Polymer 50 (2009) 1378–1388

Contents lists available at ScienceDirect

Polymer

journal homepage: www.elsevier.com/locate/polymer

A supramolecular gene carrier composed of multiple cationic α-cyclodextrins threaded on a PPO–PEO–PPO triblock polymer

Chuan Yang [a], Xin Wang [b], Hongzhe Li [b], Jeak Ling Ding [c], De Yun Wang [d], Jun Li [a,b,*]

[a] Division of Bioengineering, Faculty of Engineering, National University of Singapore, 7 Engineering Drive 1, Singapore 117574, Singapore
[b] Institute of Materials Research and Engineering, A*STAR (Agency for Science, Technology and Research), 3 Research Link, Singapore 117602, Singapore
[c] Department of Biological Science, National University of Singapore, 14 Science Drive 4, Singapore 117543, Singapore
[d] Department of Otolaryngology, National University of Singapore, 5 Lower Kent Ridge Road, Singapore 119074, Singapore

ARTICLE INFO

Article history:
Received 15 October 2008
Received in revised form
2 January 2009
Accepted 8 January 2009
Available online 14 January 2009

Keywords:
Cyclodextrins
Polymers
Gene delivery

ABSTRACT

Cationic polymers have been studied as promising nonviral gene delivery vectors. In contrast to the conventional polycations with long sequences of covalently bonded repeating units, this work reports a supramolecular gene carrier where many cationic cyclic units are threaded over a polymer chain to form a chain-interlock structured gene carrier. A series of novel supramolecular cationic polyrotaxanes consisting of multiple α-cyclodextrin (α-CD) rings grafted with various linear or nonlinear oligoethylenimine (OEI) chains, which are threaded and capped over a reverse Pluronic poly(propylene oxide)–poly(ethylene oxide)–poly(propylene oxide) (PPO–PEO–PPO) amphiphilic triblock copolymer chain, were synthesized and characterized in term of their molecular and supramolecular structures, DNA binding and condensation ability, cytotoxicity, and *in vitro* gene transfection efficiency in cultured cells. The supramolecular cationic polyrotaxanes were found to contain 8 cationic α-CD rings that are threaded on a PPO–PEO–PPO triblock copolymer chain. They demonstrated strong ability to bind and condense plasmid DNA into nano-sized particles which are suitable for gene delivery. In both HEK293 and COS7 cells, these polyrotaxanes show low cytotoxicity and high transfection efficiency. In particular, the cationic polyrotaxanes displayed sustained gene delivery capability in HEK293 cells in both serum and serum free condition with the increasing expression duration.

1. Introduction

Over the last few decades, supramolecular architectures have intrigued researchers because of their unique structures and remarkable properties such as molecular recognition, self-assembly, self-organization, and kinetic and thermodynamic complementarity [1–3]. Polyrotaxanes, formed by multiple macrocycles threaded over a polymeric chain, are such an example [4]. Cyclodextrins (CDs) are a series of cyclic oligosaccharides composed of 6, 7, or 8 D(+)-glucose units linked by α-1,4-linkages, and named α-, β-, or γ-CD, respectively [5,6], and have been widely used in pharmaceutics for controlled drug release [7–9]. Since the first polyrotaxane was synthesized in 1992 with multiple α-CDs threaded and trapped over a polymer chain [10,11], growing interest has been focused on the studies of such supramolecular structures [12–24], and their potential applications as novel functional materials [25–33].

Recently, cationic polymers have been widely studied as nonviral gene carriers. Comparing with other gene delivery systems such as viral vectors and cationic lipids, cationic polymers for gene delivery are generally economical and stable, and they can be produced in a large scale and show low host immunogenicity [34]. By now a great number of polycations have been reported to be able to deliver gene [35–40], including homopolymers or derivatives of polyethylenimine (PEI) [36], poly(L-lysine) [37], polyamidoamine [38], poly(L-glutamic acid) [39], polyphosphoester [40], and chitosan [34].

In contrast to the conventional polycations containing long sequences of covalently bonded repeating units, we recently designed a new class of cationic supramolecules composed of multiple oligoethylenimine-grafted (OEI-grafted) β-CDs threaded on a polymeric chain, as an integrated supramolecular entity capable of condensing plasmid DNA into small nanoparticles for efficient gene delivery [31–33]. One important feature of the supramolecular gene carriers is that the macrocycles in polyrotaxanes can rotate and/or slide along the polymeric chain freely,

* Corresponding author. Division of Bioengineering, Faculty of Engineering, National University of Singapore, 7 Engineering Drive 1, Singapore 117574, Singapore. Tel.: +65 6516 7273; fax: +65 6872 3069.
E-mail address: bielj@nus.edu.sg (J. Li).

0032-3861/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.polymer.2009.01.010

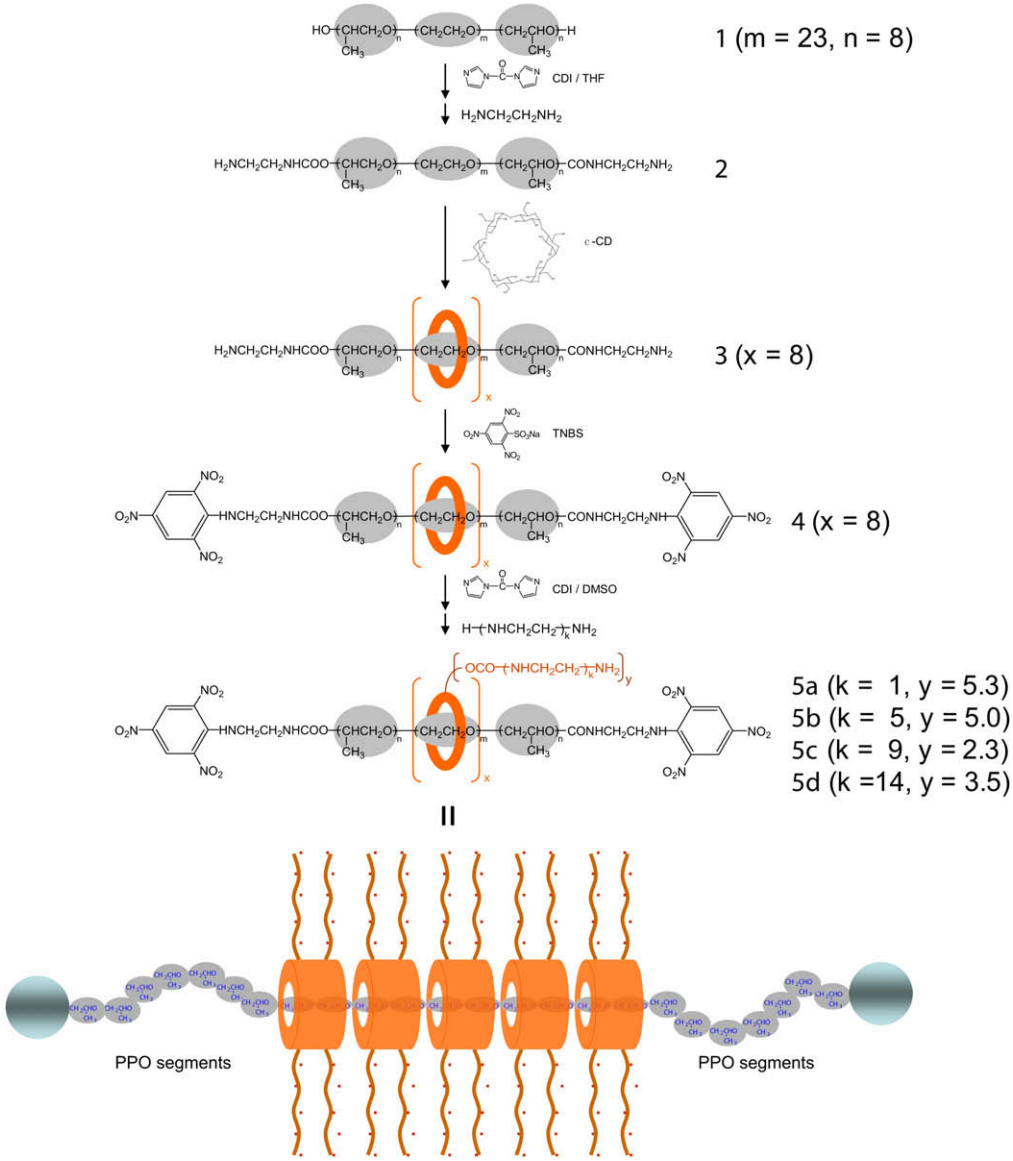
which can improve the mobility of cationic side chains linked to the macrocycles. The increased flexibility of cationic side chains substantially reduces spatial mismatching and enhances the interaction possibility of the cations and DNA [41].

In this study, we synthesized a series of cationic polyrotaxanes consisting of multiple cationic α-CDs with various linear or nonlinear OEI side chains threaded on an amphiphilic poly(propylene oxide)–poly(ethylene oxide)–poly(propylene oxide) (PPO–PEO–PPO) triblock copolymer. The PPO–PEO–PPO triblock copolymer is also known as Pluronic-R or "reverse" Pluronic copolymer, with two PPO blocks flanking a middle PEO block. It was reported that α-CD will selectively recognize the middle PEO block, forming a complex with α-CD residing over the PEO block, while the flanking PPO blocks remain free of complexation [22]. Therefore, in the cationic polyrotaxane synthesized in this work, there are a lot of free PPO segments. Pluronic block copolymers have been reported to able to act as biological adjuvants to increase expression of genes delivered into cells [42]. Therefore, the gene carriers described in this work may be a novel supramolecular gene delivery system combining the advantages of cationic polymers and Pluronic copolymers.

2. Experimental

2.1. Chemicals

Pluronic-R PPO–PEO–PPO triblock copolymer ($M_n = 1990$, $M_w/M_n = 1.04$) was supplied by Aldrich. This polymer has chain composition of $PO_8EO_{23}PO_8$. The molecular characteristics were determined by combination of GPC and ^1H NMR results, which were found to be within the specification of the supplier. 2,4,6-Trinitrobenzene sulfonic acid solution and pentaethylenehexamine were obtained from Fluka. 1,1′-Carbonyldiimidazole (CDI) and α-cyclodextrin were purchased from Tokyo Kasei incorporation. Ethylenediamine, linear PEI with molecular weight of 423 (OEI-9), branched PEI with molecular weight of 600 (OEI-14) and branched PEI (25 K) were also supplied by Aldrich. DMSO-d_6 and D_2O used as solvent in the NMR measurements were also obtained from Aldrich. Qiagen kit and Luciferase kit were purchased from Qiagen and Promega, respectively. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazodium bromide (MTT), penicillin, and streptomycin were obtained from Sigma.



Scheme 1. Synthesis procedures and the structures of multiple OEI-grafted cationic α-CD–PPO–PEO–PPO polyrotaxanes **5a**, **5b**, **5c**, and **5d**.

2.2. Synthesis of supramolecular gene carriers

A schematic illustration on the synthesis procedures and the structures of the OEI-grafted cationic α-CD–PPO–PEO–PPO polyrotaxanes **5a**, **5b**, **5c**, and **5d** are shown in Scheme 1. The detailed procedures for their preparation are described below.

2.3. Preparation of PPO–PEO–PPO bis(amine) **2**

Pluroic-R PPO–PEO–PPO triblock copolymer **1** ($M_n = 1990$, 2.0 g, 1 mmol) was heated in a flask at 80 °C in vacuum overnight. When the flask cooled, 15 mL of anhydrous DMF was injected under nitrogen. After all **1** was dissolved, the DMF solution of **1** was added dropwise during a period of 6 h under nitrogen to 15 mL of anhydrous DMF solution in which CDI (1.622 g, 10 mmol) was dissolved, and the mixture was stirred overnight under nitrogen at room temperature. Then, the resulting solution was slowly added dorpwise during a period of 3 h into 24.0 g (400 mmol) of ethylenediamine which was dissolved in 15 mL of anhydrous DMF with stirring at room temperature, followed by stirring the mixture overnight. Excess ethylenediamine and DMF were removed by vacuum evaporation. Then, the resulting viscous solution was purified by size exclusion chromatography (SEC) on a Sephadex LH-20 column using methanol as eluent. Finally, 1.80 g viscous liquid **2** was yielded (83%). ^{1}H NMR (400 MHz, DMSO-d_6, 22 °C): δ 3.25–3.71 (m, 92H and 48H, $-CH_2CH_2O-$ of PEO block and $-CH_2CHO-$ of PPO block), 3.22 (s, 4H, $CONCH_2$ of ethylenediamine), 2.04 (s, 4H, NCH_2 of ethylenediamine), 1.04 (s, 48H, $-CH_3$ of PPO block).

2.4. Preparation of polyrotaxane **4**

The resulting PPO–PEO–PPO bis(amine) **2** (0.5 g) was added to 59 mL α-CD saturated solution (0.145 g α-CD/mL H_2O), and 0.75 g $NaHCO_3$ was added to adjust the pH value of the solution. The reaction mixture was ultrasonicated for 20 min and stirred at room temperature overnight. Then, 3.15 g of sodium salt of picrylsulfonic acid was added and stirred overnight. And then, 200 mL H_2O was poured into the reaction mixture to precipitate the product. The precipitate was centrifuged and washed with water for 3 times. The resulting wet solid was dissolved in 30 mL DMSO and poured into 500 mL $CH_3COOCH_2CH_3$ to precipitate the product. The precipitate was centrifuged and washed with $CH_3COOCH_2CH_3$ for 3 times. The resulting wet solid was dissolved in 30 mL DMSO again and poured into 500 mL H_2O to precipitate the product. The resulting precipitate was centrifuged and washed with H_2O for 3 times. Finally, the resulting wet solid was dried by freeze (liquid nitrogen) *in vacuo* and 1.76 g pure polyrotaxane **4** was yielded (89%). ^{1}H NMR (400 MHz, DMSO-d_6, 22 °C): δ 8.89 (s, 2H, meta H of phenyl), 8.77 (s, 2H, meta H of phenyl), 5.62 (s, 47H, O(2)H of CD), 5.47 (m, 47H, O(3)H of CD), 4.75 (s, 47H, H(1)H of CD), 4.38 (s, 47H, O(6)H of CD), 2.80–4.00 (m, 282H, H(3), H(6), H(5), H(2) and H(4) of CD, 92H, $-CH_2CH_2O-$ of PEO block, 48H, $-CH_2CHO-$ of PPO block), 1.04 (s, 48H, $-CH_3$ of PPO block).

2.5. Preparation of cationic polyrotaxane ***5b***

The resulting polyrotaxane **4** (0.2028 g, 0.02 mmol) was dried at 40 °C in vacuum overnight. When the flask cooled, 40 mL dry DMSO was injected under nitrogen. After all **4** was dissolved, the DMSO solution of **4** was added dropwise during a period of 6 h under nitrogen to 40 mL of anhydrous DMSO solution in which CDI (2.27 g, 14 mmol) was dissolved, and the mixture was stirred overnight under nitrogen at room temperature. Then, the mixture of 300 mL THF and 600 mL Et_2O was poured in the resulting solution to precipitate the product. The precipitate was centrifuged and

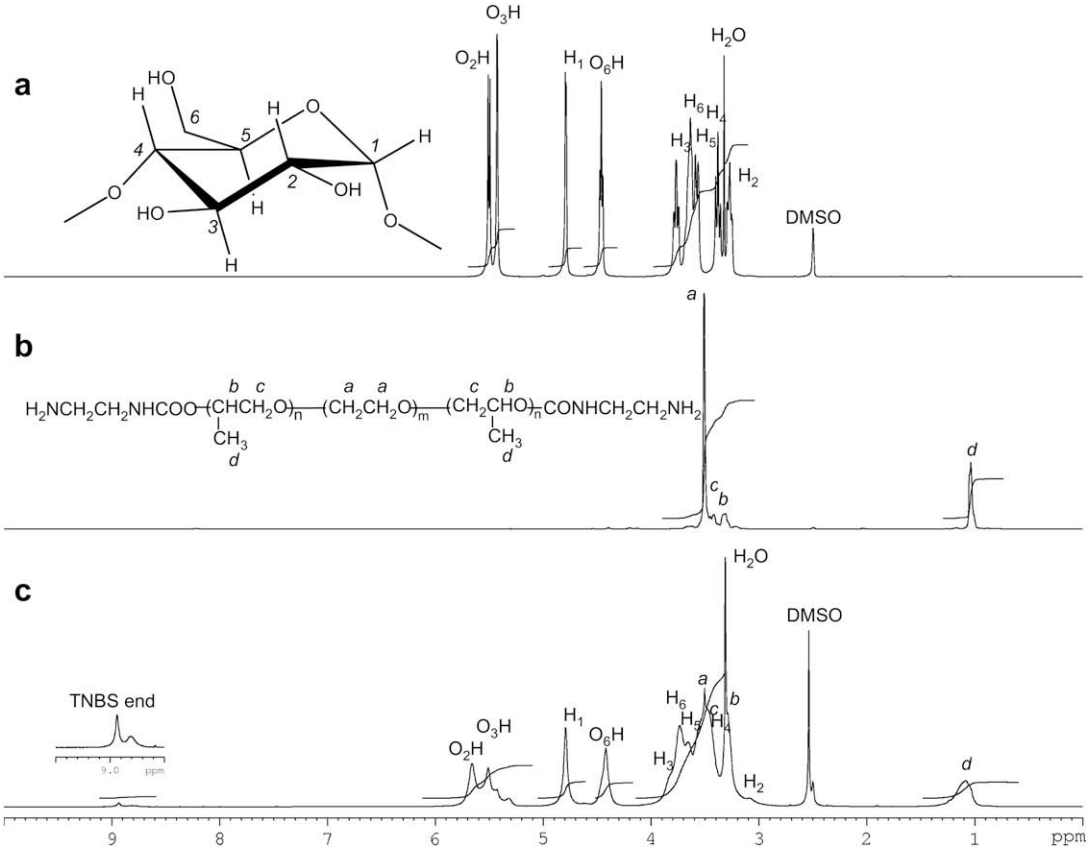


Fig. 1. ^{1}H NMR spectra of α-CD (a), PPO–PEO–PPO bis(amine) (b), and α-CD–PPO–PEO–PPO polyrotaxane (c) in DMSO-d_6.

washed with THF for 3 times. Then, the resulting wet solid was dissolved in 40 mL DMSO and this solution was slowly added dorpwise during a period of 3 h into 4.90 mL (16.8 mmol) of pentaethylenehexamine which was dissolved in 40 mL of DMSO with stirring at room temperature, followed by stirring the mixture overnight. 900 mL THF was poured in the reaction mixture to precipitate the product. The precipitate was centrifuged and washed with THF for 3 times, and the resulting crude product was purified by size exclusion chromatography (SEC) on a Sephadex G-50 column using DI water as eluent. Finally, 0.186 g brown solid **5b** was yielded (46%). ^{1}H NMR (400 MHz, D_2O, 22 °C): δ 8.43 (s, 2H, meta H of phenyl), 8.04 (s, 2H, meta H of phenyl), 5.07 (d, broad, 47H, H(1)H of CD), 3.00–4.57 (m, broad, 282H, H(3), H(6), H(5), H(2) and H(4) of CD, 92H, $-CH_2CH_2O-$ of PEO block, 48H, $-CH_2CHO-$ of PPO block, 78H, $CONCH_2$ of pentaethylenehexamine), 2.74 (m, 937H, NCH_2 of pentaethylenehexamine), 1.11 (s, 48H, $-CH_3$ of PPO block).

2.6. Cationic polyrotaxane **5a**

Cationic polyrotaxane **5a** was prepared in similar procedures to **5b**. The yield and analytical data for all **5a** are given below. Yield, 77%. ^{1}H NMR (400 MHz, D_2O, 22 °C): δ 8.38 (s, 2H, meta H of phenyl), 8.01 (s, 2H, meta H of phenyl), 5.03 (d, broad, 47H, H(1) of CD), 2.92–4.59 (m, broad, 282H, H(3), H(6), H(5), H(2) and H(4) of CD, 92H, $-CH_2CH_2O-$ of PEO block, 48H, $-CH_2CHO-$ of PPO block, 124H, $CONCH_2$ of ethylenediamine), 2.74 (s, 124H, NCH_2 of ethylenediamine), 1.12 (s, 48H, $-CH_3$ of PPO block).

2.7. Cationic polyrotaxane **5c**

Cationic polyrotaxane **5c** was prepared in similar procedures to **5b**. The yield and analytical data for all **5c** are given below. Yield, 54%. ^{1}H NMR (400 MHz, D_2O, 22 °C): δ 8.39 (s, 2H, meta H of phenyl), 8.02 (s, 2H, meta H of phenyl), 5.00 (d, broad, 47H, H(1)H of CD), 2.96–4.61 (m, broad, 282H, H(3), H(6), H(5), H(2) and H(4) of CD, 92H, $-CH_2CH_2O-$ of PEO block, 48H, $-CH_2CHO-$ of PPO block, 36H, $CONCH_2$ of OEI-9), 2.72 (m, 823H, NCH_2 of OEI-9), 1.11 (s, 48H, $-CH_3$ of PPO block).

2.8. Cationic polyrotaxane **5d**

Cationic polyrotaxane **5d** was prepared in similar procedures to **5b**. Yield, 66%. ^{1}H NMR (400 MHz, D_2O, 22 °C): δ 8.34 (s, 2H, meta H of phenyl), 7.96 (s, 2H, meta H of phenyl), 4.96 (d, broad, 47H, H(1)H of CD), 2.94–4.62 (m, broad, 282H, H(3), H(6), H(5), H(2) and H(4) of CD, 92H, $-CH_2CH_2O-$ of PEO block, 48H, $-CH_2CHO-$ of PPO block, 55H, $CONCH_2$ of OEI-14), 2.57 (m, 1827H, NCH_2 of OEI-14), 1.08 (s, 48H, $-CH_3$ of PPO block).

2.9. Analytical methods

Gel permeation chromatography (GPC) analysis for PPO–PEO–PPO triblock copolymer was carried out with a Shimadzu SCL-10A and LC-10ATVP system equipped with two Phenogel 5 μm, 50 and 1000 Å columns (size: 300 × 4.6 mm) in series and a Shimadzu RID-10A refractive index detector. THF was used as eluent at a flow rate of 0.30 mL/min at 40 °C. Monodispersed poly(ethylene glycol) standards were used to obtain a calibration curve.

GPC analysis for cationic polyrotaxanes was carried out with a Shimadzu SCL-10A and LC-10AT system equipped with a Sephadex G-75 column (size: 2.5 × 32 cm), a Shimadzu RID-10A refractive index detector. 1 × PBS buffer solution was used as the eluent. Fractions were collected per 1 mL and were detected with a HORIBA SEPA-300 high speed accurate polarimeter at wavelength 589 nm with cell length 10 cm and response 2 s.

The ^{1}H NMR spectra were recorded on a Bruker AV-400 NMR spectrometer at 400 MHz at room temperature. The ^{1}H NMR measurements were carried out 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 = 4.70 ppm for D_2O and 2.50 ppm for DMSO-d_6).

The ^{13}C NMR spectra were recorded on a Bruker AV-400 NMR spectrometer at 100 MHz at room temperature. The ^{13}C NMR measurements were carried out using composite pulse decoupling with an acquisition time of 0.82 s, a pulse repetition time of 5.0 s, a 30° pulse width, 20,080-Hz spectral width, and 32 K data points.

2.10. Plasmid

The plasmid used was pRL-CMV (Promega, USA), encoding *Renilla luciferase*, which was originally cloned from the marine organism *Renilla reniformis*. All plasmid DNAs were amplified in *Escherichia coli* and purified according to the supplier's protocol (Qiagen, Hilden, Germany). The quantity and quality of the purified plasmid DNA were assessed by optical density at 260 and 280 nm and by electrophoresis in 1% agarose gel. The purified plasmid DNA

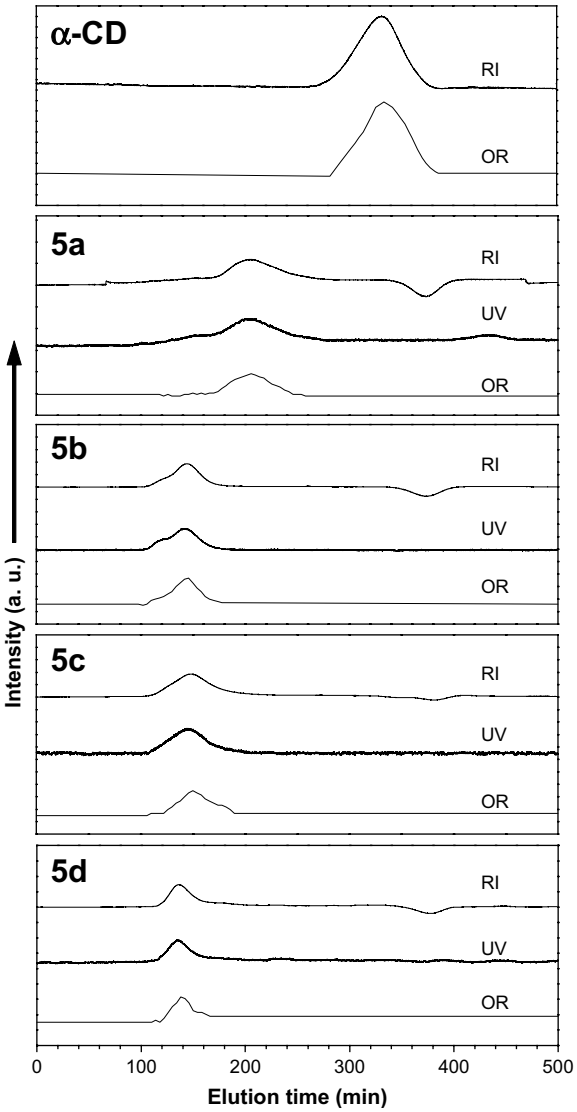


Fig. 2. GPC traces of α-CD and cationic polyrotaxane **5a**, **5b**, **5c**, and **5d** detected using refractive index (RI), UV absorption at 419 nm, and optical rotation (OR).

was resuspended in TE buffer (10 mM Tris–Cl, pH 7.5, 1 mM EDTA) and kept in aliquots at a concentration of 0.5 mg/mL.

2.11. Cells and media

All cell lines were purchased from ATCC (Rockville, MD). COS7 and HEK293 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mg penicillin, 100 μg/mL streptomycin at 37 °C and 5% CO_2. Opti-MEM reduced-serum medium, DMEM medium was purchased from Gibco BRL (Gaithersburg, MD).

2.12. Gel retardation experiments

Each polymer was examined for its ability to bind pRL-CMV through gel electrophoresis experiments. All polymer stock solutions were prepared at a nitrogen concentration of 1 mM in distilled water and the pH was adjusted to 7.4. Solutions were sterile filtered (0.2 μm) and stored at 4 °C. pRL-CMV (0.2 μg; 2 μL of a 0.1 μg/μL in TE buffer) was mixed with an equal volume of polymer at nitrogen/phosphate (N/P) ratios between 0 and 10. Each mixture was vortexed and incubated for approximately 30 min at room temperature and then analyzed on 1% agarose gel containing 0.5 μg/mL ethidium-bromide (EtBr). Gel electrophoresis was carried out in TAE running buffer (40 mM Tris-acetate, 1 mM EDTA) with a current of 80 V for 40 min in a Sub-Cell system (Bio-Rad Laboratories, CA). DNA bands were visualized and photographed by a UV transilluminator and BioDoc-It imaging system. Both of them were purchased from UVP Inc., USA.

2.13. Cell viability assay

Two cell lines (COS7 and HEK293) were cultured in DMEM medium supplemented with 10% FBS at 37 °C, 5% CO_2, and 95% relative humidity. For cell viability assay, the cells (10,000 cells/well for COS7; 15,000 cells/well for HEK293) were seeded into 96-well microtiter plates (Nunc, Wiesbaden, Germany). After 24 h, culture media were replaced with serum-supplemented culture media containing serial dilutions of the polymer and the cells were incubated for 24 h. 10 μL sterile filtered MTT (5 mg/mL) stock solution in PBS was added to each well, reaching a final concentration of 0.5 mg MTT/mL. After 5 h, unreacted dye was removed by aspiration. The formazan crystals were dissolved in 100 μL/well DMSO and measured spectrophotometrically in a microplate reader (Spectra Plus, TECAN) at a wavelength of 570 nm. Six wells were treated together as a group. The relative cell growth (%) related to control cells cultured in media without polymer was calculated by $[A]_{test}/[A]_{control} \times 100$.

2.14. In vitro *transfection and luciferase assay*

Transfection studies were performed in COS7 and HEK293 cells using the plasmid pRL-CMV as reporter gene. In brief, 24-well plates were seeded with cells at a density of 5×10^4/well 24 h before transfection. The polymer/DNA complexes at various N/P ratios were prepared by adding the polymer into DNA solutions dropwise (2 μg pRL-CMV/well), followed by vortexing and incubation for 30 min at room temperature before the transfection. At the time of transfection, the medium in each well was replaced with reduced-serum medium or normal medium. The

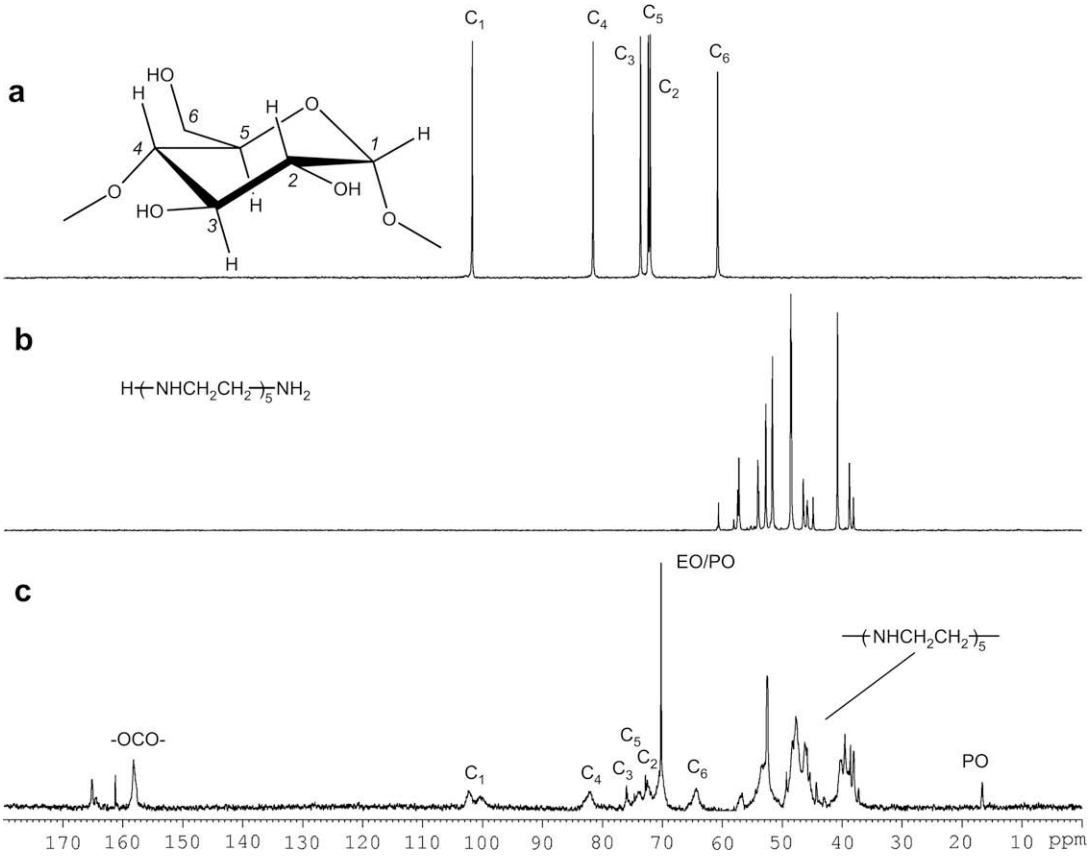


Fig. 3. ^{13}C NMR spectra of α-CD (a), pentaethylenehexamine (b), and cationic polyrotaxanes **5b** (c) in D_2O.

complexes were added into the transfection medium and incubated with cells for 4 h under standard incubator conditions. After 4 h, the medium was replaced with 500 μl of fresh medium supplemented with 10% FBS, and the cells were further incubated for an additional 20 h (longer incubation duration of 44 h and 68 h for sustained gene delivery study) under the same conditions, resulting in an expression duration of 24 h (longer expression duration of 48 h and 72 h for sustained gene delivery study). Cells were washed with PBS twice, lysed in 100 μl of cell culture lysis reagent (Promega, Cergy Pontoise, France). Luciferase gene expression was quantified using a commercial kit (Promega, Cergy Pontoise, France) and a luminometer (Berthold Lumat LB 9507, Germany). Protein concentration in the samples was analyzed using a bicinchoninic acid assay (Biorad, CA, USA). Absorption was measured on a microplate reader (Spectra Plus, TECAN) at 570 nm and compared to a standard curve calibrated with BSA samples of known concentration. Results are expressed as relative light units (RLUs) per milligram of cell protein lysate (RLU/mg protein).

2.15. Dynamic light scattering and zeta potential

Measurements of particle size and zeta potential of the complexes were performed using a Zetasizer Nano ZS (Malvern Instruments, Southborough, MA). Complex solutions (100 μl) containing 3 μg of DNA were prepared at various N/P ratios ranging from 2 to 30. The mixture was vortexed for 20 s, incubated for 30 min at room temperature and diluted in 1 mL of the distilled water before being analyzed on a Zetasizer. The size measurement was performed at 25 °C at a 90° scattering angle. The mean hydrodynamic diameter was determined by cumulative analysis. The zeta potential measurements were performed using a capillary zeta potential cell in automatic mode.

2.16. Confocal microscopy

For confocal microscopy, the plasmid pEGFP-N1 (Clontech Laboratories Inc., USA), encoding a red-shifted variant of wild-type green fluorescence protein (GFP), was used to examine the GFP expression in HEK293 cells. HEK293 cells were seeded onto lab-Tek 4-chambered coverglass (Nalge-Nane international, USA) at density of 5×10^4 cells/well in 500 μl of complete DMEM. After 24 h, transfection was undertaken with 2 μg EGFP plasmid. Each chamber was transfected in 0.3 mL reduced-serum Opti-MEM media. 20 μl of cationic polyrotaxane **5b**/DNA suspension was added per well. After 4 h, the transfection media were removed and the cells washed. After 20 h of further incubation in serum-containing media, the wells were washed with phosphate-buffered saline (PBS) and imaged under a laser scanning confocal microscope (LSM 410, Carl Zeiss, USA). GFP fluorescence was excited at 488 nm and emission was collected using a 515 nm filter.

2.17. AFM

A Digital Instruments D3000 Atomic Force Microscopy in a tapping mode was employed to image the nanoparticle samples. Briefly, silicon disks were soaked in 50% acetone for a minimum of 2 h and rinsed with distilled water. When the silicon disks were completely dry, 20 μl of cationic polyrotaxane **5b**/DNA complexes containing 1 μg of pRL-CMV at N/P ratios 0, 2 and 10 were placed on the silicon surface for 2 min after which the complexes were carefully removed with a piece of tissue paper. All the AFM images

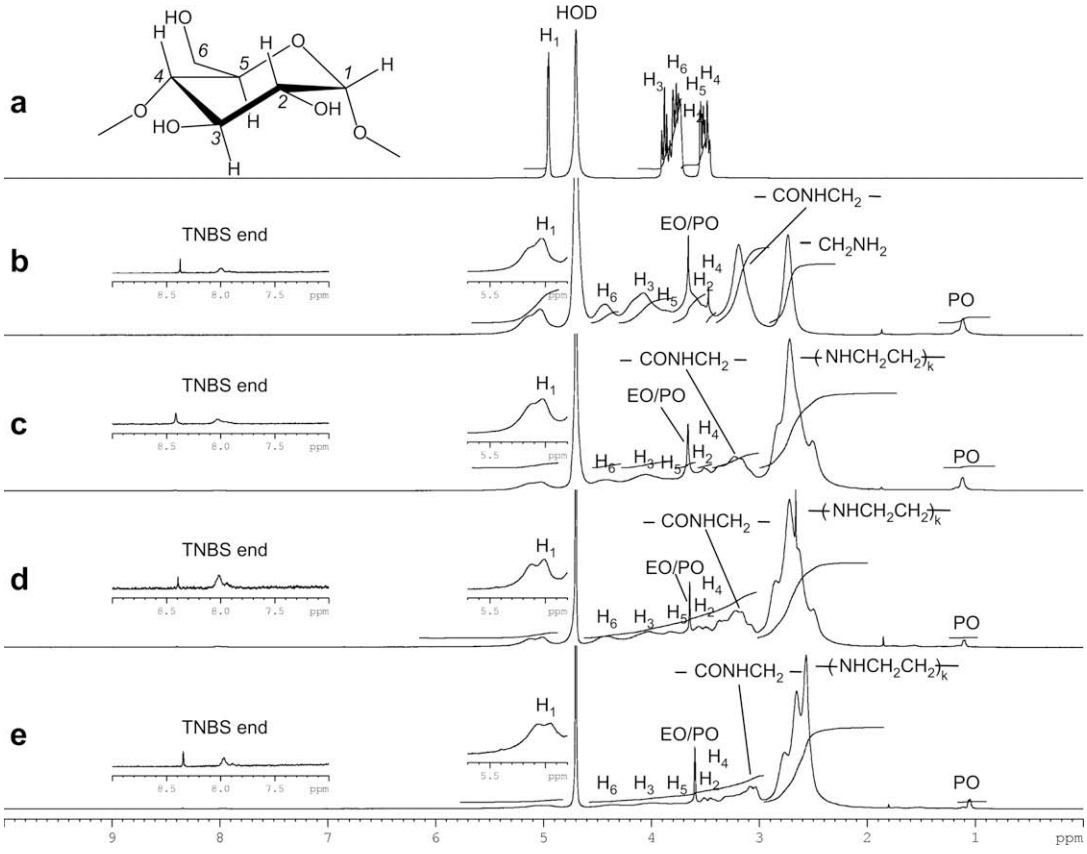


Fig. 4. ^{1}H NMR spectra of α-CD (a), and cationic polyrotaxanes **5a** (b), **5b** (c), **5c** (d), and **5d** (e) in D_2O.

were obtained with a scan rate of 0.5 or 1 Hz over a selected area of 5×5 μm or 2×2 μm. Image analysis was performed using Nanoscope software after removing the background slope by flatting images.

3. Results and discussion

3.1. Synthesis and characterization of cationic polyrotaxanes

Scheme 1 shows the synthesis procedures and the structures of the cationic polyrotaxanes (**5a**, **5b**, **5c**, and **5d**). Firstly, for conversion of both of the terminal hydroxyl groups of the PPO–PEO–PPO triblock copolymer **1** to amino groups, the hydroxyl groups were activated with CDI, followed by reaction with large excess of ethylenediamine to give PPO–PEO–PPO bis(amine) **2**. These copolymers were allowed to react with saturated solution of α-CD and large excess 2,4,6-trinitrobenzene sulfonate (TNBS) in sequence to form polyrotaxanes **4**. Finally, various linear or nonlinear OEIs with different molecular weight were grafted to polyrotaxane **4** to give the corresponding cationic polyrotaxanes (**5a**, **5b**, **5c** and **5d**).

Fig. 1 shows the ^{1}H NMR spectra of polyrotaxane **4** in comparison with α-CD and PPO–PEO–PPO bis(amine) **2** in DMSO-d_6. In Fig. 1c, the peaks for α-CD, EO and PO segments of the triblock copolymer, and the 2,4,6-trinitrophenyl end groups were all observed, while they were broadened as compared with the respective free counterparts in Fig. 1a and b. This is due to the restricted molecular movement of the components in the polyrotaxane. Quantitative comparisons between the integral intensities of the peaks of α-CD and those of threading copolymer segments gave the compositions of the polyrotaxanes. It was found that 8 α-CD rings, on average, were covered and blocked on the PPO–PEO–PPO triblock copolymer in one polyrotaxane **4**.

Fig. 2 shows the size exclusion chromatograms of the cationic polyrotaxanes in contrast to free α-CD. α-CD has relative small molecular size, which was eluted out at the low molecular weight region of the column, and was detected by refractive index (RI) and optical rotation (OR) changes. There was no detection by ultraviolet (UV) absorption at 419 nm because α-CD has no UV absorption. In contrast, all four cationic polyrotaxanes were detected by RI, UV (419 nm), and OR at the same time. It is found that they eluted out at higher molecular weight region of the column due to their large molecular size. Comparing to the other synthetic polyrotaxanes, **5a** eluted out late, which is in agreement with its smaller molecular size. Each cationic polyrotaxane showed a nearly unimodal peak and this peak eluted out at the same position in all three spectra. This result indicates that these polyrotaxanes are pure and there are limited intra- or intermolecular crosslinking byproducts.

Fig. 3 shows the ^{13}C NMR spectra of the cationic polyrotaxane **5b** in comparison with free α-CD and pentaethylenehexamine. In Fig. 3c, all peaks attributed to α-CD and grafting OEI were observed significantly. The peak at δ 158.2 ppm corresponds to the carbon of carbonyl groups, which conjugated OEI chains to α-CD rings.

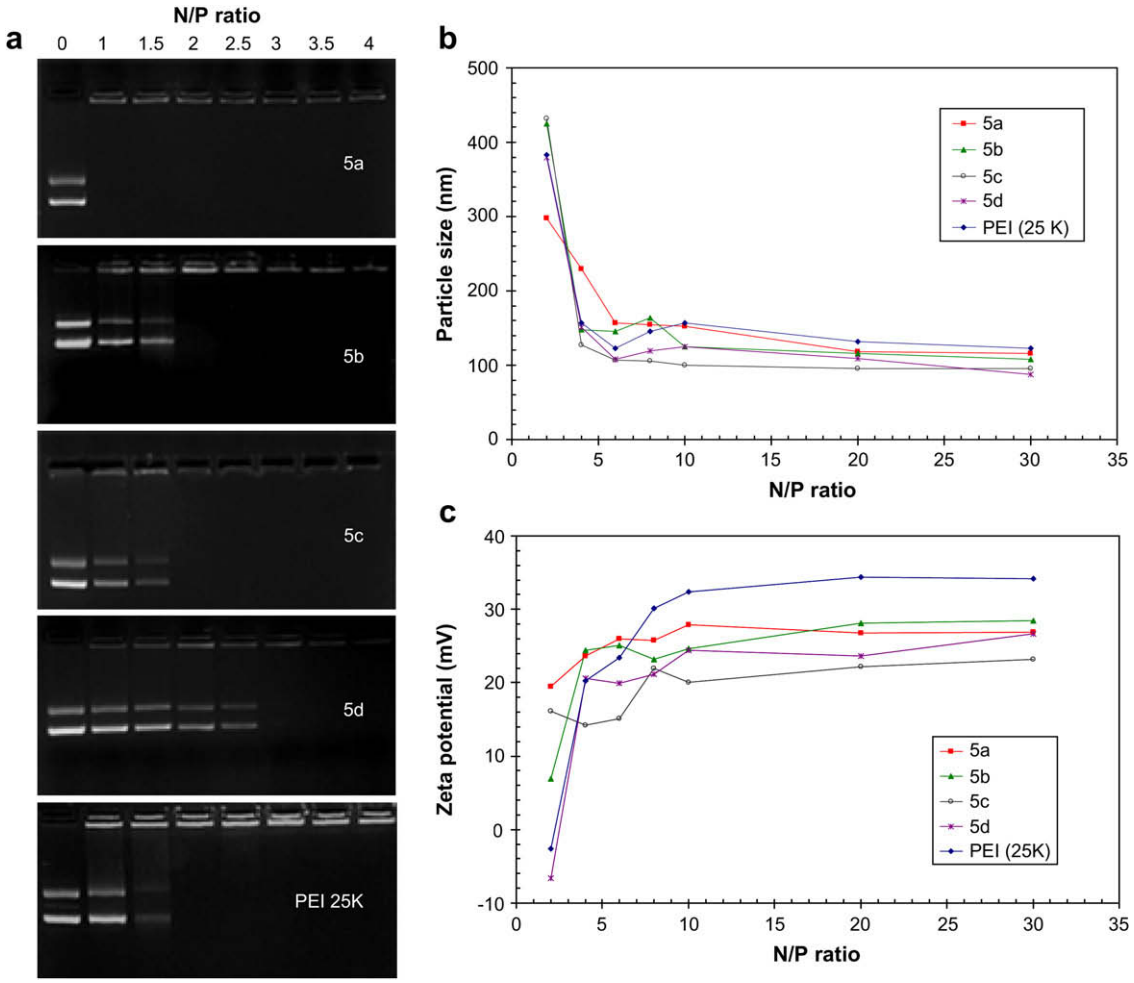


Fig. 5. Binding ability of cationic polyrotaxanes to DNA and the particle size and zeta potential of their complexes with DNA in comparison with PEI (25 K). (a) Electrophoretic mobility of plasmid DNA in the complexes; (b) Particle size of the complexes; and (c) Zeta potential of the complexes.

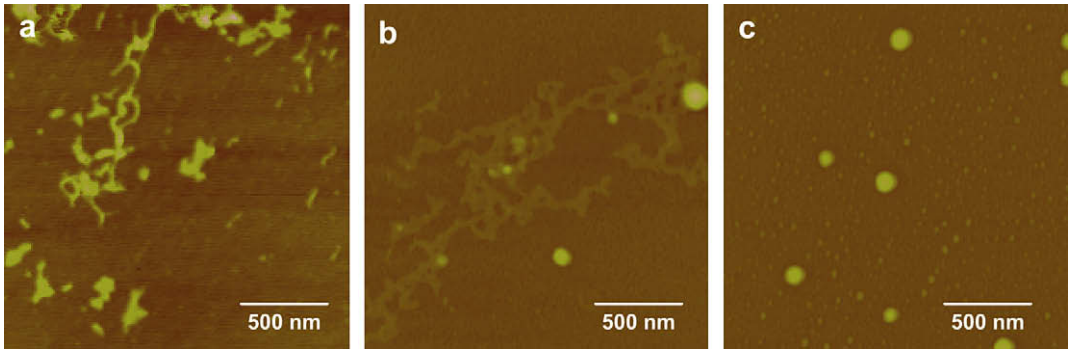


Fig. 6. Atomic force microscopy (AFM) images of the supercoiled plasmid DNA (a), and cationic polyrotaxane **5b**/DNA complex at N/P = 2 (b) and N/P = 10 (c).

Additionally, comparing to free α-CD, the peak of C-6 on the α-CD rings of **5b** was shifted from 60.7 ppm to 64.3 ppm. It may be mainly attributed to the grafting of OEI. In fact, of the three types of hydroxyl groups of α-CD, those at the 6-position (primary hydroxyl groups) are the most nucleophilic and are thought to be modified under the weak basic conditions [43].

Fig. 4 shows the ^{1}H NMR spectra of the cationic polyrotaxanes in comparison with α-CD. In the spectra of Fig. 4b–e, the signals for both α-CD and grafting OEI were observed, while the peaks were much broadened due to the restriction of the molecular motion by the grafting OEI units. From the ^{1}H NMR spectra, the average number of OEI chains conjugated to each α-CD (y) was estimated. About 5.3 molecules of ethylenediamine were grafted onto each α-CD in **5a** and about 5.0 molecules of pentaethylenehexamine were grafted onto each α-CD in **5b**, near to one OEI chain per glucose unit of α-CD. About 2.3 molecules of OEI-9 were grafted on each α-CD in **5c** and about 3.5 molecules of OEI-14 were grafted on each α-CD in **5d**. From **5a**, **5b** to **5c**, it is clear that the longer the OEI chain, the less number of OEI chains could be conjugated to each α-CD. It can be attributed to the influence of the steric hindrance of OEI chains on the conjugating reaction. But, for **5d**, more primary amino groups from branched structure of OEI-14 participated in the conjugating reaction, leading to the increasing number of OEI chains conjugating to each α-CD.

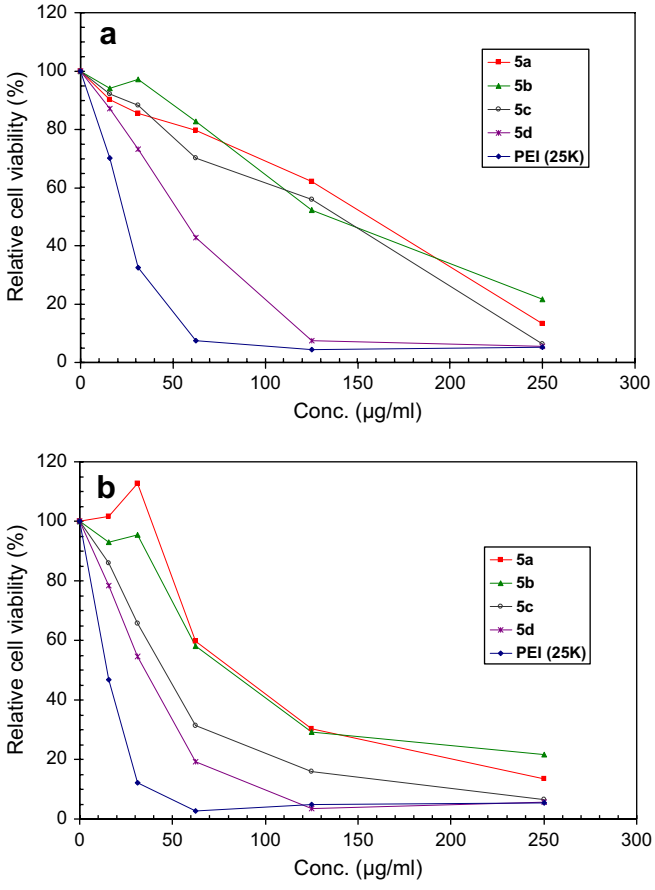


Fig. 7. Cell viability of cationic polyrotaxanes in (a) COS7 and (b) HEK293 cells in comparison with PEI (25 K).

3.2. Formation of cationic polyrotaxane/DNA complexes

The ability of the cationic polyrotaxanes to condense plasmid DNA (pDNA) into particulate structures was confirmed by agarose gel electrophoresis, particle size and zeta potential measurements, as well as AFM images. It is known that the DNA condensation capability of cationic polymers is one of the prerequisites to be gene carriers. To confirm the formation of the synthesized cationic polyrotaxane/DNA complexes, agarose gel electrophoresis was performed and retardation of DNA mobility was examined. Fig. 5a shows the gel retardation results of cationic polyrotaxane/DNA complexes with increasing N/P ratios in comparison with branched PEI (25 K). Cationic polyrotaxane **5a** could compact pDNA entirely at the low N/P ratio of 1, while **5b** and **5c** could inhibit the migration of pDNA at N/P ratio of 2 and above. It indicates that the cationic polyrotaxanes with linear OEI units have similar or slightly better DNA condensation ability compared to PEI (25 K). In the meanwhile, **5d** could merely complex pDNA completely at N/P ratio of 3 and above, which could probably be attributed to the effect of the branched OEI units in this cationic polyrotaxane.

Fig. 5b and c shows the particle size and zeta potential of cationic polyrotaxane/DNA complexes in comparison with PEI (25 K)/DNA complex at various N/P ratios. In Fig. 5b, all four cationic polyrotaxanes could efficiently compact pDNA into small nanoparticles. Generally, their mean particle size decreased sharply with the increase of N/P ratio from 2 to 6. After N/P ratio reached 6, the particle size varied within 85–165 nm. In the case of the complex formed by PEI (25 K), its hydrodynamic size reached a level of around 120–160 nm at N/P ratio of 4 and above. Within the range of N/P ratio 10–30, the particle size of PEI (25 K)/DNA complex was higher than that of the cationic polyrotaxanes at the same N/P ratio.

Zeta potential measurements are indicative of the surface charge of polymer/DNA particles, and a positive surface charge of untargeted polymer is necessary for binding to anionic cell surface, which consequently facilitates cell uptake [44]. As shown in Fig. 5c,

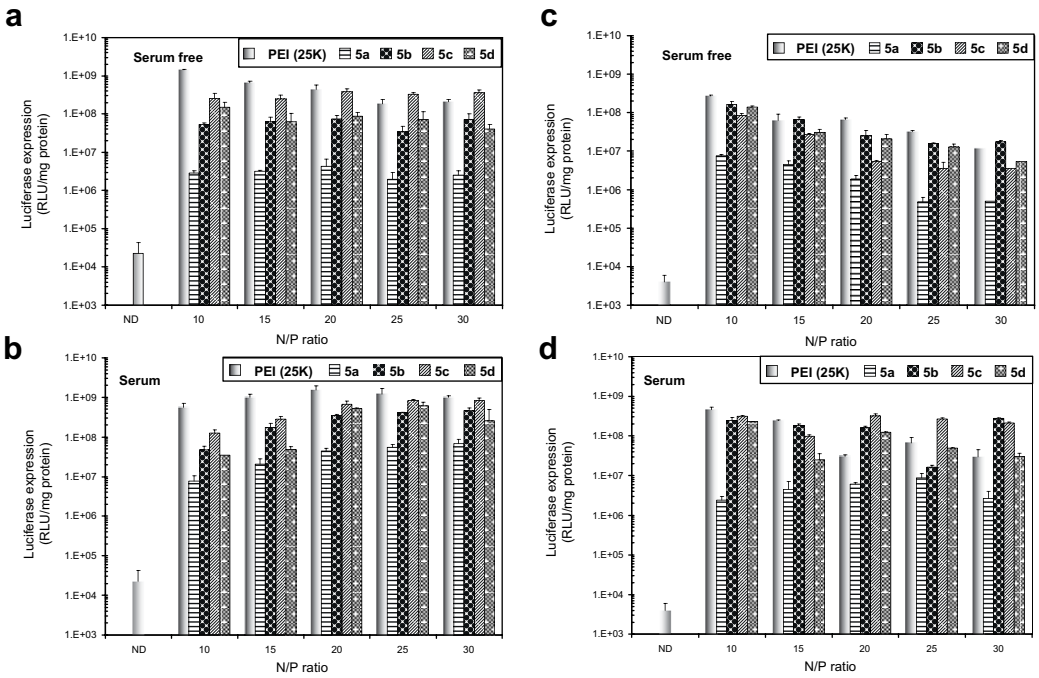


Fig. 8. *In vitro* gene transfection efficiency of the complexes of cationic polyrotaxanes in comparison with that of PEI (25 K) and naked DNA (ND), in COS7 cell in the absence and presence of serum (a) and (b); in HEK293 cell in the absence and presence of serum (c) and (d). Data represent mean ± standard deviation ($n = 3$).

the surface net charge of the complexes of pDNA with PEI and **5d** increased abruptly from negative to positive as the N/P ratio increased from 0 to 4 and stabilized at N/P ratio of 10 and above. Also, though the surface net charge of the complex of **5a**, **5b**, and **5c** with pDNA was positive and beyond 8 mV at N/P ratio 2, they stabilized within 20 ~ 28 mV and were lower than that of PEI (25 K)/DNA complex, which reached an almost constant value around 33 mV, from N/P ratio of 8–30.

Fig. 6 showed representative taping mode AFM images of naked DNA and cationic polyrotaxane **5b**/DNA complexes at N/P ratio of 2 and 10. Results from the AFM study showed that the complexation of DNA by **5b** led to the formation of compact nanoparticle. In Fig. 6a, loose, supercoiled structure of pDNA could be found when the pDNA was not condensed by polymer. At N/P ratio of 2, supercoiled plasmid DNA could still be identified under AFM while some of the pDNA was condensed to nanoparticles by **5b**. Compared to this partial condensation at N/P ratio of 2, the same amount of pDNA could be tightly packed and formed pDNA complexes at N/P ratio of 10 completely. Moreover, it was found that the diameter of the nanoparticles in Fig. 5c ranged within 110–160 nm, which is in agreement with the dynamic light scattering results.

3.3. Cytotoxicity of cationic polyrotaxanes

Cytotoxicity of polymeric gene vector may be an important factor that affects the transfection efficiency. Fig. 7 showed the results of *in vitro* cytotoxicity of the cationic polyrotaxanes analyzed by MTT method in two cell lines (COS7 and HEK293). As shown in Fig. 7a, all the synthesized cationic polymers and PEI (25 K) showed a strong dose-dependent effect on cytotoxicity and the cytotoxicity of the polymers was much lower than that of PEI (25 K). For example, at concentration of 62.5 μg/ml, COS7 cells only showed approximately 7% cell viability when incubated with PEI (25 K). In the meanwhile, in the case of **5a**, **5b**, and **5c**, which were grafted with linear OEI chains, their relative growth rate in COS7 cells showed more than 70% viability under the same condition. **5d**, which was grafted with branched OEI-14, exhibited nearly 50% cell viability at this concentration. LD_{50} value was also calculated to further compare the cytotoxicity of the cationic polymers with PEI

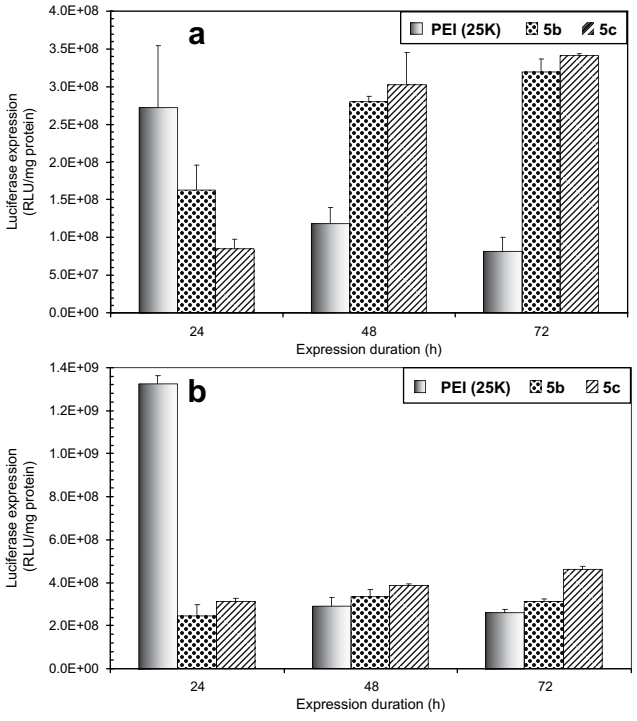


Fig. 9. *In vitro* gene transfection efficiency of the complexes of cationic polyrotaxane **5b** and **5c** in comparison with that of PEI (25 K) at N/P ratio of 10 in the absence (a) and presence (b) of serum at different expression duration (24 h, 48 h and 72 h) in HEK293 cells. Data represent mean ± standard deviation ($n = 3$).

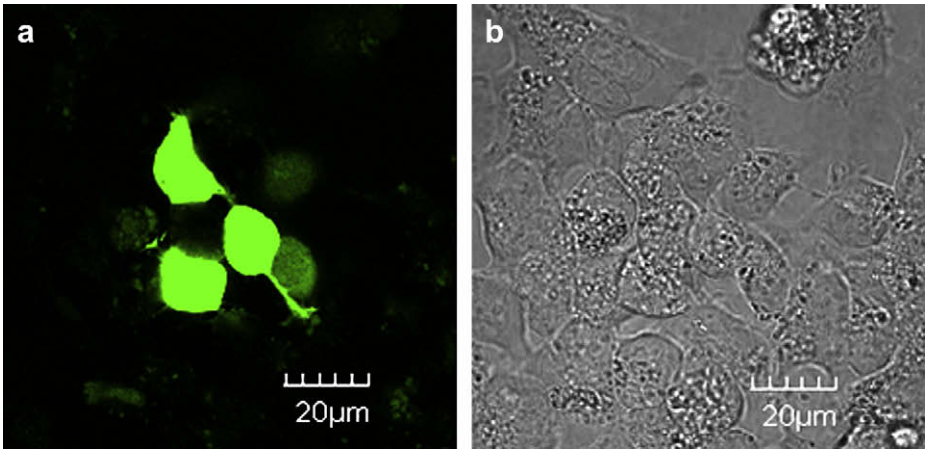


Fig. 10. The confocal images of transfected HEK293 cells. The transfection was mediated by **5b** at N/P ratio of 10 in the absence of serum using green fluorescence protein (GFP) gene as a reporter gene. The same field of cells was observed with fluorescence microscope (a) to visualize GFP expression and Nomarski optics (b).

(25 K). The LD_{50} value of PEI (25 K) in COS7 was 25 μg/ml, while those of cationic polyrotaxanes **5a**, **5b**, **5c**, and **5d** were 155, 135, 140 and 55 μg/ml, respectively, which were much lower than that of the PEI (25 K). These results may be attributed to the reduction of amino density resulted from the supramolecular structure of the polyrotaxanes since high amino density and the high molecular weight may be the reasons of the toxicity of PEI (25 K). A similar trend was also observed in HEK293 cells (Fig. 7b).

3.4. Transfection efficiency of cationic polyrotaxanes

In vitro transfection efficiency of complexes formed between pDNA and cationic polyrotaxanes was assessed utilizing a transient expression of luciferase reporter in both HEK293 and COS7 cells. Fig. 8 shows the gene transfection efficiency of cationic polyrotaxanes for DNA delivery compared with those of branched PEI (25 K) and naked pDNA in the absence and presence of serum (ND), in both COS7 and HEK293 cells. The structure of polymers plays an important role in the transfection efficiency. In COS7 cells the transfection efficiency mediated by cationic polyrotaxanes was dependent upon the chain length of the OEI conjugated to α-CD. Among the polymers examined, increased OEI length produced greater transfection efficiency following the order **5c** > **5b** > **5a**, but the transfection capability of **5d** could not follow this rule probably attributing to its branched OEI chains.

The relationship of transfection efficiency with the chain length of OEI was not strong in HEK293 cells. The transfection efficiency mediated by **5b** was quite similar to PEI at various N/P ratios in the absence of serum. Compared with PEI (25 K), **5b** and **5c** showed a similar or higher gene delivery capability in the presence of serum. Especially, when the transfection was conducted at N/P ratio of 30, the transfection efficiency of **5b** and **5c** in the presence of serum was 10-fold and 7-fold more than that of PEI (25 K), respectively.

Kinetics of expression is also important for gene delivery. Fig. 9 shows the time-dependent changes of gene expression of the cationic polyrotaxane **5b** and **5c** in comparison with that of PEI (25 K) at N/P ratio of 10 in HEK293 cells, and the transfection efficiency was monitored for 3 days. While the transfection efficiency mediated by PEI (25 K) decreased with the increasing expression duration no matter in the absence or in the presence of serum, **5b** and **5c** showed a sustained gene delivery capability. In both serum and serum free condition, increases in transfection efficiency could be found in HEK293 cells transfected with **5b** and **5c**/pRL-CMV when the expression duration increased from 24 h to 48 h, and then to 72 h. These results may be mainly attributed to the supramolecular structure of these polymers: in these cationic polyrotaxanes, the OEI-grafted α-CD rings can rotate and/or move along the polymeric chain freely, and this flexibility may enhance the interaction of cations with DNA and/or cellular membrane [41].

Confirmation of the gene delivery capability of the cationic polyrotaxane **5b** was also obtained by fluorescence microscopy (Fig. 10). Plasmid pEGFP-N1 encoding green fluorescence protein (GFP) was used to examine the GFP expression in HEK293 cells. Strong fluorescence signal could be observed when transfection was mediated by **5b** at N/P ratio of 10. GFP expression could not be detected when the transfection was mediated by naked DNA, which was used as a negative control.

4. Conclusions

In this study, a series of water soluble cationic polyrotaxanes containing PPO–PEO–PPO triblock copolymer, α-CD and various OEI chains were synthesized and investigated for gene delivery. Cytotoxicity studies showed that these cationic polyrotaxanes displayed significantly low cytotoxicity in comparison with branched PEI (25 K), owing to the low positive charge density resulted from the supramolecular structure of the polyrotaxanes. For the cationic polyrotaxanes with linear OEI chains, the increased OEI length produced greater transfection efficiency in COS7 cells. In HEK293 cells, **5b** and **5c** showed a similar or higher gene delivery capability in the presence of serum, and even 10-fold and 7-fold more than that of branched PEI (25 K) at N/P ratio of 30, respectively. More interestingly, in both serum and serum free condition, the cationic polyrotaxane **5b** and **5c** displayed the sustained gene delivery capability in HEK293 cells, while the transfection efficiency of PEI (25 K) decreased dramatically with the increasing expression duration. These transfection results can be attributed to the structure of the above cationic polyrotaxanes. Such a supramolecular structure enables the OEI-grafted α-CD rings to rotate and/or move along the polymeric chain freely, and this flexibility may enhance the interaction of the polyrotaxanes with DNA and/or cellular membrane.

Therefore, these cationic polyrotaxanes have a high potential as novel nonviral gene carriers, possibly for continuous, large dose of *in vivo* administration, due to their low cytotoxicity, high and sustained gene delivery capability, which are crucial factors in clinical uses.

Acknowledgements

The authors acknowledge the financial support from the Academic Research Fund, Ministry of Education, Singapore (WBS: R-397-000-031-112) and the Institute of Materials Research and Engineering, A*STAR, Singapore.

References

[1] Lehn JM. Supramolecular chemistry: concepts and perspectives. New York: Weinheim; 1995.
[2] Steed JW, Atwood JL. Supramolecular chemistry. New York: John Wiley & Sons; 2000.
[3] Ciferri A. Supramolecular polymers. 2nd ed. Boca Raton: Taylor & Francis; 2005.
[4] Nepogodiev SA, Stoddart JF. Chem Rev 1998;98:1959–76.
[5] Bender ML, Komiyama M. Cyclodextrin chemistry. Berlin: Springer-Verlag; 1978.
[6] Szejtli J. Cyclodextrins and their inclusion complexes. Budapest: Akademiai Kiado; 1982.
[7] Uekama K, Hirayama F, Irie T. Chem Rev 1998;98:2045–76.
[8] Hirayama F, Uekama K. Adv Drug Deliv Rev 1999;36:125–41.
[9] Irie T, Uekama K. Adv Drug Deliv Rev 1999;36:101–23.
[10] Harada A, Li J, Kamachi M. Nature 1992;356:325–7.
[11] Wenz G, Keller B. Angew Chem Int Edit 1992;31:197–9.
[12] Raymo FM, Stoddart JF. Chem Rev 1999;99:1643–63.
[13] Wenz G, Han BH, Muller A. Chem Rev 2006;106:782–817.
[14] Herrmann W, Schneider M, Wenz G. Angew Chem Int Edit 1997;36:2511–4.
[15] Kidd TJ, Loontjens TJA, Leigh DA, Wong JKY. Angew Chem Int Edit 2003;42:3379–83.
[16] Ooya T, Ito A, Yui N. Macromol Biosci 2005;5:379–83.
[17] Jiao H, Goh SH, Valiyaveettil S. Macromolecules 2002;35:3997–4002.
[18] Chan SC, Kuo SW, Chang FC. Macromolecules 2005;38:3099–107.
[19] Rusa CC, Bridges C, Ha SW, Tonelli AE. Macromolecules 2005;38:5640–6.
[20] Li J, Li X, Zhou ZH, Ni XP, Leong KW. Macromolecules 2001;34:7236–7.
[21] Li J, Toh KC. J Chem Soc Perkin Trans 2002;2:35–40.
[22] Li J, Ni XP, Leong K. Angew Chem Int Edit 2003;42:69–72.
[23] Li J, Ni XP, Zhou ZH, Leong KW. J Am Chem Soc 2003;125:1788–95.
[24] Li J, Chen B, Wang X, Goh SH. Polymer 2004;45:1777–85.
[25] Cacialli F, Wilson JS, Michels JJ, Daniel C, Silva C, Friend RH, et al. Nat Mater 2002;1:160–4.
[26] Feng ZG, Zhao SP. Polymer 2003;44:5177–86.
[27] Yu HQ, Feng ZG, Zhang AY, Sun LG, Qian LJ. Soft Matter 2006;2:343–9.
[28] Ooya T, Choi HS, Yamashita A, Yui N, Sugaya Y, Kano A, et al. J Am Chem Soc 2006;128:3852–3.
[29] Li J, Ni XP, Leong KW. J Biomed Mater Res A 2003;65A:196–202.
[30] Li J, Li X, Ni XP, Wang X, Li HZ, Leong KW. Biomaterials 2006;27:4132–40.
[31] Li J, Yang C, Li HZ, Wang X, Goh SH, Ding JL, et al. Adv Mater 2006;18:2969–74.
[32] Yang C, Wang X, Li HZ, Goh SH, Li J. Biomacromolecules 2007;8:3365–74.
[33] Li J, Loh XJ. Adv Drug Deliv Rev 2008;60:1000–17.
[34] Leong KW, Mao HQ, Truong-Le VL, Roy K, Walsh SM, August JT. J Control Release 1998;53:183–93.
[35] Yang C, Li HZ, Goh SH, Li J. Biomaterials 2007;28:3245–54.
[36] Boussif O, Lezoualch F, Zanta MA, Mergny MD, Scherman D, Demeneix B, et al. Proc Natl Acad Sci U S A 1995;92:7297–301.
[37] Zauner W, Ogris M, Wagner E. Adv Drug Deliv Rev 1998;30:97–113.
[38] KukowskaLatallo JF, Bielinska AU, Johnson J, Spindler R, Tomalia DA, Baker JR. Proc Natl Acad Sci U S A 1996;93:4897–902.
[39] Dekie L, Toncheva V, Dubruel P, Schacht EH, Barrett L, Seymour LW. J Control Release 2000;65:187–202.
[40] Wang J, Mao HQ, Leong KW. J Am Chem Soc 2001;123:9480–1.
[41] Yui N, Ooya T. Chem Eur J 2006;12:6730–7.
[42] Kabanov A, Zhu J, Alakhov V. Pluronic block copolymers for gene delivery. In: Huang L, Hung M-C, Wagner E, editors. Non-viral vectors for gene therapy. New York: Academic Press; 2005. p. 231–61.
[43] Khan AR, Forgo P, Stine KJ, D'Souza VT. Chem Rev 1998;98:1977–96.
[44] Mislick KA, Baldeschwieler JD. Proc Natl Acad Sci U S A 1996;93:12349–54.