2$)$_2$), 3.68–3.81 (m, 4H, (OCH_2CH_2NH)$_2$).

MP6 (Yield 35.11%): ^{1}H NMR (400 MHz, D_2O): δ = 2.47–3.11 (m, 61H, (OCCH_2CH_2)$_2$, (OCH$_2CH_2$)$_2$NH, (CH_2CH_2NH)$_n$), 3.34–3.55 (m, 4H, (OCH_2CH_2)$_2$NH).

2.4. Acid–base titration

In this assay, briefly, **MP1–MP6** (0.25 mmol of amino groups) was dissolved in 5 mL of 150 mM NaCl aqueous solution, and 1 N HCl was added to adjust the pH to 2.0. Aliquots (50 mL for each) of 0.1 M NaOH were added, and the solution pH was measured with a pH meter (pHS-25) after each addition. For comparison, 25 KDa PEI was used under same experimental conditions. The buffering capacity defined as the percentage of amine groups becoming protonated from pH 5.1 to 7.4, was calculated from the equation:

$$\text{buffer capacity}(\%) = 100[(\Delta V_1\text{NaOH} - \Delta V_2\text{NaOH}) \times 0.1\,\text{M}]/\text{Nmol}$$

wherein ΔV_1NaOH is the volume of NaOH solution (0.1 M) required to bring the PH value of the polymer solution from 5.1 to 7.4, ΔV_2NaOH is the volume of NaOH solution (0.1 M) required to bring the pH value of the NaCl solution from 5.1 to 7.4, and N is the total moles (0.25 mmol) of protonable amine groups in the polymer.

2.5. Cell culture

HEK (human embryonic kidney) 293 cells, human osteosarcoma (U2OS) cells, HeLa and HepG2 cells were incubated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1‰ antibiotics (penicillin–streptomycin, 10,000 U mL^{-1}) at 37 °C in a humidified atmosphere containing 5% CO_2.

2.6. Amplification and purification of plasmid DNA

pGL-3 and pEGFP-N1 plasmids were used. The former one as the luciferase reporter gene was transformed in *Escherichia coli* JM109 and the latter one as the enhanced green fluorescent protein gene was transformed in *E. coli* DH5α. Both plasmids were amplified in Luria–Bertani broth at 37 °C overnight. The plasmids were purified by an EndoFree Tiangen TM Plasmid Kit. Then the purified plasmids were dissolved in TE buffer solution and stored at −20 °C. The integrity of plasmids was confirmed by agarose gel electrophoresis. The purity and concentration of plasmids were determined by ultraviolet (UV) absorbance at 260 and 280 nm.

2.7. Agarose gel electrophoresis (binding capacity and stability of polymers/pDNA complexes)

MP1–MP6/DNA complexes at different weight ratios ranging from 0.2 to 6.4 were prepared by adding an appropriate volume of **MP1–MP6** to 5 μL of Puc-19 (0.025 mg/mL). The obtained complex solution was diluted to a total volume of 15 μL, and then the complexes were incubated at 37 °C for 30 min. After that the complexes were electrophoresed on a 1% (W/V) agarose gel containing GelRed™ and with Tris–acetate (TAE) running buffer at 150 V for 30 min. DNA was visualized under an ultraviolet lamp using a Vilber Lourmat imaging system. In the experiments with serum, the complexes solution (20 μL contained 10% or 50% serum) was obtained by adding PBS buffer and serum (2 μL or 10 μL) and incubating for a certain time. Stabilities of **MP4**, **MP6**/DNA polyplexes were evaluated by testing the abilities of them to protect pDNA against DNase degradation. Generally, **MP4**, **MP6** and pDNA (0.125 μg) were mixed at a weight ratio of 1.6 for 30 min at room temperature. Then 2 μL DNase (2unit/uL) was added and the mixtures of **MP4**, **MP6**, pDNA, and DNase were incubated at 37 °C for 2 h. The mixtures were then heated in a water bath at 65 °C for 10 min to denature DNase I. Stability of the polyplex was further analyzed by apolyanion competition assay, 5 μL heparin sodium (1.6 mg/mL) was added to the **MP4**, **MP6**/DNA polyplex solution and the mixture was incubated for 10 min at room temperature. The samples were run at the same electrophoresis condition as described above [30].

2.8. Ethidium bromide displacement assay

The ability of **MP1–MP6** to condense DNA was studied using ethidium bromide (EB) exclusion assays. Fluorescence spectra were measured at room temperature in air by a Horiba Jobin Yvon Fluoromax-4 spectrofluorometer and corrected for the system response. EB (5 μL, 1 mg mL^{-1}) was put into quartz cuvette containing 2.5 mL of 10 mM Hepes solution. After shaking, the fluorescence intensity of EB was measured. Then CT DNA (10 μL, 1 mg mL^{-1}) was added to the solution and mixed symmetrically, and the measured fluorescence intensity is the result of the interaction between DNA and EB. Subsequently, the solutions of **MP1–MP6** (0.2 mg mL^{-1}, 5 μL for each addition) were added to the above solution for further measurement. All the samples were excited at 520 nm and the emission was measured at 600 nm. The pure EB solution and DNA/EB solution without cationic polymer were used as negative and positive controls, respectively. The percent relative fluorescence (%F) was determined using the equation %F = (F − FEB)/(F0 − FEB), wherein FEB and F0 denote the fluorescence intensities of pure EB solution and DNA/EB solution, respectively.

2.9. Particle size and ζ-potential measurements in water

Particle size and zeta potential measurements of polyplexes were carried out using a Nano-ZS 3600 (Malvern Instruments, USA) with a He–Ne Laser beam (633 nm, fixed scattering angle of 901) at 25 °C. **MP1**–**MP6**/DNA polyplexes at weight ratios ranging from 0.2 to 12.8 were prepared by adding 1 μg of pUC-19 to the appropriate volume of the polymer solution (in ultrapure water). Then the solution of the polyplexes was incubated at 37 °C for 30 min and then diluted with deionized water to 1 mL prior to measurement. Data were shown as mean ± standard deviation (SD) based on triplicate independent experiments.

2.10. Transmission electron microscopy (TEM)

The morphologies of the polyplexes were observed by TEM (JEM-100CXa) with an acceleration voltage of 100 kV. 2 μg of pUC-19 was added to the appropriate volume of the polymer solution (weight ratio of polymer relative to pDNA, w/w = 6.4: 1), then diluted to the total volume of 50 μL. The solution of the polyplexes was incubated at 37 °C for 0.5 h. 15 min before measurement, the polyplex solution was diluted with deionized water or water containing 10% fetal bovine serum (FBS) to 1 mL. A drop of DNA/polymer polyplexes suspension was placed onto the copper grid. After a few minutes, the excess solution was blotted away with filter paper. Then, a drop of 0.5% (w/v) phosphotungstic acid was placed on the above grid. The grid was dried at room temperature at atmospheric pressure for several minutes before observation.

2.11. Cell viability assay

Toxicity of **MP1**–**MP6** toward U2OS cells, HEK293 cells, HeLa cells and HepG2 cells was determined by using a Cell Counting Kit-8. The U2OS cells and HepG2 cells (9000 cells/well), HEK293 cells and HeLa cells (8000 cells/well) were seeded into 96-well plates and cultured overnight for 70–80% cell confluence. The cells were then incubated in a culture medium containing **MP1**–**MP6** with a particular concentration for 24 h. After that, polymer solutions were removed, 100 μL of sterile filtered CCK-8 (0.1 mg mL^{-1}) stock solution in PBS was added to each well for additional 1 h incubation at 37 °C. Then, the absorbance of each sample was measured using an ELISA plate reader (model 680, BioRad) at a wavelength of 450 nm. The cell survival was expressed as follows: cell viability = $(OD_{treated}/OD_{control}) \times 100\%$. Besides, the cell viability of 25 KDa PEI was also performed.

2.12. Transfection procedure

Gene transfection of a series of complexes was investigated in U2OS cells, HEK293 cells, HeLa cells and HepG2 cells. Cells were seeded in 24-well plates (1.0×10^5 cells/well for U2OS and HepG2 cells, 8×10^4 cells/well for HeLa and HEK293 cells) and grown to reach 70–80% cell confluence at 37 °C for 24 h in 5% CO_2. Before transfection, the medium was replaced with a serum-free or a 10% serum-containing culture medium containing polymer/pDNA (0.8 μg) complexes at various weight ratios. After 4 h under standard incubator conditions, the medium was replaced with fresh medium containing serum and incubated for another 20 h.

For fluorescent microscopy assays, cells were transfected by complexes containing pEGFP-N1. After 24 h incubation, GFP-expressed cells were observed with an inverted fluorescence microscope (Nikon Eclipse TE 2000E) equipped with a cold Nikon camera. Control transfection was performed in each case using a commercially available transfection reagent 25 KDa PEI based on the standard conditions specified by the manufacture.

For luciferase assays, cells were transfected by complexes containing pGL-3. For a typical assay in a 24-well plate, 24 h post transfection as described above, cells were washed with cold PBS and lysed with 100 μL 1× Lysis reporter buffer (Promega). The luciferase activity was measured by microplate reader (Model 550, BioRad, USA). Protein content of the lysed cell was determined by BCA protein assay. Gene TE was expressed as the relative fluorescence intensity per mg protein (RLU/mg protein). All the experiments were done in triplicates.

2.13. Cellular uptake of plasmid DNA (flow cytometry)

The cellular uptake of the polymer/fluorescein labeled-DNA complexes was analyzed by flow cytometry. The Label IT Cy5 Labeling Kit was used to label pDNA with Cy5 according to the manufacturer's protocol. Briefly, HeLa and HepG2 cells were seeded in 12-well plates (2.0×10^5 cells/well for HeLa, 2.4×10^5 cells/well for HepG2) and allowed to attach and grow for 24 h. For transfection in the absence of serum, the medium was exchange with serum-free medium. As for transfection in the presence of serum, the medium was exchanged with serum-containing medium. Cells were incubated with Cy5 labeled DNA complexes (1.6 μg DNA/well, optimal weight ratio of each sample) in media for 4 h at 37 °C. Subsequently, the cells were washed with 1× PBS and harvested with 0.25% Trypsin/EDTA and resuspended in 1× PBS. Mean fluorescence intensity was analyzed using flow cytometer (Becton Dickinson and Company). Cy5-labeled plasmid DNA uptake was measured in the FL4 channel using the red diode laser (633 nm). The flow cytometer was calibrated for each run to obtain a background level of ~1% for control samples (i.e., untreated cells).

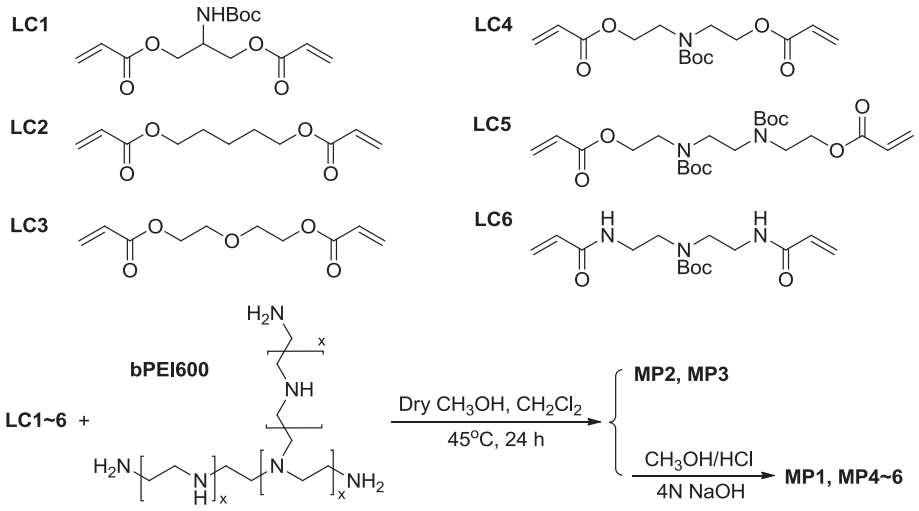
2.14. Confocal laser scanning microscopy (CLSM) analysis

HepG2 and HeLa cells were seeded at a density of 1.8×10^4 cells (HepG2 cells) and 1.5×10^4 cells (HeLa cells) per well in 35 mm confocal dish (Φ = 15 mm), 24 h prior to transfection. For transfection in the absence of serum, the medium was exchange with serum-free medium. As for transfection in the presence of serum, the medium was exchanged with serum-containing medium. Cells were incubated with Cy5 labeled DNA complexes (0.8 μg DNA/well, optimal weight ratio of each sample) in media for 2 h and 4 h at 37 °C. Subsequently, cells were rinsed twice with PBS (pH 7.4) to remove complexes that were not uptaken by cells, fixed with 4% paraformaldehyde (dissolved with PBS buffer) for 10 min, nuclear staining was done with DAPI. The CLSM observation was performed using Leica TCS SP5 at excitation wavelengths of 405 nm for DAPI (blue), 633 nm for Cy5 (red), respectively.

3. Results and discussion

3.1. Synthesis and chemical properties target polymers *MP1*–*MP6*

The preparation method of polymers **MP1**–**MP6** is shown in Scheme 1. Cross linking compounds **LC1**–**6** with different lengths and carbon-heteroatoms distributions were first prepared by relative diol or diamine and 2 equiv of acryloyl chloride. For compounds **LC1** and **LC4**–**6**, the amino groups in the middle of the chain must be protected by $(Boc)_2O$ before the acylation. Subsequent Michael addition polymerization took place between the linker **LC1**–**6** and PEI 600 Da with mole ratio of 1: 1 in anhydrous methanol and chloroform at 45 °C for 24 h. After the reaction, MeOH–HCl solution was added to remove the Boc group (if needed). The crude products were recrystallized by methanol and diethyl ether for 3 times to ensure their polydispersity. Gel permeation chromatography (GPC) was used to measure the



Scheme 1. Synthetic route of cationic polymers.

molecular weights of the polymers, and the results are listed in Table 1. Due to the lack of bulky Boc group, which would hinder the polymerization process, **MP2** and **MP3** have distinctly higher molecular weights than others.

Cationic polymers with various types of amino groups are assumed to have high buffering capacity, which is also called as "proton-sponge effect". Such effect may lead to the disruption of endosome in the transfection process, facilitating the escape of polymer/DNA complexes (polyplexes) [31]. Therefore, the buffering capacity of the polymers may be defined as the percentage of amine groups that can become protonated during endosomal acidification in the pH range from pH 7.4–5.1 (Table 2). It was shown that these polymers have comparable or slightly higher buffering capacities than 25 KDa PEI. The acid-base titration curves in Fig. S1 also shows similar results. The structure of linking group has slight effect on the buffering capacity, and the polymer with linking groups bearing primary amine (**MP1**) has lower pH buffering ability than those with secondary amine-contained linking groups (such as **MP5**).

3.2. The formation and properties of polymer/DNA polyplexes

The cationic property of the polymers facilitates their electrostatic interaction with negatively charged nucleic acids, which is the cargo in the delivery process. The formation of polymer/DNA complex (polyplex) can reduce the electrostatic repulsion between DNA and the cell surface and can protect DNA against enzymatic degradation by nucleases in cytoplasm or serum [32]. The DNA condensation ability of **MP1**–**MP6** was assessed by observing the electrophoretic mobility of DNA band in agarose gel. As shown in Fig. 1A, these polymers could effectively condense DNA from the weight ratio (polymer/DNA, w/w) of 0.8. **MP6** showed slightly higher DNA binding ability, suggesting that amide groups might benefit the interaction, and its higher molecular weight might also contribute. Similar assay was also carried out with the presence of serum, and results show that serum has little effect on the condensation ability of the polymers (Fig. 1B). Besides, the binding abilities of the polymers were also investigated by the ethidium bromide (EB) dye replacement assay [33]. The fluorescence of EB intercalating into the base pairs of DNA would be quenched by another binding agent which may exclude EB molecules. It was shown that the addition of polymers to EB pretreated DNA caused considerable decrease of the fluorescent emission intensity along with the increase of w/w ratio (Fig. S2), indicating that the EB was gradually replaced by the polymers. Such results further illustrate the strong DNA binding ability of the polymers.

Furthermore, the stability of polyplexes was studied via gel electrophoresis. **MP1**–**MP6**/DNA polyplexes were incubated with 10% and 50% serum against different times. Results in Fig. 2A show that no released or degraded DNA was found even after 2 h incubation, indicating the stability of the polyplexes. The incubation was further elongated with 50% serum. It was shown that DNA would be degraded by serum in 24 h (Fig. S3, lane 7), however, the polyplexes could remain stable after 48 h incubation (Fig. S3, lane 9). We also investigated the decomplexation of the polyplexes by heparin, which is more negatively charged than DNA and can lead to the release of DNA by its stronger interaction with cationic polymers. As shown in Fig. 2B, DNA was found to be gradually released with the increase of heparin. By comparing the polyplexes, it was found that the heparin amount needed to release the DNA from **MP6**/DNA complex was more than those from other polyplexes (**MP2**–**MP4**), indicating that **MP6** has higher DNA affinity, especially in the presence of negatively charged materials. On the other hand, excessively high DNA binding ability (e.g. PEI) may hinder the release of DNA in the cells [34], thus the balance between the DNA condensation and release abilities is essential for non-viral vectors. Fig. 2B shows that although **MP6** has higher DNA binding ability than its analogs, such ability is also lower than that of PEI.

Table 1
Molecular weights and polydispersities of target polymers.

Polymers	Mw (Da)	PDI
MP1	12,543	2.52
MP2	24,996	1.83
MP3	33,057	1.92
MP4	10,271	1.98
MP5	10,038	2.03
MP6	14,934	2.49

Table 2
The buffer capacity of synthesized polymers and 25 KDa PEI.

	MP1	MP2	MP3	MP4	MP5	MP6	PEI
Buffering capacity (%)	16.0	20.0	16.0	20.0	22.0	20.0	16.0

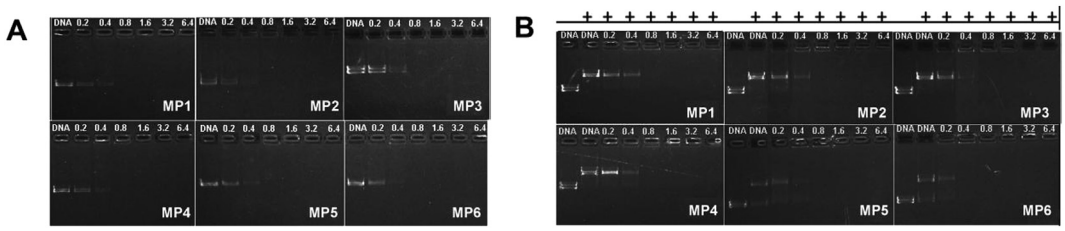


Fig. 1. DNA retardation assay by agarose gel electrophoresis in the absence (A) and presence (B) of 10% serum (+). Lanes from left to right: pDNA control, **MP1**–**MP6**/DNA w/w ratios of 0.2, 0.4, 0.8, 1.6, 3.2 and 6.4, respectively.

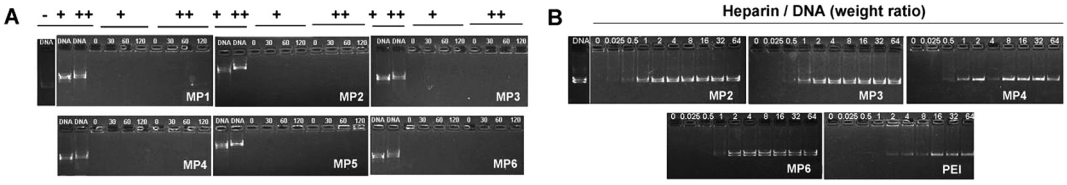


Fig. 2. Polyplex stability studies via electrophoretic gel retardation assay. (A) polyplexes (w/w = 1.6) against different incubation times (0, 30, 60, 120 min) in the presence of 10% or 50% serum (−: without serum, +: with 10% serum, ++: with 50% serum); (B) release of DNA with the addition of heparin at various concentrations (heparin/DNA: w/w = 0, 0.025, 0.5, 1, 2, 4, 8, 16, 32, 64; polymer/DNA: w/w = 0.8).

DNase I is an endonuclease that catalyzes the hydrolytic cleavage of phosphodiester linkages in the DNA backbone. Considering the abundant amounts of DNase I in tissues and blood, DNA degradation by DNase I is a major barrier for gene delivery *in vitro* and *in vivo* [35,36]. The DNA protection by **MP4** and **MP6** against DNase was studied, and results are shown in Fig. 3. Naked DNA was degraded in the presence of DNase within 2 h (Lane 3), while **MP4** and **MP6** could protect DNA from degradation, and the protected DNA could be released almost intactly by the addition of heparin (Lanes 4 and 6).

The size of nanoparticles is important for its substantial effect on their pharmacokinetics and pharmacodynamics [37]. The condensation of DNA by **MP4** and **MP6** resulted in nanoscale particles, which were directly visualized under transmission electron microscopy (TEM). As shown in Fig. 4A and B, the polyplexes in deionized water at weight ratio of 6.4 were observed as regular spherical shape with the diameters about 40 nm. Polyplex formed from **MP6** seemed to have smaller size and more regular shape than the other. Besides, with the presence of 10% serum, the size of **MP4**/DNA polyplex decreased, and the two polyplexes had similar diameter and shape (Fig. 4C and D), and still well dispersed uniformly in the medium. These results also indicate their good serum tolerance.

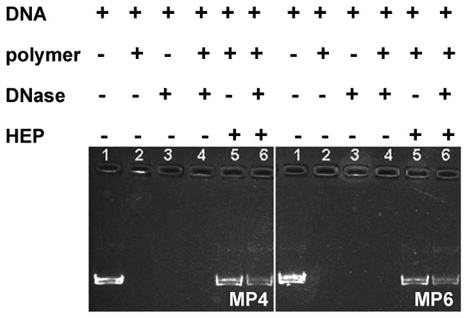


Fig. 3. DNA protection by **MP4** and **MP6** against DNase. Lane 1: naked DNA; Lane 2: polyplex (w/w = 1.6); Lane 3: pDNA with DNase for 2 h; Lane 4: Polyplex (w/w = 1.6) with DNase for 2 h; Lane 5: Polyplex (w/w = 1.6) treated with heparin; Lane 6: Polyplex (w/w = 1.6) with DNase for 2 h followed by heat-inactivation of DNase and then treated with heparin.

Dynamic light scattering (DLS) assay was subsequently carried out to study the particle sizes and zeta potentials of all formed polyplexes. Results in Fig. 5A reveal that with the increase of w/w ratio, the particle sizes decreased and tended to be constant with mean sizes of 200–350 nm. It is worth mentioning that the particle sizes determined by DLS were much larger than those obtained by TEM. This might be due to the experimental conditions: the particle sizes determined by DLS were examined in the hydrated state in solution, while those obtained by TEM had been dried after dropping complexes solution onto C-coated Cu meshes [38,39]. **MP1**/DNA shows smaller particle size than others, this might be due to the primary amines in **MP1** structure. Meanwhile, **MP6**/DNA also shows small size which could be attributed to its higher DNA binding ability. With respect to the surface charge, the excess of cationic polymer over DNA molecules generally resulted in an overall positive zeta-potentials. As shown in Fig. 5B, the zeta potential of polyplexes gave an increasing trend along with the rise of weight ratio. The values turned to be positive from w/w of 0.8, which is in accord with the w/w for complete DNA retardation observed in gel electrophoresis. Further increase of w/w led to much slighter increase of zeta potentials, which finally reached about +40 mV. This positive charge might facilitate the interaction between the polyplexes and cell membrane, leading to more efficient cellular uptake.

3.3. Cytotoxicity

CCK8-based cell viability assays were performed in HEK293, HeLa, U2OS and HepG2 cells at various concentrations which cover the range used in the gene transfection assays. 25 KDa PEI was used as control, and the results are shown in Fig. 6. In most cases, the cell viabilities of studied polymers were higher than that of 25 KDa PEI after 24 h incubation, suggesting that these polymers have better biocompatibility. The cytotoxicity was found to be affected by molecular weight and surface charge of the polymers. The polymers with higher molecular weights (**MP2**, **MP3**, **MP6**) have higher toxicity, and the cell viability were lower. **MP5** seems to have the lowest cytotoxicity, which might be attributed to its lower molecular weight and zeta potential. We speculate that the presence of ester or amide bonds in the polymer backbone may reduce their toxicity.

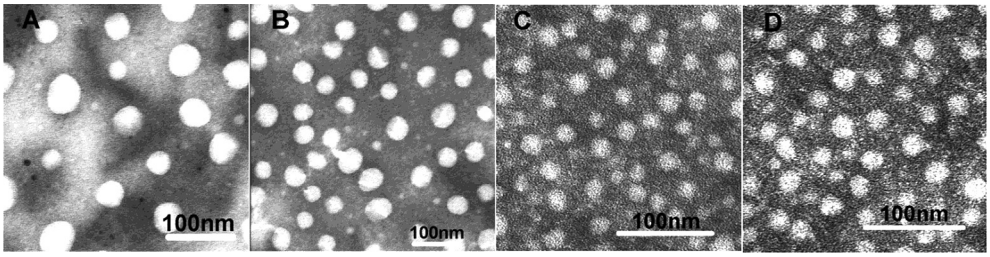


Fig. 4. TEM images of **MP4**/DNA (A and C) and **MP6**/DNA (B and D) polyplexes at w/w of 6.4 in deionized water (A and B) and in water with 10% serum (C and D).

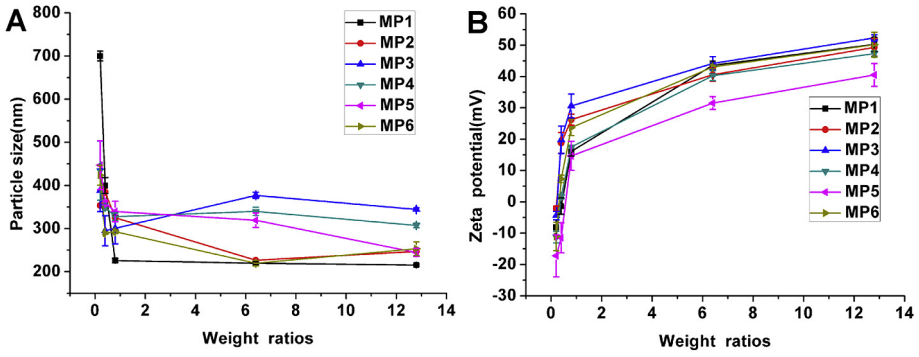


Fig. 5. Average particle size (A) and zeta-potential (B) of **MP1**–**MP6**/DNA polyplexes at weight ratio of 0.2, 0.4, 0.8, 6.4 and 12.8 (mean ± SD, n = 3).

3.4. *In vitro gene transfection*

Theoretically, satisfactory polycationic gene vectors should possess both low cytotoxicity and high TE. To inspect the transfection performance of the newly prepared polymers, gene delivery experiments were first performed in HeLa cells using pGL-3 as reporter gene. Results in Fig. 7A reveal that in the absence of serum, only **MP4** has slightly higher TE than PEI, which was used under its optimal w/w ratio. However, to our delight, with the presence of 10% serum, all of the polymers exhibited higher TE than PEI. Especially for **MP4** and **MP6**, 12 and 19 times respectively higher TEs were obtained at the optimized w/w ratio (12.8). Results clearly

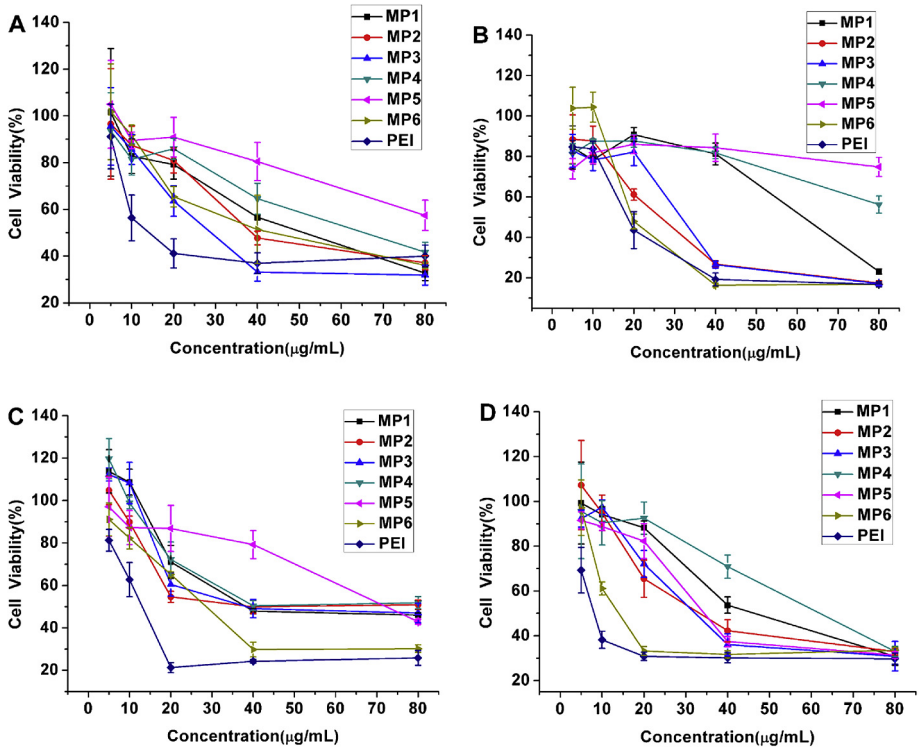


Fig. 6. Relative cell viabilities caused by **MP1**–**MP6** toward HEK293 (A), HeLa (B), U2OS (C) and HepG2 cells (D), 25 KDa PEI was used as control. Data represent mean ± SD (n = 3).

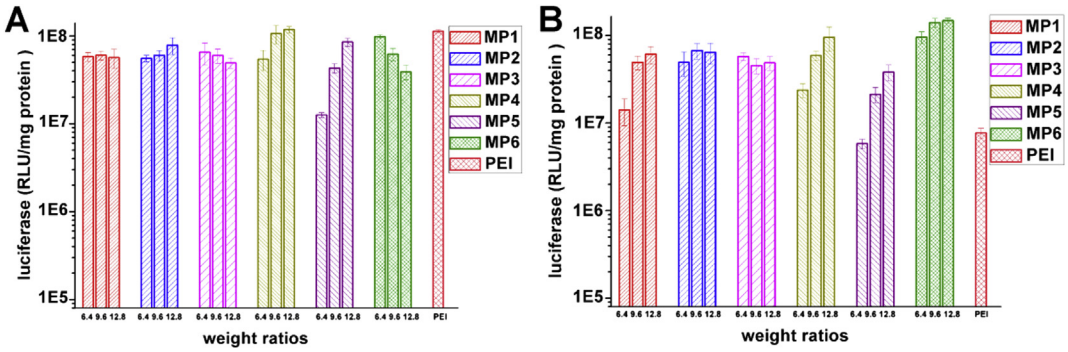


Fig. 7. Luciferase gene expression transfected by polyplexes **MP1**–**MP6** at different weight ratios in comparison with 25 KDa PEI (w/w = 1.4, N/P = 10) in HeLa cells in the absence (A) and presence (B) of serum. Data represent mean ± SD (n = 3).

indicate that these materials have much higher serum tolerance than 25 KDa PEI. Such biocompatibility might be ascribed to the cross-linking LMW PEI structure, in which the oxygen atoms may screen the positive charges on the polyplex, preventing their aggregation with negatively charged serum proteins.

The transfections mediated by **MP4** and **MP6** in other cell lines were subsequently processed. Normal cell HEK293 and tumor cells U2OS/HepG2 were applied to study the TE without or with the presence of serum. Although such two materials exhibited some difference of TE between these cell lines, we could also find that better TE could be obtained in the presence of serum, similar to the results in HeLa cells. **MP6** gave better TE than **MP4** in HEK293 and HepG2 cells, and 4.5 and 17 times higher TE than PEI were obtained in the presence of serum, respectively (Fig. 8A and C). Meanwhile, in U2OS cells, **MP4** gave better results (Fig. 8B). Results further demonstrate that this type of cationic polymers might act as promising non-viral gene vectors toward various cell lines. In addition, the effect of serum was further studied by varying its concentration in **MP6**-mediated transfection in HepG2 cells. As shown in Fig. 8D, the TE of PEI dramatically decreased with the rise of serum concentration. On the contrary, no obvious TE decrease was observed in **MP6**-mediated transfection until the serum concentration was raised to >40%, and up to 355 times higher TE than PEI was obtained at its optimal w/w ratio (9.6) with 70% serum. Results also indicate that with the increase of serum, higher polymer dose (w/w) is needed for better TE. This might be attributed to the more amount of negatively charged proteins in higher concentration of serum, thus more cationic polymers is necessary for efficient DNA binding and protection. To directly visualize the transfected cells, enhanced green fluorescent protein (eGFP)

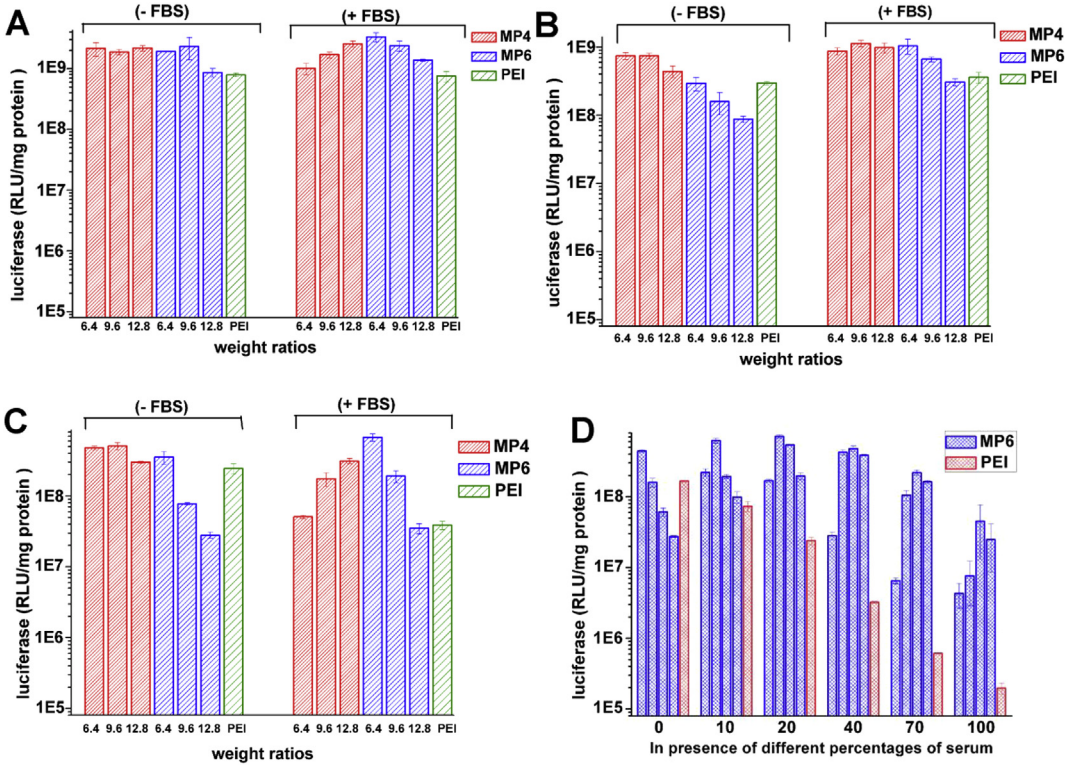


Fig. 8. Luciferase gene expression transfected by **MP4** and **MP6** at various w/w in comparison with 25 KDa PEI (w/w = 1.4, N/P = 10) in HEK293 (A), U2OS (B) and HepG2 (C) cells in the absence and presence of 10% serum. (D): TE of **MP6** in HepG2 cells in the presence of different concentrations of serum, the weight ratios in each group were 3.2, 6.4, 9.6 and 12.8. Data represent mean ± SD (n = 3).

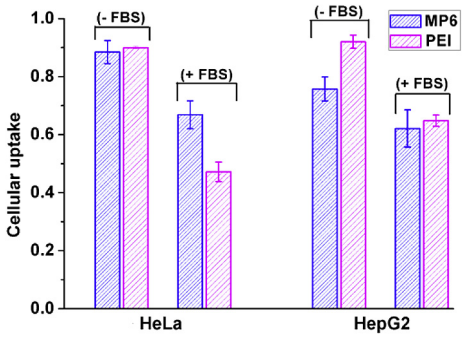


Fig. 9. Cellular uptake of **MP6**/DNA polyplexes at optimal transfection w/w ratio in HeLa and HepG2 cells (in the absence and presence of serum) quantified by flow cytomety analysis. 25 KDa PEI was used as control. Data represent mean ± SD (n = 3).

expression in HeLa and U2OS cells was also observed by an inverted fluorescent microscope, and the weight ratios were used according to the optimal w/w obtained in luciferase assay. Results also show that unlike PEI, the GFP expression mediated by these polymers with the presence of 10% serum was ever higher than those without serum (Fig. S4).

The TE of gene vector depends on a multiple factors, i.e. DNA condensation and protection, serum stability, cellular uptake efficiency, intracellular trafficking such as endosomal escape, DNA unpacking, and nuclear entry [40]. To further study the transfection mechanism by title polymers, we applied flow cytometry to investigate the cellular uptake of the **MP6**/DNA polyplexes, and the results in HeLa and HepG2 cells are shown in Fig. 9. After 4 h incubation of polyplexes with the cells, the percentage of positive cells for Cy5-labeled pDNA was calculated. It was shown that in both cell lines, serum has less negative effect on the celluar uptake of **MP6**/DNA than that of the polyplex formed from PEI. Such advantage of **MP6** may contribute to its better serum tolerance. In HeLa cells, the cellular uptake results are quite consistent with the transfection results shown in Fig. 7, indicating that cellular uptake might be crucial element to affect the TE. In HepG2 cells, although the TE of **MP6** is much higher than PEI in the presence of serum (Fig. 8C), the uptake of **MP6**/DNA is still lower. Such celluar uptake was also observed by confocal microscopy (Fig. S5). Therefore, it may be concluded that **MP6** has much more efficient intracellular DNA delivery ability in such cell line. To visually illuminate the internalization and intracellular distribution of the polyplexes, molecular probes Cy5 and DAPI were used to tag DNA and nucleus in the transfection toward HeLa cells. As shown in Fig. 10, after 2 h incubation, a small quantity of Cy5 labeled complexes were accumulated in the perinuclear region of cells, and serum had no obvious effect on the transfection. When the incubation time was elongated to 4 h, the red fluorescence evidently increased. It could also be found that serum has little effect on **MP6**-mediated transfection but has large negative effect on that involving PEI, indicating the uptake inhibition of PEI/DNA complex.

4. Conclusion

In summary, a new series of polycationic gene delivery vectors were synthesized via Michael addition from LMW PEI and linking compounds. These materials can efficiently condense DNA into stable nanoparticles with proper sizes and zeta-potentials. Compared to 25 KDa PEI, these polymers exhibited lower cytotoxicity and higher TE. More importantly, these materials have distinctly higher serum tolerance than PEI. In the presence of 10% serum, **MP6** may give 19 and 17 times higher TE than PEI in HeLa and HepG2 cells, respectively. No obvious TE decrease was observed even the serum concentration was raised to >40%, indicating that these materials may act as promising candidates for non-viral gene delivery in future *in vivo* application.

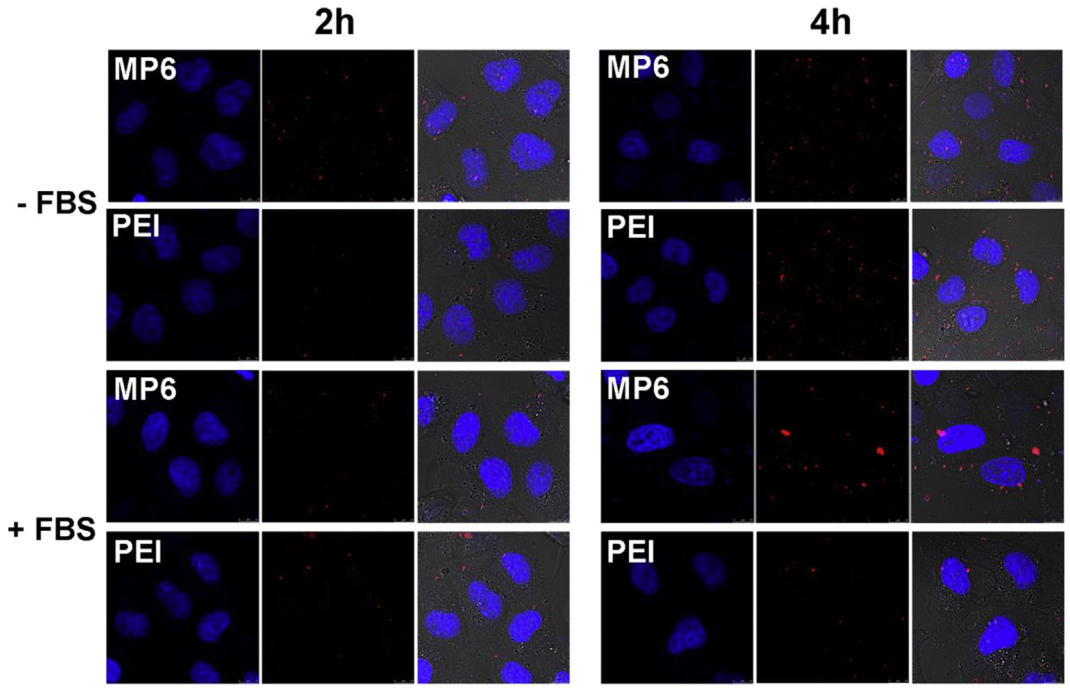


Fig. 10. Confocal microscopic images of HeLa cells treated with Cy5 labeled **MP6**/DNA polyplexes at optimal transfection w/w ratio for 2 h and 4 h in the absence and presence of serum, 25 KDa PEI was used as control (w/w = 1.4). For each triad image, left: cell nuclei stained by DAPI (blue); middle: Cy5-labeled pDNA (red); right: merged image. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.polymer.2015.03.070.

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