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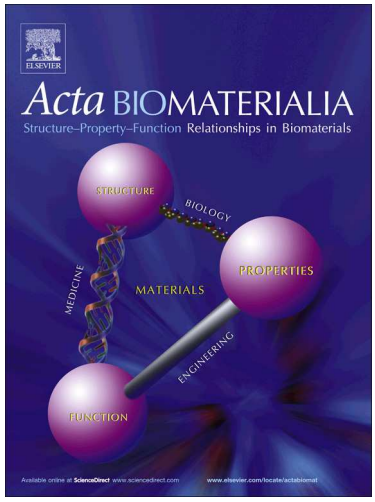

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Biodegradable cationic poly(carbonates): effect of varying side chain hydrophobicity on key aspects of gene transfection

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Abstract

The degree of hydrophobicity in cationic polymers plays an important but often underappreciated role in the safety and efficacy of gene delivery processes. In order to further elucidate structure-activity relationships of biodegradable cationic poly(carbonate) gene carriers, we synthesized a series of narrowly dispersed homo-polymers *via* metal-free organocatalytic living ring-opening polymerization (ROP) of cyclic carbonate monomers bearing either alkyl (propyl, hexyl or nonyl) or 4-methyl benzyl halide side chains. The polymers were then quaternized using bis-tertiary amines to install both quaternary ammoniums and tertiary amines for DNA binding and endosomal escape, respectively. Among the polymers with similar molecular lengths and charge densities, it was found that an increase in side chain alkyl spacer length from 3 to 6 carbons significantly enhanced cellular uptake and luciferase gene expression in HepG2 and HeLa cell lines without causing overt hemolysis and cytotoxicity. A further increase of side chain alkyl length to 9 carbons, however, led to a drastic decline in gene expression due to increased cellular toxicity, which was correlated with an increased disruption and lysis of red blood cell membranes. Interestingly, the incorporation of an aromatic 4-methyl benzyl spacer increased DNA binding strength, reduced particle sizes of resultant DNA complexes, and enhanced cellular uptake, leading to improved luciferase gene expression, albeit with higher levels of hemolysis and cytotoxicity. Taken together, the findings of this study demonstrate that a delicate balance between cationic charge density and hydrophobicity could be achieved by utilizing a hexyl

spacer in the side chains of cationic poly(carbonates), hence providing insights on the future development of non-viral cationic polymeric gene delivery systems.

1. Introduction

Cationic polymers have been the focus of intense research as non-viral gene carriers due to a greater flexibility in their synthesis to vary chemical composition, molecular weight, architecture, and functionality [1]. The use of biodegradable polymers which undergo degradation in biological milieus into non-toxic excretable side products has been recognized to not only be advantageous for reducing toxicity and preventing the long-term accumulation of polymers in the body, but also in facilitating the release of the sequestered DNA molecules following cellular uptake [2-5]. For successful clinical applications of biodegradable cationic polymers, key challenges including poor control of molecular weights, large polydispersities, low gene transfection efficiencies, and poor colloidal stabilities remain to be overcome. In recent years, cationic poly(carbonates) have emerged as a highly promising class of materials for gene delivery due to their inherent biodegradability, low cytotoxicities at effective doses, and efficient gene transfection abilities [6-13]. Advances made in the synthesis of functional cyclic carbonate monomers [14, 15] and metal-free living organocatalytic ring-opening polymerization (ROP) [16, 17], in particular, have afforded a facile and cost effective method to obtain well-defined and narrowly dispersed cationic poly(carbonates) with a wide range of functionalities that are desirable for biomedical applications. For instance, we have previously reported the synthesis of a novel cationic poly(carbonate) prepared via random copolymerization of cyclic carbonate monomers bearing ethyl and propyl bromide side chains, followed by quaternization with a bis-

tertiary amine, and demonstrated that the cationic poly(carbonate) mediated high gene expression levels, which were comparable or higher than that induced by the "gold" standard branched 25 kDa poly(ethylene imine) (PEI) in various cell lines without causing significant cytotoxicities [6]. The subsequent incorporation of various functionalities such as poly(ethylene glycol) [7] and galactose ligands [8] led to enhancements in colloidal stability as well as conferred liver cell targeting capabilities.

In order to fully realize the potential of cationic poly(carbonates) as non-viral gene carriers in therapeutic applications, it is imperative to further elucidate structure-function relationships so as to obtain the most optimal balance between gene transfection efficiencies and toxicity. The degree of hydrophobicity and chemical structure of hydrophobes in cationic polymers has a profound, but often underappreciated role in influencing many aspects of gene delivery including DNA binding and condensation, promoting polyplex charge inversion to enhance adsorption to cell membranes, overcoming serum inhibition, and facilitating dissociation of DNA from polycations [18, 19]. For instance, the modification of low molecular weight PEI (1.8 kDa) with different hydrophobic moieties including ethyl, octyl, deodecyl, benzyl and phenylurea using an established methylcarboxytrimethylene carbonate (MTC) monomer platform revealed that as little as a single hydrophobic modification per polymer is sufficient to reduce polyplex sizes, increase surface charges, and enhance gene transfection efficiencies [20]. A molecular dynamics simulation study has also showed that oleic acid-modified PEI associated with DNA in a more energetically favored manner to form more stable polyplexes with lipid tails on the periphery, which enhances interaction with cell membranes to promote cellular internalization [21]. Using a library of end-modified poly(β-amino esters) synthesized by Michael addition of various diacrylate and primary amine combinations, Sunshine *et al.* has also shown that while increasing

base polymer backbone and/or side chain hydrophobicity tended to significantly enhance gene transfection, an increase in side chain hydrophobicity produced even more dramatic improvements in gene transfection as compared to increasing base polymer diacrylate hydrophobicity, plausibly due to the greater spacing between charged nitrogens as the diacrylate alkyl length increases [22].

From these studies, it is obvious that a balance between hydrophobicity and charge density needs to be achieved for optimal gene transfection. In our efforts to improve the gene transfection efficiencies of cationic poly(carbonates), and further define structure-activity relationships influencing key aspects of gene transfection, we synthesized a series of polymers with a same-centered structure composed of a poly(carbonate) backbone, and with side chains comprised of a hydrophobic spacer (propyl, hexyl, 4-methyl benzyl or nonyl) and hydrophilic moiety comprised of quaternary ammonium for DNA binding and tertiary amine for endosomal escape (**Fig. 1a**). We hypothesized that a moderate increase in side chain hydrophobicity from the typical propyl side chain utilized in our previous studies [6, 7] would enhance the gene transfection capability of the cationic poly(carbonate) and serve to inform future designs of gene transfection polymers. Furthermore, the procedure for the incorporation of hydrophobic segments used in this study does not consume amino groups which are essential for DNA binding and condensation. As such, any potential reductions in cationic charge densities, stabilities, and gene transfection efficiencies typically observed with a high degree of hydrophobe substitution via primary amines present on cationic gene delivery polymers [20, 23] can be circumvented, hence allowing for better delineation of hydrophobic-hydrophilic effects. The effects of side chain hydrophobicity of the cationic poly(carbonates) bearing similar charge densities on DNA binding abilities, physicochemical properties, cellular uptake, gene

transfection capabilities, and cytotoxicities were investigated in the human hepatocellular carcinoma HepG2 and human cervical cancer HeLa cell lines.

2. Materials and methods

2.1. Materials

N-(3,5-trifluoromethyl)phenyl-*N*'-cyclohexylthiourea (TU) was prepared as previously reported [16]. TU was dried using CaH_2 in dry THF, recovered by filtration and vacuum dried to remove THF. 1,8-Diazabicyclo[5,4,0]undec-7-ene (DBU) was dried over CaH_2 and vacuum distilled before use. All other chemicals were bought from Sigma-Aldrich and used as received unless especially mentioned. Ultra-pure (HPLC grade) water was provided by J.T. Baker (U.S.A.), phosphate-buffered saline (PBS) and tris-borate-EDTA (TBE) buffers purchased from 1st BASE (Singapore). Ethidium bromide solution was bought from Biorad Laboratories (U.S.A.), 1 kb DNA ladder obtained from New England Biolabs, reporter lysis buffer and luciferin substrate purchased from Promega (U.S.A.), and bicinchoninic acid (BCA) protein assay reagent bought from Pierce (U.S.A.). Plasmid DNA encoding the 6.4 kb firefly luciferase gene driven by the cytomegalovirus (CMV) promoter was provided by Carl Wheeler, Vical (U.S.A.), amplified in *Escherichia coli* DH5α and purified using Endofree Giga plasmid purification kit from Qiagen. Fetal bovine serum (FBS), sodium pyruvate, non-essential amino acids, penicillin, streptomycin, ultrapure agarose and minimum essential medium (MEM) were obtained from Invitrogen (U.S.A.). HeLa and HepG2 cell lines were bought from ATCC (U.S.A.), and cultured according to the recommended protocols.

2.2. Synthesis of functional carbonate monomers

Synthesis of 5-methyl-5-(3-bromopropyl)oxycabonyl-1,3-dioxan-2-one (MTC-PrBr) [21] and 4-(chloromethyl)benzyl 5-methyl-2-oxo-1,3-dioxane-5-carboxylate (MTC-BnCl) [22] was reported in our previous work. In a similar protocol [21], 5-methyl-5-(6-bromohexyl)oxycabonyl-1,3-dioxan-2-one (MTC-HexBr) and 5-methyl-5-(9-bromononyl)oxycabonyl-1,3-dioxan-2-one (MTC-NonBr) were synthesized. ^{1}H NMR of MTC-HexBr (400 MHz, $CDCl_3$, 22 °C): δ 4.69 (d, 2H, -CH_2OCOO-), 4.21 (m, 2H, -CH_2OCOO-), 4.18 (s, 2H, -OCH_2-), 3.41 (t, 2H, -CH_2Br), 1.86 (m, 2H, -CH_2-), 1.69 (m, 2H -CH_2-), 1.48 (m, 2H, -CH_2-), 1.38 (m, 2H, -CH_2-), 1.32 (s, 3H, -CH_3). ^{1}H NMR of MTC-NonBr (400 MHz, $CDCl_3$, 22 °C): δ 4.68 (d, 2H, -CH_2OCOO-), 4.20 (m, 2H, -CH_2OCOO-), 4.18 (s, 2H, -OCH_2-), 3.41 (t, 2H, -CH_2Br), 1.85 (m, 2H, -CH_2-), 1.66 (m, 4H -CH_2-), 1.42 (m, 2H, -CH_2-), 1.32 (m, 9H, -CH_2- and -CH_3).

2.3. Synthesis of poly(carbonates)

ROP of MTC-HexBr using 4-methylbenzyl alcohol (4-MBA) as the initiator is given as a typical example. All polymerizations were carried out in a nitrogen-filled glove box. 4-MeBnOH (5.0 mg, 0.0409 mmol), MTC-HexBr (0.397 g, 1.23 mmol), and TU (18.9 mg, 0.0615 mmol) were first dissolved in 1.5 mL of DCM. DBU (9.18 µL, 0.0614 mmol) was added to initiate the polymerization and the reaction mixture was stirred at room temperature for 1 h. Subsequently, acetic anhydride (120 µL, 1.27 mmol) was added and the solution was stirred at room temperature for a further 48 h. Finally, the crude polymer solution was precipitated into cold methanol twice and the precipitate was dried *in vacuo*. P(MTC-HexBr) Yield: 0.306 g (76 %), PDI 1.26. ^{1}H NMR (400 MHz, $CDCl_3$, 22 °C): δ 5.10 (s, 2H, -PhCH_2O- of 4-MBA), 4.29 (s, 120H, -CH_2OCOO- and -OCH_2-), 4.13 (t, 60H, -OCH_2-), 3.42 (t, 60H, -CH_2Br), 2.35 (s, 3H, -

CH_3 of 4-MBA), 2.05 (s, 3H, H of acetyl end), 1.87 (quin, 60H, -CH_2-), 1.65 (m, 60H, -CH_2-), 1.46 (m, 60H, -CH_2-), 1.38 (m, 60H, -CH_2-),1.27 (s, 90H, -CH_3).

P(MTC-PrBr) Yield: 0.207 g (74 %), PDI 1.26. ^{1}H NMR (400 MHz, $CDCl_3$, 22 °C): δ 7.22 (dd, 4H, Ph-H of 4-MBA), 5.11 (s, 2H, -PhCH_2O- of 4-MBA), 4.29 (m, br, 192H, -CH_2OCOO-, -OCH_2- and -OCH_2-), 3.45 (t, 64H, -CH_2Br), 2.35 (s, 3H, -CH_3 of 4-MBA), 2.19 (quin, 64H, -CH_2-), 2.06 (s, 3H, H of acetyl end), 1.27 (s, 96H, -CH_3).

P(MTC-BnCl) Yield: 0.289 g (78 %), PDI 1.28. ^{1}H NMR (400 MHz, $CDCl_3$, 22 °C): 7.31 (dd, 124H, Ph-H of 4-(chloromethyl)benzyl alcohol), 7.22 (dd, 4H, Ph-H of 4-MBA), 5.12 (s, 62H, -OCH_2-), 5.08 (PhCH_2O- of 4-MBA), 4.55 (s, 62H, -CH_2Cl), 4.27 (s, 124H, -CH_2OCOO- and -OCH_2-), 2.34 (s, 3H, -CH_3 of 4-MBA), 1.95 (s, 3H, H of acetyl end), 1.23 (s, 93H, -CH_3).

P(MTC-NonBr) Yield: 0.130 g (70 %), PDI 1.26. ^{1}H NMR (400 MHz, $CDCl_3$, 22 °C): 7.22 (dd, 4H, Ph-H of 4-MBA), 5.10 (s, 2H, -PhCH_2O- of 4-MBA), 4.28 (s, 124H, -CH_2OCOO- and -OCH_2-), 4.11 (t, 62H, -OCH_2-), 3.41 (t, 62H, -CH_2Br), 2.35 (s, 3H, -CH_3 of 4-MBA), 2.05 (s, 3H, H of acetyl end), 1.85 (quin, 62H, -CH_2-), 1.62 (m, 124H, -CH_2-), 1.42 (m, 62H, -CH_2-), 1.31 (s, 279H, -CH_2- and -CH_3), 1.25 (s, 93H, -CH_3).

2.4. Quaternization of poly(carbonates)

The amination of the P(MTC-HexBr) polymer is given as a typical example. The polymer (0.306 g, 0.93 mmol Br^-) was dissolved in DMSO (5 mL) and *N,N,N′,N′*-tetramethylethylenediamine (TMEDA; 580 µL, 3.87 mmol) was added. The reaction mixture was allowed to stir overnight, and the crude product was purified via dialysis using a 1,000 Da molecular weight cutoff regenerated cellulose membrane tubing against an acetonitrile/isopropanol (1:1, v/v) mixture for 48 h. Finally, the solvent was evaporated and the

final product was freeze-dried to give a white solid (0.269 g, 65 %). P[MTC-$O(CH_2)_6N^+(CH_3)_2Br^-(CH_2)_2N(CH_3)_2$] ^{1}H NMR (400 MHz, $(CD_3)_2SO$, 22 °C): δ 7.21 (dd, 4H, Ph-H of 4-MBA), 5.09 (s, 2H, -$PhCH_2O$- of 4-MBA), 4.19-4.28 (m, br, 120H, H of -CH_2OCOO- and -OCH_2-), 4.05 (t, 60H, -OCH_2-), 3.45 (m, br, 60H, -CH_2N^+-), 3.09 (s, 180H, -N^+CH_3), 2.62 (t, 60H, -NCH_2-), 2.31 (s, 3H, -CH_3 of 4-MBA), 2.19 (s, 180H, -NCH_3), 2.01 (s, 3H, H of acetyl end), 1.66 (s, 60H, -CH_2-), 1.58 (m, 60H, -CH_2-), 1.33 (m, br, 120H, -CH_2-), 1.18 (s, 90H, -CH_3).

P[MTC-$O(CH_2)_3N^+(CH_3)_2Br^-(CH_2)_2N(CH_3)_2$] (0.201 g, 70 %) ^{1}H NMR (400 MHz, $(CD_3)_2SO$, 22 °C): δ 7.21 (dd, 4H, Ph-H of 4-MBA), 5.09 (s, 2H, -$PhCH_2O$- of 4-MBA), 4.25-4.31 (m, br, 128H, -CH_2OCOO- and -OCH_2-), 4.12 (s, 64H, -OCH_2-), 3.48 (s, br, 128H, -CH_2N^+-), 3.14 (s, 192H, -N^+CH_3), 2.64 (s, 64H, -NCH_2-), 2.37 (s, 3H, -CH_3 of 4-MBA), 2.31 (s, 192H, -NCH_3), 2.20 (s, br, 64H, -CH_2-), 2.01 (s, 3H, H of acetyl end), 1.21 (s, 96H, -CH_3).

P[MTC-OBn$N^+(CH_3)_2Cl^-(CH_2)_2N(CH_3)_2$] (0.326 g, 82 %) ^{1}H NMR (400 MHz, $(CD_3)_2SO$, 22 °C): δ 7.33 (dd, 124H, Ph-H of 4-(chloromethyl)benzyl alcohol), 7.17 (dd, 4H, Ph-H of 4-MBA), 5.17 (s, 62H, -OCH_2-), 5.05 (s, 2H, -$PhCH_2O$- of 4-MBA), 4.70 (s, 62H, -CH_2N^+-), 4.27 (s, 124H, H of -CH_2OCOO- and -OCH_2-), 3.49 (s, br, 62H, H of -CH_2N^+-), 3.03 (s, 186H, -N^+CH_3), 2.74 (s, 62H, -NCH_2-), 2.35 (s, 3H, -CH_3 of 4-MBA), 2.21(s, 186H, -NCH_3), 1.94 (s, 3H, H of acetyl end), 1.21(s, 93H, -CH_3).

P[MTC-$O(CH_2)_9N^+(CH_3)_2Br^-(CH_2)_2N(CH_3)_2$] (0.143 g, 84 %) ^{1}H NMR (400 MHz, $(CD_3)_2SO$, 22 °C): δ 7.19 (dd, 4H, Ph-H of 4-MBA), 5.08 (s, 2H, -$PhCH_2O$- of 4-MBA), 4.17-4.26 (m, br, 124H, H of -CH_2OCOO- and -OCH_2-), 4.04 (s, 62H, -OCH_2-), 3.41 (s, br, 62H, -CH_2N^+-), 3.35 (s, 62H, -CH_2N^+-), 3.07 (s, 186H, -N^+CH_3), 2.61 (s, 62H, -NCH_2-), 2.30 (s, 3H, -CH_3 of 4-MBA), 2.19 (s, 186H, -NCH_3), 1.99 (s, 3H, H of acetyl end), 1.65 (s, 62H, -CH_2-), 1.53 (m, 62H, -CH_2-), 1.27 (s, 310H, -CH_2-), 1.16 (s, 93H, -CH_3).

2.5. Molecular characterization of cationic poly(carbonates)

2.5.1. Gel permeation chromatography (GPC)

The GPC elution curve for the homopolymers were obtained using a Waters HPLC system equipped with a 2690D separation module with two Styragel HR1 and HR4E (THF) 5 mm columns (size: 300 × 7.8 mm) in series, and a Waters 410 differential refractometer detector. THF was used as the mobile phase (flow rate: 1 mL/min). Number-average molecular weights and polydispersity indices were obtained from a calibration curve of a series of polystyrene standards (Molecular weights: 1,350 - 151,700 Da).

2.5.2. ^{1}H NMR spectroscopy

The ^{1}H NMR measurements were performed using a Bruker Advance 400 MHz NMR spectrometer with an acquisition time of 3.2 s, a pulse repetition time of 2.0 s, a 30° pulse width, 5208-Hz spectral width, and 32 K data points. Chemical shifts were referred to the solvent peaks (δ = 7.26 and 2.50 ppm for $CDCl_3$ and DMSO-d_6, respectively).

2.6. Degradation of cationic poly(carbonate) in physiological conditions

To investigate the degradation of the synthesized cationic poly(carbonate), 5.0 mg of polymer **b** was dissolved in 1.0 mL of 1 × PBS (pH 7.4) and incubated for 26 days at 37 °C prior to freeze-drying. As a control, a second 5.0 mg sample of polymer **b** was dissolved in 1.0 mL of 1 × PBS and freeze-dried immediately. Subsequently, the lyophilized samples were separately dissolved in 0.7 mL of deuterated DMSO and analyzed for compositional changes using ^{1}H NMR spectroscopy as described above.

2.7. Water-to-octanol partition coefficient (log P) determination

The dansyl-conjugated polymers were individually prepared at 62.5 and 125 mg/L in 500 μL of PBS, followed by adding an equal volume of octanol to the microfuge tube. The tube was vortexed for 5 min and allowed to equilibrate overnight at room temperature in the dark. Following incubation, a known aliquot of the aqueous and lipid phase was separately diluted 10-fold in methanol, and the fluorescent intensities of the dansyl-conjugated polymers were determined using a microplate reader with excitation and emission wavelengths of 336 and 520 nm, respectively. The concentration of cationic polycarbonate in each phase was determined by comparing the fluorescent intensities against that obtained from a calibration curve prepared from known polymer concentrations in methanol for each type of polymer. The water-to-octanol partition coefficient was defined as log P = log [(Polymer concentration)$_{\text{in octanol layer}}$/(Polymer concentration)$_{\text{in aqueous layer}}$].

2.8. Preparation of cationic poly(carbonate)/DNA or PEI/DNA complexes

To form the poly(carbonate)/DNA complexes at the intended N/P ratios (molar ratio of nitrogen content in the polymer to the DNA phosphorus content), equal volumes of DNA solution was added drop-by-drop into the poly(carbonate) solutions prepared at various concentrations in 10 mM sodium phosphate buffer (pH 6.0), which was found in our lab to be optimal for gene transfection using cationic polycarbonates bearing pendant tertiary and quaternary amines due to the greater protonation of the former in slightly acidic conditions under gentle vortexing for approximately 10s. The mixture was then incubated at room temperature (23 °C) for 30 min before use.

2.9. Particle size measurement

The particle sizes of the poly(carbonate)/DNA complexes prepared in 10 mM sodium phosphate buffer (pH 6.0) were measured using the Zetasizer Nano (Malvern Instrument Ltd., Worcestershire, UK) equipped with a 633 nm He-Ne laser. Scattered light was detected at an angle of 173˚ and at a set temperature of 25 °C. Particle size measurements were repeated for 3 runs per sample and reported as the mean ± standard deviation of 3 readings.

2.10. Gel retardation assay

The poly(carbonate)/DNA complexes prepared at N/P ratios from 0 to 10 were electrophoresed on 0.7% agarose gel which was pre-stained with ethidium bromide in 0.5 × TBE buffer at 80 V for 50 min. The relative positions of the complexed to naked DNA were evaluated under an UV illuminator (Chemi Genius, Evolve, Singapore).

2.11. Heparin displacement assay

Poly(carbonate)/DNA complexes were first formed at N/P ratio 21, and then incubated with equal volumes of heparin (final concentration ranging from 0 to 0.8 mg/mL) in 10 mM sodium phosphate buffer (pH 6.0 and 7.4) for 1 h at room temperature. Subsequently, an equal volume of SYBR Safe Green dye (diluted 1:20,000 using water), which intercalates with free DNA to give a fluorescent signal was added, and the relative amount of DNA displaced from the poly(carbonate)/DNA complexes was determined through fluorescent intensity measurements using the TECAN microplate reader. Relative fluorescence (%) = $(F_{sample}-F_{blank})/(F_{free\ DNA}/F_{blank})$ × 100.

2.12. Hemolysis testing

In this experiment, rat blood was obtained from Biological Resource Center (BRC), Agency for Science, Technology and Research (A*STAR), Singapore, and the protocol was approved by the Institutional Animal Care and Use Committee (IACUC), BRC, A*STAR, Singapore. A 2% v/v of fresh rat red blood cells diluted in PBS (600 μL) was added into each tube containing 60 μL of the poly(carbonate)/DNA complexes prepared at different N/P ratios. Following 1 h incubation at 37 °C, the tubes were centrifuged at 1000 × g for 5 min. 100 μL of the supernatant was transferred into each well of a 96-well plate, and analyzed for hemoglobin absorption at 576 nm using a TECAN microplate reader. Red blood cells suspension containing sodium phosphate buffer was used as negative control, while that lyzed with 0.1 % v/v Triton X-100 gave 100% hemolysis. Percentage of hemolysis was calculated using the following formula:

% Hemolysis = [(O.D.$_{576nm}$ of poly(carbonate)/DNA complexes − O.D.$_{576nm}$ of sodium phosphate buffer)/(O.D.$_{576nm}$ of 0.1% v/v Triton X-100 − O.D.$_{576nm}$ of sodium phosphate buffer)] × 100.

2.13. Cytotoxicity testing

HepG2 and HeLa cells were respectively maintained in MEM and DMEM growth media that were supplemented with 10% FBS, 1 mM sodium pyruvate, 100 U/mL penicillin and 100 mg/mL streptomycin, and cultured at 37 ^{o}C, under an atmosphere of 5% CO_2 and 95% humidified air.

HepG2 and HeLa cells were seeded onto 96-well plates at a density of 15, 000 and 6,000 cells per well, respectively, one day before treatment. The cells were then incubated with 10 μL of poly(carbonate)/DNA complexes in 100 μL of fresh media for 4 h at 37 ^{o}C. After removing the

incubation medium, and replacing each well with fresh media, the cells were returned to the incubator for a further 68 h. The MTT assay was then performed as previously reported [6].

2.14. In vitro luciferase gene expression

The *in vitro* gene transfection efficiency of the cationic poly(carbonate)/DNA complexes was investigated using HepG2 and HeLa cell lines using a luciferase reporter gene. HepG2 and HeLa were seeded onto 24-well plates at a density of 8×10^4 and 6×10^4 cells per well, respectively. The gene transfection procedure and analysis of luciferase expression was performed as previously reported [6].

2.15. Cellular uptake studies

Cellular uptake of the poly(carbonate)/DNA complexes were evaluated by flow cytometry using fluorescently labelled plasmid DNA (pCMV-luciferase) accordingly to a previously reported protocol [24]. Briefly, HeLa cells seeded into 12-well plates at a density of 1.8×10^5 cells per well the day before were incubated with 100 μL of poly(carbonate)/DNA complexes in 1 mL of growth media for 2.5 h. After incubation, the cells were trypsinized, and rinsed once with 1 × PBS. The percentage of cells that have taken up the poly(carbonate)/DNA complexes were analyzed using a flow cytometer (BD LSR II; 10,000 events), and reported as mean ± standard deviation.

2.16. Statistical analysis

Statistical analysis was performed using the two-tailed Student's *t*-test (Microsoft Excel) and the difference between mean readings was considered to be statistically significant when $p < 0.05$.

3. Results and Discussion

3.1. Polymer synthesis and characterization

A series of cationic poly(carbonates) was designed to have a same-centered structure where all the repeat units contain a hydrophobic spacer (propyl, hexyl, 4-methyl benzyl or nonyl) between the polymer backbone and quaternary ammonium group (Fig. 1a). Organocatalytic ROP of alkyl bromide or 4-methyl benzyl chloride functionalized MTC monomers was performed using 4-MBA as the initiator in the presence of a co-catalyst (TU/DBU) system at a monomer to initiator feed ratio of 30 according to a previously reported procedure [6, 14]. To avoid scission of the polymers via possible transesterification in the subsequent amination step, the hydroxyl ends of the precursor polymers were acetylated with acetic anhydride after polymerization. After purification, the polymers were aminated using a large excess of *N,N,N′,N′*-tetramethylethylenediamine (TMEDA) to prevent intra- or inter-molecular crosslinking. The excess TMEDA was subsequently removed by dialysis to give the final cationic poly(carbonate). Using gel permeation chromatography, all polymers were found to have a narrow molecular weight distribution with polydispersity indices (PDI) ranging between 1.26-1.28 (Fig. 1b and Table 1). Polymer lengths and compositions were verified by comparing ^{1}H NMR integration values of the respective protons associated with each carbon atom with 4-methyl benzyl alcohol initiator's methyl protons (3H, –CH3), which are described in detail under Sections 2.3 and 2.4; and with a summary provided in Table 1. All peaks ascribed to 4-MBA, MTC monomers,

TMEDA and the acetyl end groups were clearly observed in the ^{1}H NMR and ^{13}C NMR spectra (Fig.1c, Experimental Section, Fig. S1, and Fig. S2). By comparing the integral intensities of grafted TMEDA methyl peaks (-NCH_3) with that of 4-MBA methyl protons (-CH_3), all MTC monomer units in the final polymers were found to be fully quaternized, and no cross-linking between polymer chains was observed. In this study, the synthesized cationic poly(carbonates) were designed to have similar molecular weights and charge densities (Table 1), which would offer a more valid comparison of the effects of side chain hydrophobicity on key aspects of gene transfection.

3.2. Biodegradation of cationic poly(carbonate)

The biodegradability of the synthesized cationic poly(carbonate) under physiological conditions was investigated by incubating the cationic poly(carbonate) in 1 × PBS at 37 °C for 26 days, followed by ^{1}H NMR analysis after freeze-drying. As seen from Fig. S3, the peaks of H-g and H-h, which correspond to the methylene and methyl groups of the poly(carbonate) backbone disappeared completely, hence indicating a full degradation of the poly(carbonate) backbone. In contrast, no changes in the poly(carbonate) composition was observed with the control sample, which was dissolved in 1 × PBS and freeze-dried immediately. This result was also consistent with the work of Renxi Zhuo and his co-workers who have previously demonstrated the biodegradability and biocompatibility of poly(carbonate)-based polymers in biomedical applications [9, 25].

3.3. Physicochemical properties, DNA binding ability, and binding strength

In order to evaluate the overall hydrophobicity of the cationic polycarbonates, the water-to-octanol partition coefficient (log P), where P refers to the ratio of polymer concentration in the octanol and water phases at equilibrium, was first determined. For this experiment, a series of dansyl-labelled cationic polycarbonates was synthesized (Scheme S1), and their relative fluorescent intensities and hence corresponding concentrations in the octanol and water phases were respectively determined against standard curves of known polymer concentrations. A low log P value indicates hydrophilicity while a higher value indicates relative hydrophobicity. As expected, an increase in the pendant alkyl chain length from propyl to hexyl and nonyl correlated with a step-wise increase in log P values (Fig. S4), hence confirming enhanced polymer hydrophobicity. The polymer bearing the aromatic 4-methyl benzyl side chains (polymer **c**) had a similar log P value to the hexyl containing polymer (polymer **b**; log P = -1.6 and -1.5, respectively at 125 mg/L polymer concentration), hence demonstrating a similar level of hydrophobicity.

As the incorporation of hydrophobic moieties within polycations is expected to contribute to the cooperative binding of genetic materials with electrostatic forces and promote polyplex charge inversion which could affect polyplex stability, interaction with cell membranes, cellular uptake, and the eventual release of DNA from polyplexes [21, 26-28], we first investigated the effects of varying polymer side chain hydrophobicity on key physicochemical properties and DNA binding abilities. As seen from Table 2, the addition of negatively charged DNA to the cationic poly(carbonates) in 10 mM sodium phosphate buffer (pH 6.0) led to the formation of small positively charged complexes with hydrodynamic diameters ranging from 57.4 to 190.1 nm (PDIs of $\leq$ 0.2), and zeta-potentials between + 27.1 to 34.6 mV. While complexes formed from cationic poly(carbonates) with hydrophobic alkyl spacers (polymers **a**, **b** and **d**) have

similar particle sizes among them, that formed from the aromatic 4-methyl benzyl group containing cationic poly(carbonate) (polymer **c**) were found to be significantly smaller at all N/P ratios evaluated ($p < 0.001$). These results suggest that the presence of aromatic 4-methyl benzyl groups in the side chains of the cationic poly(carbonate) may act in concert with electrostatic forces to enhance DNA binding and condensation possibly via π-π stacking with aromatic rings present in nucleotides [29], leading to the formation of more compact polyplexes.

The DNA binding ability of the cationic poly(carbonates) was studied using agarose gel electrophoresis. As seen from Fig. 2a, the cationic poly(carbonates) effectively bound and condensed DNA to completely retard its movement across the agarose gel at a low N/P ratio of 2. To evaluate the binding strength of the cationic poly(carbonates), the heparin displacement assay was performed. In this experiment, the amount of DNA displaced from the cationic poly(carbonate)/DNA complexes by different concentrations of polyanionic heparin was estimated using the SYBR Green dye, which increases in fluorescence over a hundred fold once bound to free double-stranded DNA. From Fig. 2b, it can be observed that the fluorescent intensities for all the cationic poly(carbonate)/DNA complexes increased slightly at low heparin concentrations, with a drastic rise in fluorescent intensities above 0.2 mg/mL heparin indicating dose-dependent displacement of DNA from the complexes. Close to 100% relative fluorescence was attained at a heparin concentration of 0.8 mg/mL, hence indicating complete DNA displacement from the cationic poly(carbonate)/DNA complexes. Interestingly, an increase in cationic poly(carbonate) pendant alkyl chain length from 3 or 6 carbons to 9 carbons resulted in a significantly lower degree of DNA displacement from the cationic poly(carbonate)/DNA complexes at an intermediate heparin concentration of 0.4 mg/mL ($p < 0.05$). This result suggests that the longer and thus more hydrophobic alkyl moieties present in the side chains of

polymer **d** contributed to a greater degree of interaction with the DNA molecules possibly *via* Van der waal's interactions, which have also been reported to facilitate interactions between the hydrophobic vinyl polymer backbones in neutral poly(vinyl alcohol) and poly(vinyl pyrrolidone) with DNA [27, 30]. The amount of DNA displaced from the aromatic 4-methyl benzyl group containing polymer **c**/DNA complexes was similar to that formed from polymer **d**, and was significantly lower than DNA complexes formed from the less hydrophobic polymers **a** and **b**, hence indicating a high degree of DNA interaction or binding which is consistent with the smaller DNA complex sizes observed in Table 2.

The effect of pH on DNA binding by the cationic poly(carbonate) was also investigated by forming the DNA complexes in 10 mM sodium phosphate buffer at pH 7.4 before repeating the heparin displacement assay as described above. At a higher pH, the cationic poly(carbonates) were found to bind DNA more weakly compared to the use of a lower pH of 6.0. This could be seen from the higher initial relative fluorescent intensities of the poly(carbonate)/DNA complexes at 0 mg/mL heparin concentration (~37-52% *vs.* 11-25% at pH 6.0), and the much lower heparin concentrations required to yield DNA displacements of more than 70% relative fluorescent intensities for DNA complexes formed at pH 7.4 (Fig. 2b and Fig. S5). The stronger DNA binding at a lower pH of 6.0 could possibly be attributed to the greater degree of protonation of the tertiary amines of the cationic poly(carbonates), leading to enhanced electrostatic interactions with the DNA molecules when compared to the use of a higher pH at 7.4. Importantly, a similar trend for the effect of cationic poly(carbonate) side chain hydrophobicity on DNA binding strength was also observed at pH 7.4; with polymers **a** and **b** achieving a greater degree of DNA displacement (≥ 90% relative fluorescent intensities)

compared to polymers **c** and **d** (80 and 72% relative fluorescent intensities, respectively) at 0.2 mg/mL heparin concentration (Fig. S5).

3.4. Hemolytic activity and cytotoxicity

As the effect of hydrophobic modifications in polycations on membrane interactions and cytotoxicity is highly dependent on the degree of hydrophobicity and chemical structures of the incorporated hydrophobic moieties [18], the structural-toxicity relationship of the synthesized cationic poly(carbonates) was investigated. The hemolytic activity of the cationic poly(carbonates) was studied using rat red blood cells. As seen from Fig. 3a, the degree of hemolysis generally increases with an increase in alkyl side chain hydrophobicity in the following order: nonyl > hexyl > propyl. Although an increase in pendant alkyl chain length from 3 to 6 carbons in the cationic poly(carbonates) slightly enhanced their hemolytic activity, the level of hemolysis induced by polymer **b** could still be considered low, with HC_{10} values (defined as the lowest peptide concentration that induces > 10% hemolysis) of more than 1000 mg/L. On the other hand, the 4-methyl benzyl alcohol side chain-containing cationic polycarbonate (polymer c) was found to induce significantly higher levels of hemolysis compared to the hexyl side chain containing polymer (polymer b) despite having similar log P values or degree of hydrophobicity ($P < 0.01$). A similar trend in hemolytic potentials was also observed with the cationic poly(carbonate)/DNA complexes, with that formed from the aromatic polymer **c** or more hydrophobic polymer **d** mediating greater red blood cell lysis from N/P ratios 28 onwards (Fig. 3b).

The cytotoxicity of the cationic poly(carbonate)/DNA complexes was evaluated against HepG2 and HeLa cell lines. Consistent with the trend in hemolytic potentials observed earlier, an

increase in hydrophobicity of the pendant hydrocarbon chains in the cationic poly(carbonates) significantly increases the cytotoxicity of the resultant DNA complexes towards both cell lines (Fig. 4). Interestingly, an increase in pendant alkyl chain length from 3 to 6 carbons (polymers **a** and **b**) induced minimal cytotoxicity to mammalian cells with more than 83% cell viability at the highest N/P ratio tested (N/P 42). A further increase to 9 carbons (polymer **d**), however, led to cytotoxicity at low polymer concentrations particularly in HeLa cells for which only 53% cell viability was observed at the lowest N/P ratio tested, hence suggesting a greater degree of interaction between the more hydrophobic nonyl side chains of the cationic poly(carbonate) with membrane lipid bilayers. On the other hand, the cationic poly(carbonate) incorporating bulkier aromatic 4-methyl benzyl moieties in the side chains mediated intermediate levels of cytotoxicity that fall between that of the two poly(carbonates) bearing longer alkyl side chains (n = 6 and 9) with 100% cell viability in HepG2 and more than 75% cell viability in HeLa cells up to N/P ratio 21. These results taken together clearly indicate that the level of membrane disruption and toxicity can be tuned by varying the hydrophobicity and chemical nature of hydrophobic moiety (i.e. linear vs. aromatic) incorporated.

3.5. Cellular uptake study

The cellular uptake propensities of the various cationic poly(carbonate)/DNA complexes were investigated in HeLa cells using DNA labelled with fluorescent YOYO-1 iodide. Due to the acute cellular toxicity observed with the more hydrophobic polymer **d**, cellular uptake studies were performed with DNA complexes formed from polymers **a**-**c** at N/P ratios 14 and 21 at which more than 75% cell viabilities were observed (Fig. 4b). The cellular uptake of the cationic

poly(carbonate)/DNA complexes significantly increased in the following order: polymer **a** < **b** < **c** ($p < 0.05$; Fig. 5), with the more hydrophobic side chains imparting a greater propensity for cellular entry. The results taken together suggest that an increase in pendant alkyl chain length from 3 to 6 carbons or the introduction of an aromatic ring, enhanced membrane permeation without inducing overt cytotoxicity, leading to a greater entry of cationic poly(carbonate)/DNA complexes by adsorptive endocytosis into the cells, where the genetic cargoes can be released to mediate protein expression. The consistently enhanced membrane permeability observed with polymer **c** could be related to the non-covalent cationic-π interactions that have been observed between an electron-rich system such as the benzyl groups and the cationic headgroups of lipids such as phosphatidylcholine in the cell membrane [31], hence leading to greater membrane interaction and the eventual entry of the cationic polycarbonate and its genetic cargoes across the cell membrane.

3.6. In vitro luciferase gene expression

The gene transfection abilities of the cationic poly(carbonates) were compared in HepG2 and HeLa cell lines using a luciferase reporter gene in the presence of serum proteins. In both cell lines, polymers **a**-**c** mediated luciferase expression profiles that were dependent on N/P ratio. The luciferase activity increased with increasing N/P ratios and reached a plateau from N/P 21 onwards (Fig. 6). This result correlated well with the size results. From N/P ratio 21, the size was relatively constant (Table 2). An increase in pendant alkyl chain length from 3 to 6 carbons was found to enhance the luciferase gene expression across most N/P ratios, while the incorporation of the bulky 4-methyl benzyl group in cationic poly(carbonate) side chain mediated moderately higher luciferase gene expression than the hexyl-containing polymer in both cell lines. These

results were consistent with the step-wise increase in cellular uptake of the cationic poly(carbonate)/DNA complexes with increasing polymer hydrophobicities (Fig. 5). A further increase in the pendant alkyl chain length to 9 carbons, however, resulted in a drastic decrease of luciferase expression to levels which were similar to that of the propyl-containing polymer. This observation could be attributed to the high levels of cytotoxicity seen earlier with polymer **d**/DNA complexes in both cell lines (Fig. 4). Importantly, the luciferase gene expression levels induced by polymers **b** and **c** were in the same order of magnitude or higher than the commercially available PEI benchmark. The high levels of gene transfection mediated by both polymers, coupled with the minimal cytotoxicity observed across all N/P ratios tested for polymer **b** (hexyl), and at optimal gene transfection N/P ratio, i.e. N/P 21, for polymer **c** (4-methyl benzyl) demonstrate that the incorporation of these two hydrophobic moieties conferred a delicate balance between charge and hydrophobicity for favorable gene transfection, and further highlight the importance for such investigations in the future development and optimization of cationic polymers for gene delivery applications.

4. Conclusions

A series of narrowly dispersed poly(carbonate) homo-polymers with similar charge densities each possessing pendant groups of varying alkyl lengths and aromatic ring was synthesized by organocatalytic ROP. The subsequent quaternization of the alkyl bromide or 4-methyl benzyl chloride side chains with a bis-tertiary amine served to introduce quaternary and tertiary amines for DNA binding and endosomal escape, respectively. An increase in side chain alkyl length from 3 to 6 carbons was found to improve luciferase gene expression in serum-containing media with minimal toxicity towards mammalian cells at effective concentrations. A further increase to

9 carbons reduced the luciferase gene expression levels to that of the propyl-containing cationic poly(carbonates), concurrent with higher toxicity. In comparison to the polymers bearing linear alkyl side chains, the incorporation of an aromatic 4-methyl benzyl group improved gene transfection, albeit with higher hemolytic properties and cytotoxicity compared to the propyl and hexyl group containing polymers. Cellular uptake studies revealed that the effect of the various hydrophobic moieties in cationic poly(carbonates) to mediate cellular uptake of DNA complexes increases in the following order: propyl < hexyl < 4-methyl benzyl. These results thus clearly demonstrate that the optimization of side chain hydrophobicity and/or aromaticity in cationic polymers is necessary to achieve a fine balance in cellular uptake and gene delivery capabilities, while minimizing unwanted cytotoxic effects arising from hydrophobic interactions and perturbations of membrane phospholipid bilayers.

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Captions of Tables

Table 1 Chemical composition and polydispersity indices (PDIs) of the synthesized cationic poly(carbonates).

Table 2 Particle size, zeta-potential, and polydispersity of cationic poly(carbonate)/DNA complexes in 10 mM sodium phosphate buffer (pH 6.0) determined using dynamic light scattering.

Captions of Figures

Fig. 1. (a) Synthetic scheme and structures of cationic poly(carbonate) homo-polymers, (b) GPC elution curve of synthesized poly(carbonates), and (c) ^{1}H NMR spectra of P(MTC-HexBr) in $CDCl_3$ and its cationic derivative in DMSO-d_6.

Fig. 2. Evaluation of DNA binding ability and binding strength of cationic poly(carbonates). (a) Electrophoretic mobility of DNA in cationic poly(carbonate)/DNA complexes and (b) displacement of DNA from cationic poly(carbonate)/DNA complexes (N/P ratio 21) with increasing concentrations of heparin. Poly(carbonate)/DNA complexes were formed in sodium phosphate buffer at pH 6.0.

Fig. 3. Hemolytic activity of (a) cationic poly(carbonates) and (b) their DNA complexes against rat red blood cells.

Fig. 4. Cytotoxicity of cationic poly(carbonate)/DNA complexes against (a) human hepatocellular carcinoma HepG2 and (b) human cervical cancer HeLa cell lines.

Fig. 5. Cellular uptake of cationic poly(carbonate)/DNA complexes in HeLa cell line at N/P ratios at which minimal cytotoxicities were observed.

Fig. 6. Luciferase gene expression levels in (a) HepG2 and (b) HeLa cell lines mediated by the cationic poly(carbonate)/DNA complexes at various N/P ratios indicated. PEI/DNA complexes were prepared at N/P ratio 10.

Table 1 Chemical composition and polydispersity indices (PDIs) of the synthesized cationic poly(carbonates).

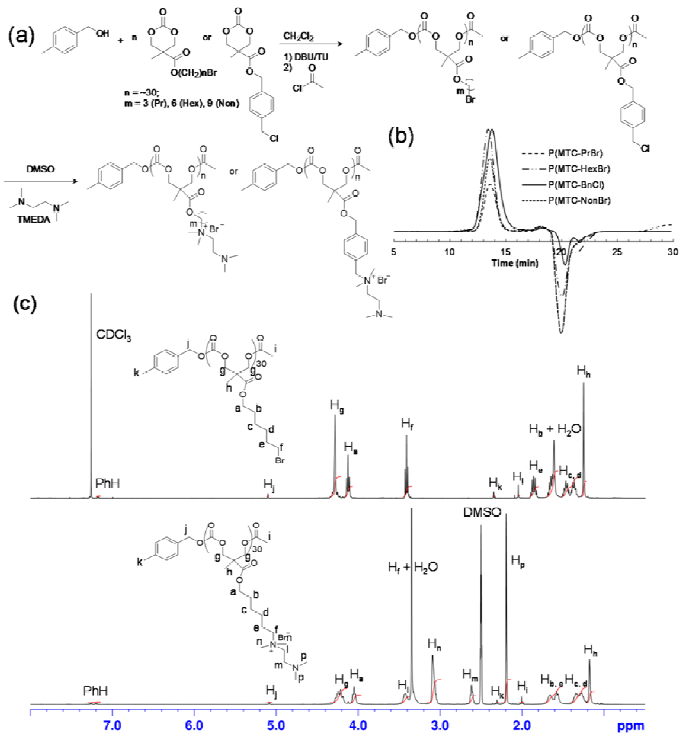
Polymer	Monomer	Degree of polymerization[a]	Mn[a] (g/mol)	PDI[b]	N content (%)[a]
a	MTC-PrBr	32	12 958	1.26	5.54
b	MTC-HexBr	30	13 347	1.26	5.01
c	MTC-BnCl	31	12 783	1.28	5.22
d	MTC-NonBr	31	15 235	1.26	4.55

[a] Calculated from integrations on ^{1}H NMR spectra.

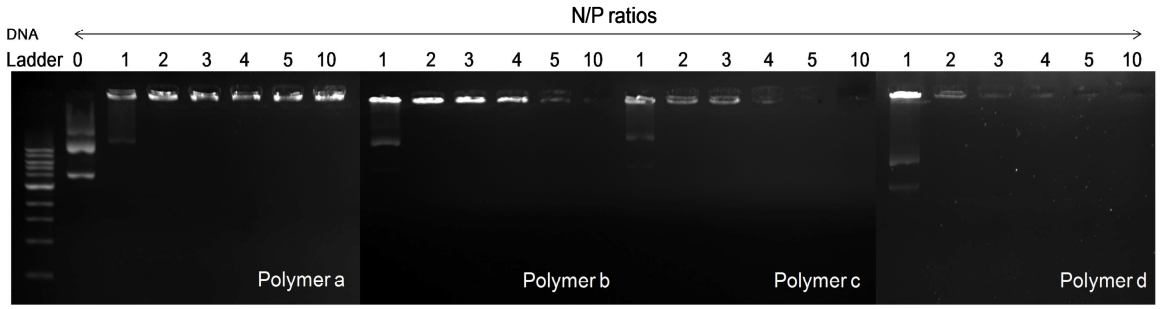
[b] Before quaternization. Determined by gel permeation chromatography (THF) using polystyrene standards.

Table 2 Particle size, zeta-potential, and polydispersity of cationic poly(carbonate)/DNA complexes in 10 mM sodium phosphate buffer determined using dynamic light scattering.

Polymer	N/P ratio	Z-average diameter ± S.D. (nm)	Zeta-potential ± S.D. (mV)	PDI
a	7	113.3 ± 0.9	28.0 ± 0.9	0.082
	14	84.7 ± 0.2	28.7 ± 1.0	0.078
	21	74.8 ± 1.0	29.4 ± 1.8	0.102
	28	71.4 ± 0.3	30.6 ± 0.9	0.125
	35	70.3 ± 1.0	30.2 ± 0.7	0.122
	42	72.1 ± 0.5	32.5 ± 0.9	0.134
b	7	146.5 ± 0.8	32.0 ± 2.5	0.114
	14	101.5 ± 0.9	33.8 ± 1.5	0.101
	21	88.4 ± 0.5	32.9 ± 1.2	0.095
	28	82.3 ± 1.0	34.5 ± 1.6	0.094
	35	79.0 ± 0.3	34.6 ± 0.7	0.122
	42	78.0 ± 0.8	33.1 ± 1.9	0.122
c	7	72.7 ± 0.5	28.0 ± 1.0	0.134
	14	60.2 ± 0.2	27.5 ± 1.0	0.131
	21	58.7 ± 0.5	27.3 ± 0.8	0.181
	28	57.7 ± 0.1	27.1 ± 1.4	0.158
	35	58.4 ± 0.6	29.4 ± 0.4	0.202
	42	57.4 ± 0.6	28.7 ± 1.5	0.206
d	7	190.1 ± 1.7	28.6 ± 0.7	0.186
	14	116.2 ± 1.8	29.6 ± 0.3	0.139
	21	97.6 ± 1.9	29.5 ± 0.7	0.162
	28	87.9 ± 0.8	30.1 ± 0.4	0.158
	35	82.8 ± 1.3	28.5 ± 1.3	0.168
	42	78.3 ± 1.6	28.6 ± 0.9	0.173



(a)


N/P ratios
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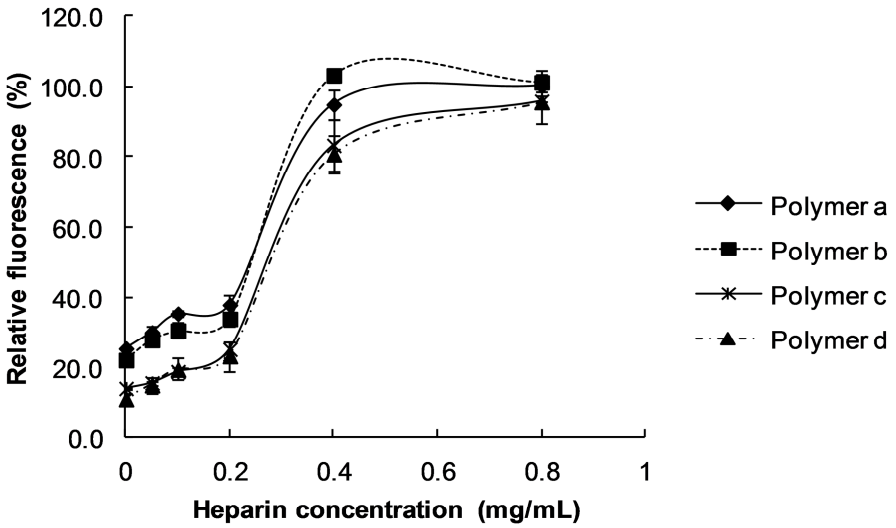
Polymer a
Polymer b
Polymer c
Polymer d

(b)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60.0
40.0
20.0
0.0
0
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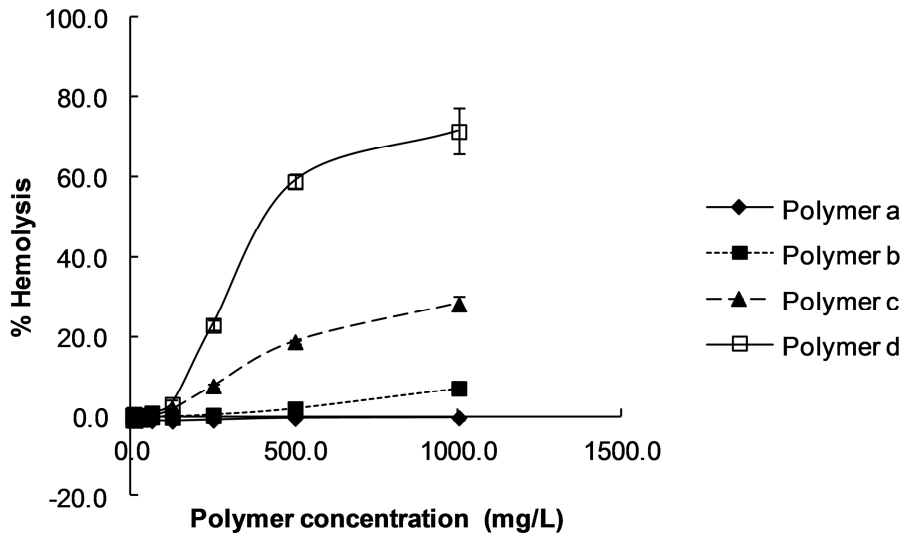
0.4
0.6
0.8
1
Heparin concentration (mg/mL)
Polymer a
Polymer b
Polymer c
Polymer d

(a)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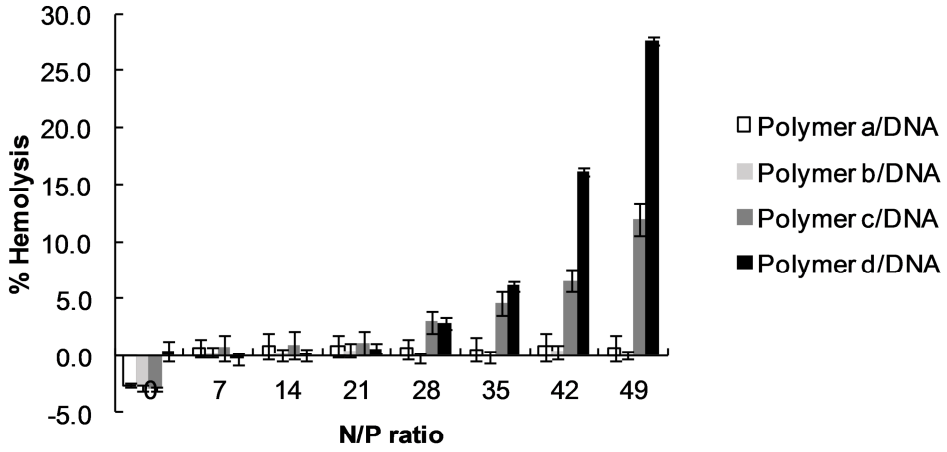
Polymer concentration (mg/L)
Polymer a
Polymer b
Polymer c
Polymer d

(b)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15.0
10.0
5.0
0.0
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% Hemolysis
0
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14
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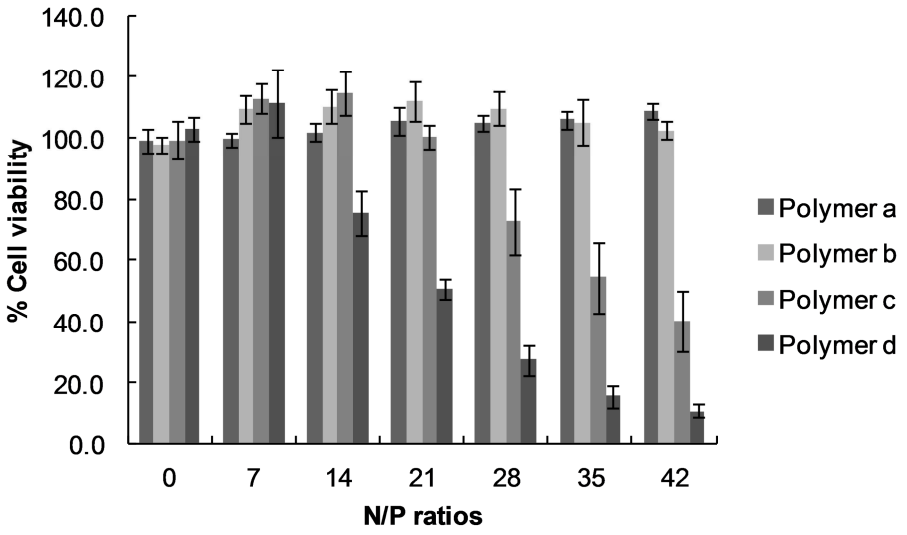
28
35
42
49
N/P ratio
Polymer a/DNA
Polymer b/DNA
Polymer c/DNA
Polymer d/DNA

(a)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100.0
80.0
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21
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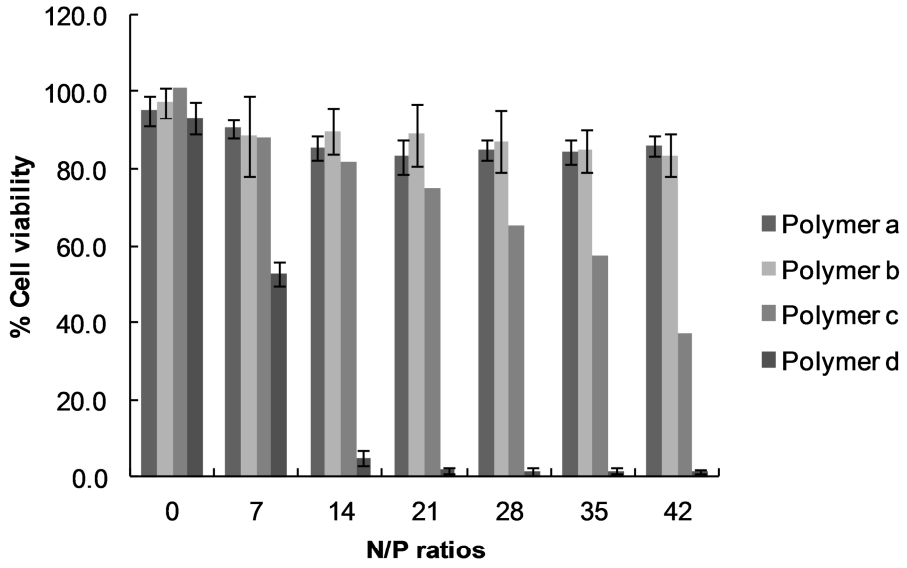
42
N/P ratios
Polymer a
Polymer b
Polymer c
Polymer d

(b)


120.0
100.0
80.0
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7
14
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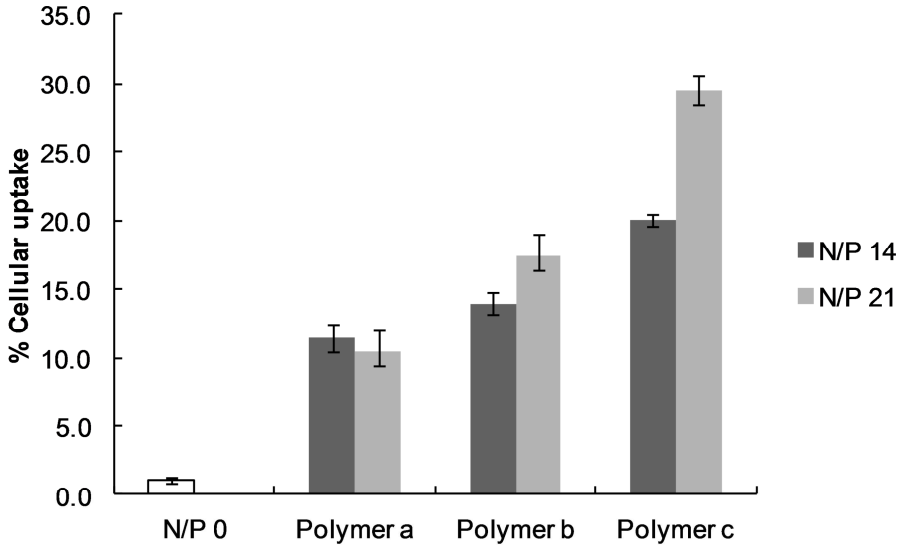
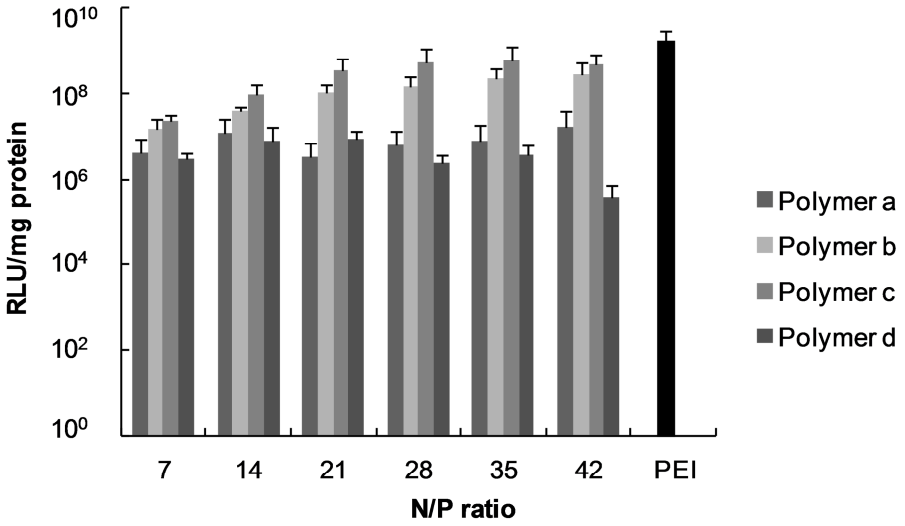
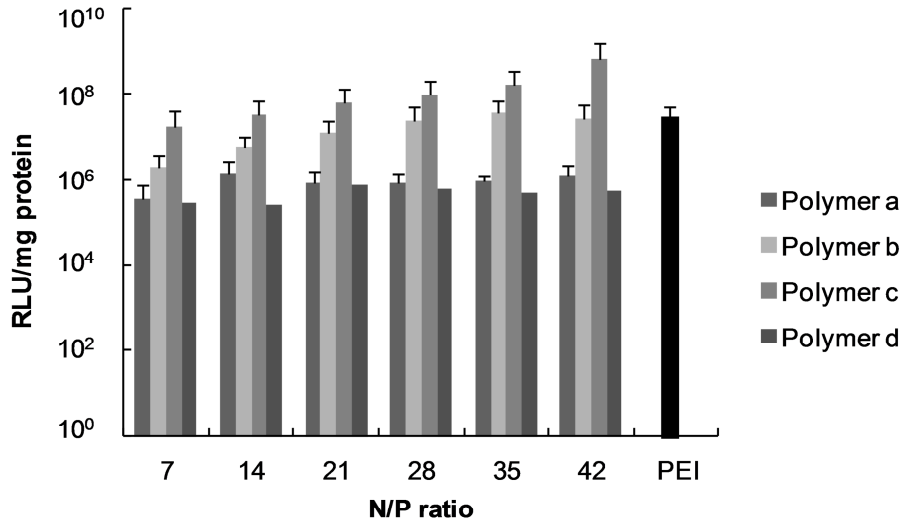
N/P ratios
Polymer a
Polymer b
Polymer c
Polymer d



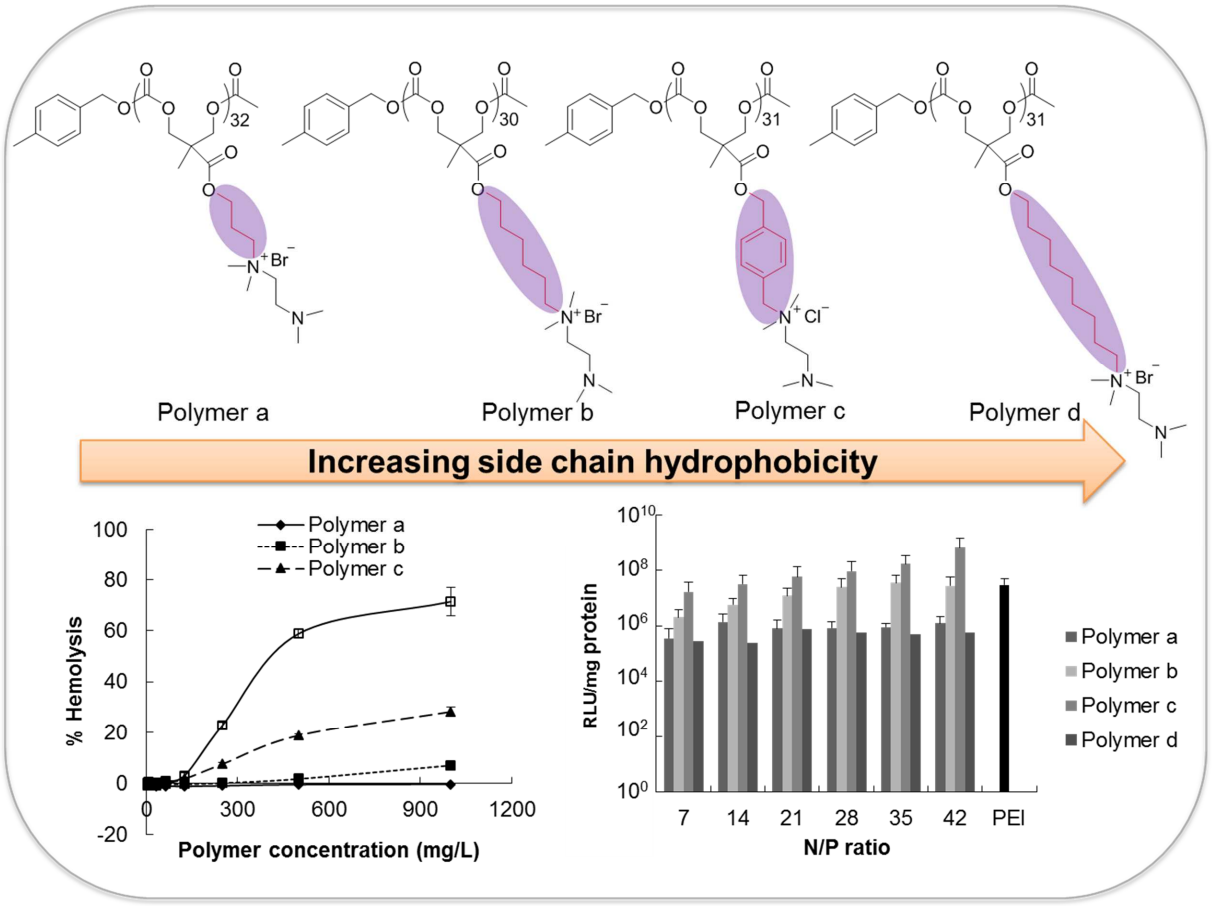
(a)


RLU/mg protein
10^10
10^8
10^6
10^4
10^2
10^0
7
14
21
28
35
42
PEI
N/P ratio
Polymer a
Polymer b
Polymer c
Polymer d

(b)


RLU/mg protein
10^10
10^8
10^6
10^4
10^2
10^0
7
14
21
28
35
42
PEI
N/P ratio
Polymer a
Polymer b
Polymer c
Polymer d

Owing to their ease of synthesis and well-controlled polymerization, biodegradable cationic poly(carbonates) have emerged as a highly promising class of biomaterials for gene delivery. The hydrophobicity of side chains in cationic polymers plays an important but often underappreciated role in influencing key aspects of gene transfection. In our efforts to improve gene transfection and understand structure-activity relationships, we synthesized a series of cationic polymers bearing a common poly(carbonate) backbone, and with side chains containing various hydrophobic spacers (propyl, hexyl, 4-methyl benzyl or nonyl) before the cationic moiety. A moderate degree of hydrophobicity was optimal as the cationic poly(carbonate) with hexyl side chains mediated high gene transfection efficiencies while causing low cytotoxicities. (111 words)


Polymer a
Polymer b
Polymer c
Polymer d
Increasing side chain hydrophobicity
% Hemolysis
Polymer concentration (mg/L)
RLU/mg protein
N/P ratio
7
14
21
28
35
42
PEI