

Folic Acid Modified Cationic γ -Cyclodextrin-oligoethylenimine Star Polymer with Bioreducible Disulfide Linker for Efficient Targeted Gene Delivery

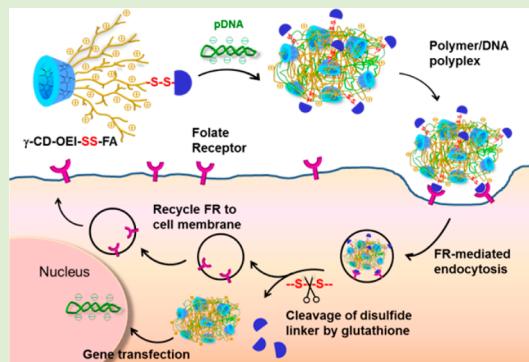
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S Supporting Information

ABSTRACT: For an efficient folate-targeted delivery, while the interaction between the folate on the carriers and the folate receptor (FR) on the cells is necessary, the recovering and recycling of FR to maintain a high density level of FR on the cellular membrane is also important. Herein, we demonstrate a design and synthesis of a new star-shaped cationic polymer containing a γ -cyclodextrin (γ -CD) core and multiple oligoethylenimine (OEI) arms with folic acid (FA) linked by a bioreducible disulfide bond for efficient targeted gene delivery. The newly synthesized cationic polymer, named γ -CD-OEI-SS-FA, could be cleaved efficiently, and FA was readily released under reductive condition similar to intracellular environment. The γ -CD-OEI-SS-FA polymer was well-characterized and studied in terms of its gene delivery properties in FR-positive KB cells and FR-negative AS49 cells under various conditions, in comparison with cationic polymers such as high molecular weight branched polyethylenimine (PEI), γ -CD-OEI star-shaped cationic polymer, γ -CD-OEI-FA polymer where FA was directly linked to the star polymer without disulfide linker. Our data have demonstrated that the new γ -CD-OEI-SS-FA gene carrier had low cytotoxicity and possessed capacity to target and deliver DNA to specific tumor cells that overexpress FRs, as well as functions to recover and recycle FRs onto cellular membranes to facilitate continuous FR-mediated endocytosis to achieve very high levels of gene expression. This study has expanded the strategy of FA-targeted delivery by combining the smart FR-recycling function to achieve the significant enhancement of gene expression. The new FA-targeted and bioreducible carrier may be a promising efficient gene delivery system for potential cancer gene therapy.



1. INTRODUCTION

In recent years, great interest has been focused on the design and synthesis of safe and effective nonviral vectors to carry and protect oligonucleotides for gene therapy. Nonviral vectors, including cationic lipids, polymers, and dendrimers, are promising due to their advantages over viral vectors, such as the safety, low cytotoxicity, easy manipulation, and ability to be readily functionalized.¹ Polyethylenimine (PEI) is one of the most prominent examples of cationic polymers that have been extensively studied for nonviral gene delivery.² The increase of gene delivery ability of PEI was observed as increasing of its molecular weight from 600 to 70000 Da, but was accompanied with high toxicity.^{3,4}

Cyclodextrins (CDs) are a series of natural cyclic oligosaccharides composed of 6, 7, and 8 D(+)-glucose units linked by α -1,4-linkages, named α -, β -, and γ -CD, respectively. These water-soluble, biocompatible oligosaccharides do not have immune responses and show low toxicity in animals and humans.⁵ CDs are usually used as solubilization and

stabilization reagents for small molecules.⁶ Their application for oligonucleotide delivery thanks to the property that the hydroxyl groups of CD rings offer opportunity for multiple modifications. In 1999, Davis' group reported the first synthesis of CD-conjugated cationic polymers for gene delivery.⁷ These CD-based polymers illustrated lower cytotoxicity and improved transfection efficiency as nonviral gene delivery vectors.^{8–10} Considering the good gene transfection efficiency of PEI, CD-modified PEI may have great potential as a promising gene delivery vector. Our group reported a series of cationic star polymers by conjugating PEI or oligoethylenimine (OEI) chains to α -CD as nonviral gene delivery vectors.^{11,12} These CD-OEI star-shaped polymers showed much lower cytotoxicity and excellent gene transfection efficiency that were comparable

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to or even higher than that of the well-studied branched PEI (25 kDa).

The strategy to employ proper targeting groups is often used to develop gene delivery vectors which can target to specific cells or tissues. Folic acid (FA) is essential for the biosynthesis of nucleotide bases. Many malignant cells overexpress folate receptors (FRs) that is a high affinity folate-binding protein in cellular membrane,¹³ which renders FA an attractive candidate ligand for tumor-specific targeted gene delivery. Therefore, vectors conjugated with FA can be efficiently taken up by malignant cells which overexpress FRs via receptor-mediated endocytosis.^{14,15} The total amount of folate-conjugated vectors internalized into a cell is roughly proportional to the overall number of FRs expressed on the cell.¹³ However, after the FRs on the cell surface are consumed during the internalization of the folate-conjugated vectors, the cells can no longer recognize the folate ligand, and the folate-conjugated vectors lose the ability of tumor-specific targeted delivery. Only if the cellular membranes can readily recover the consumed FRs, the folate-conjugated vectors can be efficiently and continuously internalized by the target tumor cells.¹⁶ Therefore, the continuous recycling of FRs after cellular endocytosis is essential.

Disulfide bond (-S-S-) is a covalent linkage that can be easily synthesized by oxidation of sulphydryl and can be cleaved in the presence of reducing agents. The oxidizing extracellular milieu and reducing intracellular space renders the disulfide bond a delivery tool, which allows the vector to be cleaved and to release the carried gene within the cells. In nonviral gene delivery systems, disulfide-based conjugates have been demonstrated to have higher gene transfection efficiency in comparison with disulfide-free precursors.^{17,18} Low molecular weight PEI linked with reducible disulfide bond could assist uncoupling of PEI from DNA to enhance gene delivery, and the cleaved low molecular weight PEI could be easily cleared from the body.^{18–22} A disulfide bond was also incorporated in some folate-mediated targeted gene delivery systems, where the disulfide linker was used to trigger cleavage of polymers followed by enhanced DNA release.^{23–25} Moreover, folate-drug conjugates with cleavable linkers (such as disulfide bond) were reported to ensure the release of drugs after endocytosis in the target cells.^{26–30}

Herein we design a folate-conjugated γ -CD-OEI star-shaped cationic polymer with disulfide bond as the linker between folate and γ -CD-OEI as a new multifunctional gene carrier. We have demonstrated that the newly designed γ -CD-OEI-SS-FA gene carrier had low cytotoxicity, and possessed capacity to target and deliver DNA to specific tumor cells that overexpress FRs, as well as function to recover and recycle FRs onto cellular membranes to facilitate continuous FR-mediated endocytosis to achieve very high levels of gene expression.

2. EXPERIMENTAL SECTION

2.1. Materials. Branched PEI (MW, 600 and 25000), *N,N'*-dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP), folic acid (FA), buthionine sulphoximine (BSO), pyridine, and anhydrous dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich. Cystamine dihydrochloride, succinic anhydride, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazodium bromide (MTT), and DL-dithiothreitol (DTT) were obtained from Alfa Aesar (MA, U.S.A.). 1,1'-Carbonyldimidazole (CDI) and α , β -, and γ -CD were purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). D₂O and DMSO-*d*₆ used as solvents in the NMR measurement were

obtained from Cambridge Isotope Laboratories, Inc. (Andover, U.S.A.).

2.2. Synthesis Procedures. The intermediates and the final cationic gene carriers were synthesized as follows.

2.2.1. FA-SS-NH₂. To a suspension of FA (265 mg, 0.6 mmol) and cystamine dihydrochloride (180 mg, 0.8 mmol) in a mixture of DMSO (4 mL) and pyridine (4 mL) was added DCC (144 mg, 0.7 mmol) and DMAP (cat.). The mixture was stirred at room temperature for 18 h under dark, then poured into acetone (80 mL). The yellow precipitate was collected and washed with acetone (40 mL) twice, and dried under vacuum to yield 330 mg of yellow solid (yield, 96%). The crude was used in the next step without further purification.

2.2.2. FA-SS-COOH. To a solution of FA-SS-NH₂ crude (330 mg, 0.6 mmol) in pyridine (5 mL) was added succinic anhydride (100 mg, 1.0 mmol), and then the mixture was stirred at room temperature for 18 h under dark. The reaction mixture was then poured into acetone (80 mL). The yellow precipitate was collected, washed with acetone (40 mL) twice, and dried under vacuum to yield 348 mg of yellow solid (yield, 90%). ¹H NMR (400 MHz, DMSO-*d*₆): δ 8.62 (s, 1H, -CH- of pyrazine), 8.01 (brs, 2H, -NH-), 7.63 (d, 2H, J = 8.4 Hz, -CH- of benzyl ring), 6.90 (brs, 2H, -NH-), 6.62 (d, 2H, J = 8.4 Hz, -CH- of phenyl ring), 4.47 (s, 2H, -CH₂-), 4.32 (m, 1H, -CH-), 2.72 (t, 4H, J = 6.8 Hz, -CH₂S-), 2.20–2.45 (m, 4H, -CH₂CO- of succinic anhydride), 1.80–2.20 (m, 4H, -CH₂ of FA).

2.2.3. γ -CD-OEI. To a solution of CDI (4.9 g, 30.0 mmol) in DMSO (30 mL) was added dropwise a solution of γ -CD (1.3 g, 1.0 mol) in DMSO (20 mL) during 1 h. After stirring for 20 h, the solution was added dropwise into a mixture of Et₂O/THF (400 mL/200 mL). The white precipitate was collected by centrifuge and washed with Et₂O/THF (20 mL/10 mL) twice, then dissolved into anhydrous DMSO (20 mL). The previous solution was added dropwise into a solution of PEI-600 (12.6 g, 21 mmol) in DMSO (40 mL) during 30 min, and then stirred at room temperature for an additional 24 h. Purification by dialysis (MWCO, 2000) against water for 5 d, freeze-dried to yield 2.1 g of colorless solid (yield, 56.0%). ¹H NMR (400 MHz, D₂O): δ 5.05 (brs, 8H, H-1 of γ -CD), 2.95–4.57 (m, 62H, H-2–6 of γ -CD, -CONHCH₂- of OEI), 1.87–2.95 (m, 395 H, -CH₂CH₂NH- of OEI).

2.2.4. γ -CD-OEI-FA_{1,2}. To a solution of γ -CD-OEI (110 mg, 20.9 μ mol) and FA (9.2 mg, 20.8 μ mol) in DMSO/pyridine (1 mL/1 mL) was added DCC (4.3 mg, 20.8 μ mol) and DMAP (cat.). The mixture was stirred at room temperature for 18 h under dark. Purification by dialysis (MWCO, 2000) against water under dark for 5 d, freeze-dried to yield 95 mg of yellow solid (73.4%). ¹H NMR (400 MHz, D₂O): δ 8.57 (s, 1.2H, -CH- of pyrazine on FA), 7.65 (d, 2.4H, -CH- of phenyl ring on FA), 6.77 (d, 2.4H, -CH- of phenyl ring on FA), 4.98 (brs, 8H, H-1 of γ -CD), 4.52 (2.4H, -CH₂NHPh- of FA), 4.25 (1.2H, -CHCOOH- of FA), 3.00–4.50 (m, 63H, H-2–6 of γ -CD, -CONHCH₂- of OEI), 2.10–2.92 (m, 395H, -CH₂CH₂NH- of OEI).

2.2.5. γ -CD-OEI-SS-FA_{0,8}. To a solution of γ -CD-OEI (57 mg, 10 μ mol) and FA-SS-COOH (7 mg, 10 μ mol) in DMSO (1 mL) and pyridine (1 mL) was added DCC (2 mg, 10 μ mol) and DMAP (cat.). The reaction mixture was stirred at room temperature for 18 h under dark. Purification by dialysis (MWCO, 2000) against water under dark for 5 d, freeze-dried to yield 42 mg of yellow solid (68%). ¹H NMR (400 MHz, D₂O): δ 8.45–8.61 (m, 0.8H, -CH- of pyrazine on FA), 7.53–7.74 (m, 1.6H, -CH- of phenyl ring on FA), 6.57–6.81 (m, 1.6H, -CH- of phenyl ring on FA), 5.02 (brs, 8H, H-1 of γ -CD), 2.95–4.57 (m, 64H, -CH₂NHPh and -CHCOOH- of FA, H-2–6 of γ -CD, and -CONHCH₂- of OEI), 1.88–2.95 (m, 395H, -CH₂CH₂NH- of OEI).

2.2.6. γ -CD-OEI-SS-FA_{1,3}. To a solution of γ -CD-OEI (57 mg, 10 μ mol) and FA-SS-COOH (10 mg, 15 μ mol) in DMSO (1 mL) and pyridine (1 mL) was added DCC (3 mg, 15 μ mol) and DMAP (cat.). The reaction mixture was stirred at room temperature for 18 h under dark. Purification by dialysis (MWCO, 2000) against water under dark for 5 d, freeze-dried to yield 54 mg of yellow solid (83%). ¹H NMR (400 MHz, D₂O): δ 8.42–8.69 (m, 1.3H, -CH- of pyrazine on FA), 7.48–7.77 (m, 2.6H, -CH- of phenyl ring on FA), 6.54–6.84 (m, 2.6H, -CH- of phenyl ring on FA), 5.00 (brs, 8H, H-1 of γ -CD), 2.95–4.57 (m, 65H, -CH₂NHPh and -CHCOOH- of FA, H-2–6 of γ -CD, and -CONHCH₂- of OEI), 1.91–2.96 (m, 395H, -CH₂CH₂NH- of OEI).

2.2.7. γ -CD-OEI-SS-FA_{1,7}. To a solution of γ -CD-OEI (57 mg, 10 μ mol) and FA-SS-COOH (16 mg, 25 μ mol) in DMSO (1 mL) and pyridine (1 mL) was added DCC (5 mg, 25 μ mol) and DMAP (cat.). The reaction mixture was stirred at room temperature for 18 h under dark. Purification by dialysis (MWCO, 2000) against water under dark for 5 d, freeze-dried to yield 57 mg of yellow solid (84%). ¹H NMR (400 MHz, D₂O): δ 8.42–8.70 (m, 1.7H, -CH- of pyrazine on FA), 7.47–7.79 (m, 3.4H, -CH- of phenyl ring on FA), 6.51–6.92 (m, 3.4H, -CH- of phenyl ring on FA), 5.00 (brs, 8H, H-1 of γ -CD), 2.98–4.57 (m, 66H, -CH₂NHPh and -CHCOOH- of FA, H-2–6 of γ -CD, and -CONHCH₂- of OEI), 1.91–2.98 (m, 395H, -CH₂CH₂NH- of OEI).

2.3. ¹H NMR Spectroscopy. ¹H NMR spectra were recorded on a Bruker AV-400 NMR spectrometer at 400 MHz. The ¹H 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 referenced to the solvent peak (δ = 4.70 ppm for D₂O, δ = 2.50 ppm for DMSO-*d*₆).

2.4. UV–Vis Spectroscopy. All absorption spectra were recorded on a Shimadzu UV 2501 spectrophotometer against a solvent blank. Absorption was measured in quartz cuvettes (frosted wall, 0.7 mL). Samples were dilute to 0.01 mg/mL for FA, and 0.1 mg/mL for cationic polymer γ -CD-OEI, γ -CD-OEI-FA_{1,2}, and γ -CD-OEI-SS-FA_{1,3} before measurement.

2.5. Release Test. The concentrations of released FA by cleavage of disulfide linker from γ -CD-OEI-SS-FA using DTT were measured by UV–vis spectroscopy. In brief, 1.0 mL of γ -CD-OEI-SS-FA_{1,3} (5 mg/mL with DTT concentrations at 0 μ M, 10 μ M, and 10 mM in PBS buffer) was loaded into dialysis tubes (MWCO 2000, Spectrum Laboratories Inc., U.S.A.). These dialysis tubes were put into centrifuge tubes immersed 50 mL of PBS solution with corresponding DTT concentrations. Then the centrifuge tubes were placed in a shaker agitated at 200 rpm and maintained the temperature at 37 °C. At appropriate intervals, 1 mL of the dissolution medium was withdrawn and analyzed by UV–vis spectroscopy (Shimadzu UV 2501) at a wavelength of 284 nm. Meanwhile, 1 mL of fresh medium was added to replace the medium that was withdrawn.

2.6. Plasmids. The pRL-CMV (Promega, U.S.A.) plasmid, encoding Renilla luciferase, originally cloned from the marine organism *Renilla reniformis* was used. This plasmid DNA (pDNA) was amplified in *Escherichia coli* and purified following the protocol of the supplier (Qiagen, Hilden, Germany). The concentration of the purified plasmid DNA was measured by optical density at 260 and 280 nm. The quality was detected by electrophoresis in 1% agarose gel. The purified pDNA was resuspended in TE buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA) and kept at a concentration of 1.0 mg/mL.

2.7. Cells and Media. All cell lines were purchased from ATCC (Rockville, MD). KB cells are FR-positive human nasopharyngeal cells, cultured in Minimum Essential Medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 0.3 g/L of L-glutamine, 0.1 g/L of sodium pyruvate, 100 units/mg of penicillin, and 100 μ g/mL of streptomycin at 37 °C and 5% CO₂. The A549 human lung epithelial carcinoma cell line was cultured in Ham's F-12 Nutrient Mixture (F-12) supplemented with 10% FBS, 100 units/mg of penicillin, and 100 μ g/mL of streptomycin at 37 °C and 5% CO₂. Roswell Park Memorial Institute (RPMI) 1640 medium (FA free) supplemented with 10% FBS, 100 units/mg of penicillin, and 100 μ g/mL of streptomycin was used during cell viability and gene transfection test. MEM, F-12, and RPMI-1640 medium was purchased from Gibco BRL (Gaithersburg, MD).

2.8. Gel Retardation Assay. The binding ability of the all cationic polymers with pRL-CMV was tested by gel electrophoresis experiments. The sample solutions were diluted as various nitrogen concentrations by distilled water. Then pRL-CMV (0.1 mg/mL of TE buffer) was mixed with an equal volume of previous polymer solution to prepare DNA/polymer complex at various nitrogen/phosphate (N/P) ratio solutions from 0 to 4. Each sample was vortexed and incubated for 30 min at room temperature before loading to 1% agarose gel containing 0.5 μ g/mL ethidium bromide (EtBr). Gel electrophoresis was done in TAE buffer (40 mM Tris-acetate, 1 mM EDTA) at 100 V for 40 min in a Sub-Cell system (Bio-Rad

Laboratories, CA). DNA bands were visualized by a UV lamp by a GelDoc system (Synoptics Ltd, U.K.).

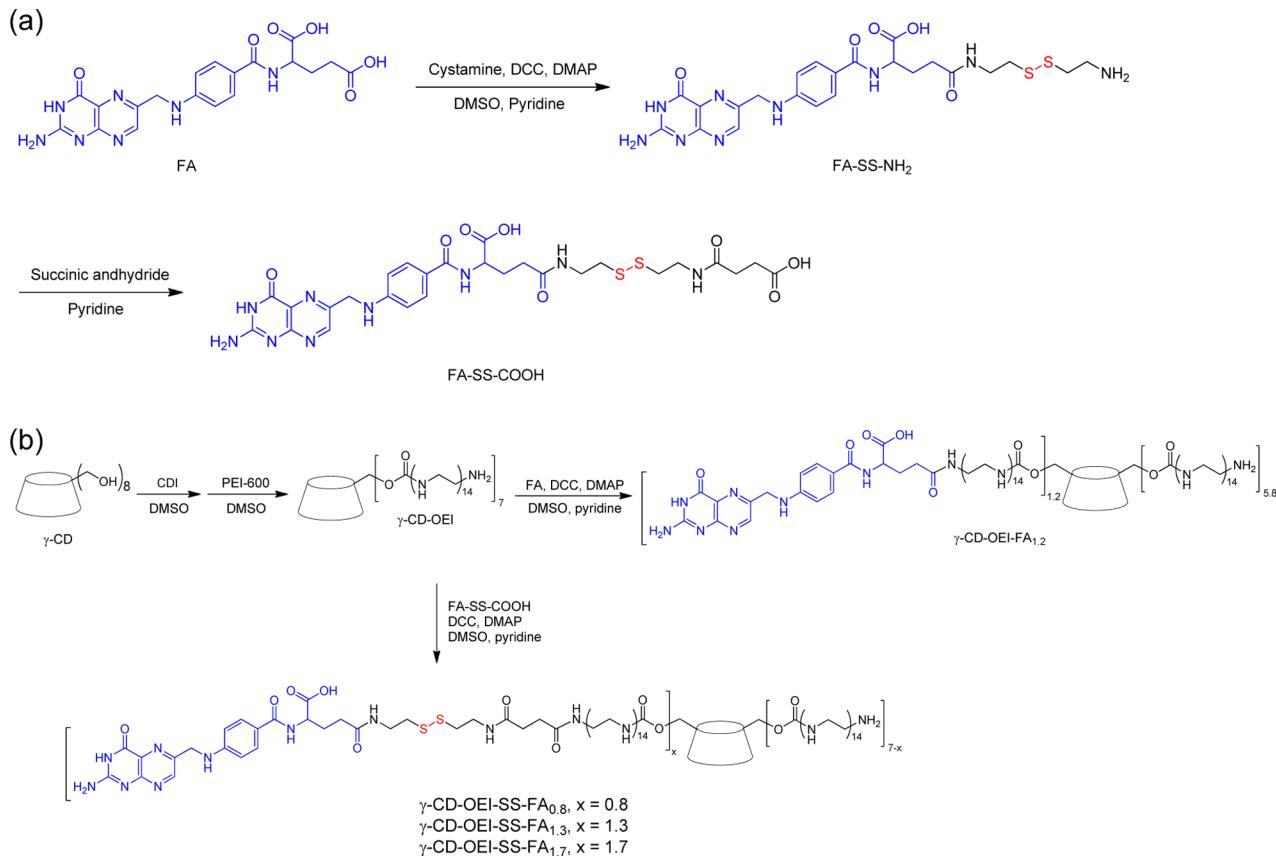
2.9. Dynamic Light Scattering and Zeta-Potential Measurements. Particle size and zeta potential of the polymer/pDNA complexes were assessed using a Zetasizer Nano ZS (Malvern Instruments, Southborough, MA, U.S.A.) with a laser light wavelength of 633 nm at a 173° scattering angle. Briefly, 100 μ L of appropriate polymers mixed with 3 μ g of pDNA to prepare various solutions with N/P ratios ranging from 10 to 100. The mixture was vortexed for 30 s and incubated for 30 min at room temperature, and then diluted into 1 mL of distilled water and vortexed for 30 s before test by the Zetasizer. The particle size measurement was carried out at 25 °C in triplicate.

The deconvolution of the measured correlation curve to an intensity size distribution was accomplished using a nonnegative least-squares algorithm. The Z-average hydrodynamic diameters of the particles were provided by the instrument. The Z-average size is the intensity weighted mean diameter derived from a Cumulants or single-exponential fit of the intensity autocorrelation function. The zeta potential measurements were carried using a capillary zeta potential cell in automatic mode with same samples measured for particle size.

2.10. Cell Viability Assay. Human KB carcinoma cell line was cultured in FA free RPMI 1640 medium supplemented with 10% FBS at 37 °C, 5% CO₂, and 95% relative humidity. A total of 100 μ L of medium with a density of 1.5 \times 10⁵ cells/mL was seeded into 96-well plates (NUNC, Wiesbaden, Germany). After 24 h, culture media were replaced with fresh culture media containing serial dilutions of polymers, in which the cells were cultured for 20 h. Then 10 μ L of sterile filtered MTT (5 mg/mL) stock solution in PBS was added to each well, reaching a final MTT concentration of 0.5 mg/mL. After 4 h, unreacted dye was discarded by aspiration. The formazan crystals were dissolved in 100 μ L/well DMSO and the absorbance was measured using a microplate reader (Spectra Plus, TECAN) at a wavelength of 570 nm. Five 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} × 100%.

2.11. In Vitro Transfection and Luciferase Assay. Gene transfection efficiency studies were carried out in KB and A549 cell lines using pRL-CMV as reporter gene. In brief, 24-well plates were seeded with cells at a density of 8 \times 10⁴/well (KB cells) or 6 \times 10⁴/well (A549 cells) for 24 h before transfection. The sample/DNA complexes at various N/P ratios were prepared by adding the samples into DNA solutions, followed by vortexing for 20 s and incubation for 30 min at room temperature before the transfection. Each well was first replaced with 400 μ L of fresh RPMI 1640 medium (FA free) for gene transfection efficiency test, or RPMI 1640 medium with FA at different concentrations (0.000, 0.001, 0.010, and 0.100 g/L) for FA competition test, or RPMI 1640 medium (FA free) with BSO at different concentrations (0, 50, 250, and 500 μ M) for disulfide inhibition assay. Then the sample/DNA complexes were added into previous medium and incubated for 4 h under standard incubator conditions. After 4 h, the medium was replaced with 500 μ L of fresh RPMI 1640 medium (FA free), and the cells were further incubated for an additional 20 h under the same conditions. Cells were washed with PBS twice and 100 μ L of cell culture lysis reagent was added (Promega, Cergy Pontoise, France), then it was shaken at 1000 rpm for 2 h at room temperature before testing. 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, U.S.A.). 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 per milligram of cell protein lysate (RLU/mg protein).

2.12. Confocal Microscopy. Confocal microscopy was used to evaluate the expression of the plasmid pEGFP-N1 (Clontech Laboratories, Inc., Mountain View, CA), encoding a red-shifted variant of wild-type green fluorescence protein (GFP) by a KB cell line. In brief, a density of 1.2 \times 10⁴/well KB cells in 0.3 mL of RPMI 1640 medium (FA free) were seeded onto a Lab-Tek four-chambered

Scheme 1. Synthesis of FA with Disulfide Linker FA-SS-COOH (a) and γ -CD-OEI-FA and γ -CD-OEI-SS-FA (b)

coverglass system (Nalge-Nane International, Naperville, IL). After 24 h, EGFP polyplexes with PEI (25 kDa) at N/P ratio of 10, and the EGFP polyplexes with γ -CD-OEI, γ -CD-OEI-FA_{1.2}, and γ -CD-OEI-SS-FA_{1.3} at N/P ratio of 50 were added into the transfection medium. After incubation for 4 h, the medium was replaced with 0.3 mL of fresh RPMI 1640 medium (FA free) and further incubated for an additional 20 h under same conditions. The cells were imaged using an Olympus Fluoview FV500 confocal laser scanning microscope (Olympus, Japan). EGFP fluorescence was excited by 488 nm laser line and detected using a 515 nm filter.

2.13. Statistical Analysis. Statistical analysis was carried out by a standard Student's *t*-test with a minimum confidence level of 0.05 as significant statistical difference. All the data are reported as mean \pm standard deviation.

3. RESULTS AND DISCUSSION

3.1. Synthesis of γ -CD-OEI-SS-FA. γ -CD has 8 primary hydroxyl groups that can be easily modified. Scheme 1 shows the synthesis procedures of FA-SS-COOH and γ -CD-OEI-SS-FA. γ -CD-OEI-FA was also synthesized as a control compound. First, coupling reaction between FA and cystamine dihydrochloride was carried out by DCC and DMAP to introduce disulfide bond. Second, the amino-terminated FA was allowed to react with succinic anhydride to give FA-SS-COOH. The feeding ratio of DCC and cystamine was less than 1:1 to make sure that only the γ -carboxyl group of FA was conjugated with disulfide bond because overmodification of α -carboxyl group of FA may result in loss of binding ability to FR. Third, the primary hydroxyl groups of γ -CD were activated by CDI, and then allowed to react with large excess of PEI-600 to give a γ -CD-OEI star-shaped cationic polymer (this step is similar to our previous report on the synthesis of cationic star-shaped α -

CD-OEI polymers).¹¹ To ensure there was no intra- or intermolecular cross-linking, the CDI-activated intermediate was purified by precipitation in Et₂O/THF and large excess of PEI was used. Finally, unmodified FA and the FA-SS-COOH were allowed to react with γ -CD-OEI to produce γ -CD-OEI-FA and γ -CD-OEI-SS-FA, respectively. By varying the feeding ratio of FA-SS-COOH to γ -CD-OEI, three samples of γ -CD-OEI-SS-FA were obtained with different amounts of FA grafted onto γ -CD-OEI (Scheme 1b).

The successful synthesis of these samples was confirmed by ¹H NMR and UV-vis spectroscopy. Figure 1 shows the ¹H NMR spectra of γ -CD-OEI-SS-FA_{1.3} in comparison with γ -CD-OEI and γ -CD-OEI-FA_{1.2}. The typical signals from H-1 of γ -

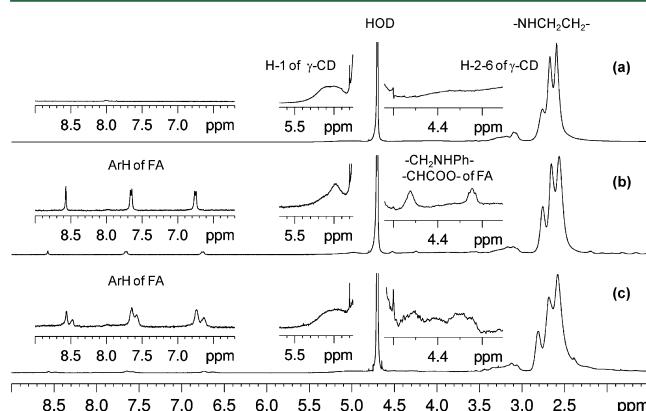


Figure 1. ¹H NMR spectra of γ -CD-OEI (a), γ -CD-OEI-FA_{1.2} (b), and γ -CD-OEI-SS-FA_{1.3} (c) in D₂O.

CD and OEI ethylene protons appeared at 5.0 and 1.9–3.0 ppm, respectively. The numbers of grafted OEI arms were calculated based on the integral ratios of these peaks. Successful linkage of FA to γ -CD-OEI was confirmed by their aromatic proton signals with chemical shifts at 6.8, 7.7, and 8.6 ppm. Meanwhile, the number of grafted FA was determined to be 1.2 for γ -CD-OEI-FA_{1,2} and 1.3 for γ -CD-OEI-SS-FA_{1,3} according to the peak integrals of FA aromatic proton signals and OEI ethylene proton signals. The numbers of grafted FA for γ -CD-OEI-SS-FA_{0,8} and γ -CD-OEI-SS-FA_{1,7} were also calculated from their ¹H NMR spectra.

UV-vis spectroscopy was used to further confirm the structure of the synthesized γ -CD-OEI-FA and γ -CD-OEI-SS-FA. As shown in Figure 2, both γ -CD-OEI-FA_{1,2} and γ -CD-

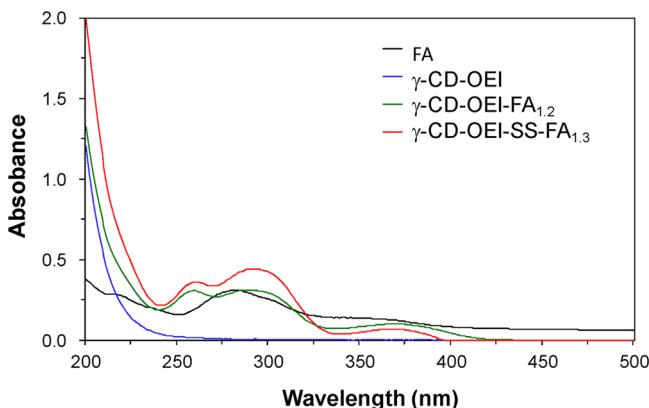


Figure 2. UV-visible spectra of FA (0.01 mg/mL), γ -CD-OEI (0.1 mg/mL), γ -CD-OEI-FA_{1,2} (0.1 mg/mL), and γ -CD-OEI-SS-FA_{1,3} (0.1 mg/mL) in H₂O.

OEI-SS-FA_{1,3} have three absorption peaks at 260, 291, and 369 nm, due to the conjugation of FA, while FA shows peaks at 282 and 362 nm. In contrast, γ -CD-OEI has no UV-vis absorption. The positively charged γ -CD-OEI withdraws electrons of unsaturated aryl rings, causing red shifts of FA absorption bands from 282 to 291 nm and 362 to 369 nm.

FA release test was used to demonstrate the cleavage of disulfide bond in the presence of DTT. γ -CD-OEI-SS-FA_{1,3} was incubated with 10 μ M and 10 mM of DTT in PBS at 37 °C to mimic the different extracellular and intracellular environments, where glutathione (GSH) concentrations should be about 10 μ M and 1–10 mM, respectively. As shown in Figure 3, the degradation of disulfide bond was very slow at low DTT concentrations (0 and 10 μ M) and only 10% of FA derived from γ -CD-OEI-SS-FA_{1,3} was detected after 120 h. However, increasing DTT concentration to 10 mM significantly increased the reduction and cleavage rate of the disulfide bond in γ -CD-OEI-SS-FA_{1,3}. As the result, more than 80% of FA released within 48 h and nearly 100% of FA released at 120 h.

3.2. Formation of γ -CD-OEI-SS-FA/DNA Complexes. It is a prerequisite for a gene delivery vector to effectively condense DNA to form nanoparticles. When negatively charged DNA encounters positively charged cationic polymer in an aqueous solution, the electrostatic interaction will cause the phase separation of DNA and the polymer, which induces the formation of condensed DNA/polymer complexes (polyplexes) as colloidal nanoparticles suitable for cellular internalization through endocytosis and/or other pathways. The ability of cationic γ -CD-OEI-SS-FA_{1,3} to condense pDNA

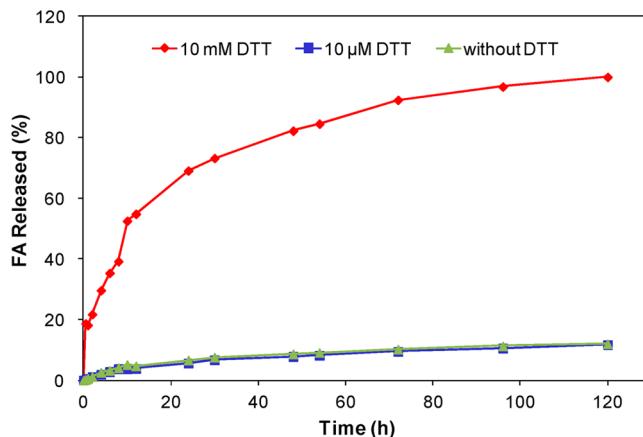


Figure 3. Release profiles of γ -CD-OEI-SS-FA_{1,3} in the absence and presence of DTT at 10 μ M and 10 mM in PBS buffer (pH 7.4, 1.0 mL) at 37 °C.

was analyzed by agarose gel electrophoresis in comparison with PEI (25 kDa), γ -CD-OEI, and γ -CD-OEI-FA_{1,2} (Figure 4). All the cationic polymers could entirely compact pDNA at N/P ratios of 2.5, which is same as the standard PEI.

The surface charge and particle size of the OEI-grafted polymers are important parameters for their biological application, which might be related to their blood circulation,

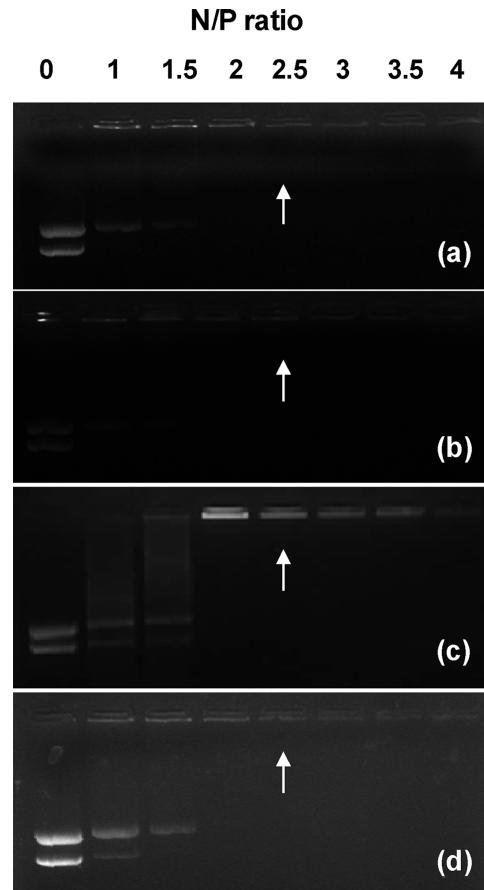


Figure 4. Electrophoretic mobility of pDNA in the polyplexes formed with PEI (a), γ -CD-OEI (b), γ -CD-OEI-FA_{1,2} (c), and γ -CD-OEI-SS-FA_{1,3} (d). The arrows indicate the N/P ratios where the DNA mobility is completely retarded.

cell encapsulation mechanism, and bioavailability.^{31,32} Figure 5 displays the results of zeta potential and particle size

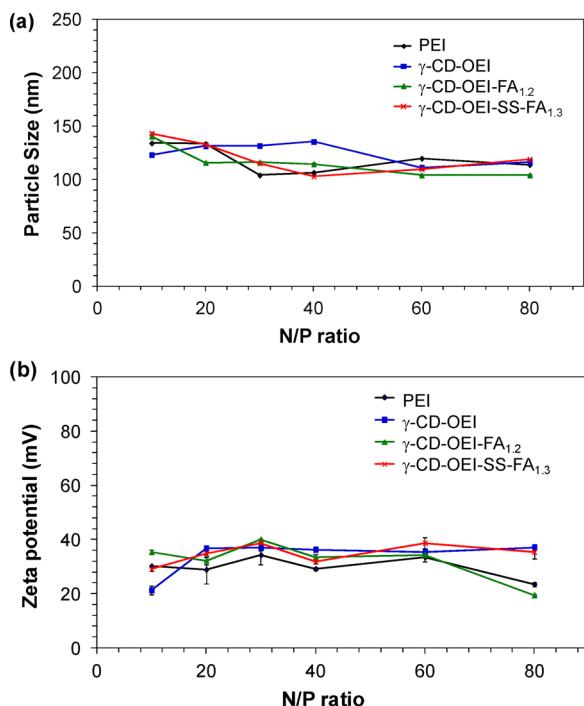


Figure 5. Particle size (a) and zeta potential (b) of the pDNA polyplexes with PEI (25 kDa), γ -CD-OEI, γ -CD-OEI-FA_{1,2}, and γ -CD-OEI-SS-FA_{1,3}, respectively, at various N/P ratios.

measurements of the pDNA complexes with PEI, γ -CD-OEI, γ -CD-OEI-FA_{1,2}, and γ -CD-OEI-SS-FA_{1,3} at various N/P ratios. Figure 5a shows that all the three cationic polymers efficiently compact pDNA into small nanoparticles with particle sizes around 100–150 nm, which is suitable for cellular uptake through endocytosis for gene delivery application.¹ The surface charge of these complexes is around 20–40 mV in Figure 5b. Unlike the negative DNA, positive charged polymer/DNA complexes are easier to bind to cell membrane and endocytosis would be one of the possible mechanisms of cellular uptake.

3.3. Cytotoxicity. Cytotoxicity is an important factor that must be considered for gene delivery materials. Kissel et al. reported that the aggregation and adherence of PEI on the cell surface is the main reason for the increased toxicity of PEI.³³ Our previous studies showed that the introduction of CDs can reduce the toxicity of PEI or OEI.^{10,11} Figure 6 shows the cell cytotoxicity of γ -CD-OEI, γ -CD-OEI-FA_{1,2}, and γ -CD-OEI-SS-FA_{1,3} compared to PEI (25 kDa) in the absence (Figure 6a) and presence (Figure 6b) of FA in the culture medium. All the synthesized cationic polymers showed lower cytotoxicity than PEI (25 kDa). Since the cytotoxicity is mostly derived from the OEI chains, modification of γ -CD-OEI by FA and disulfide bond does not significantly change the cytotoxicity. In both the absence and presence of FA in the culture medium, the introduction of disulfide linker resulted in slight decrease of cytotoxicity (Figure 6a,b). The addition of FA in the cell culture medium slightly decreased the cytotoxicity of all the samples (Figure 6b). The possible reason is that FA benefits cell growth of the control group (cells without polymers).³⁴

3.4. In Vitro Gene Transfection. In vitro gene transfection efficiency was assessed using luciferase as a marker gene in FR-

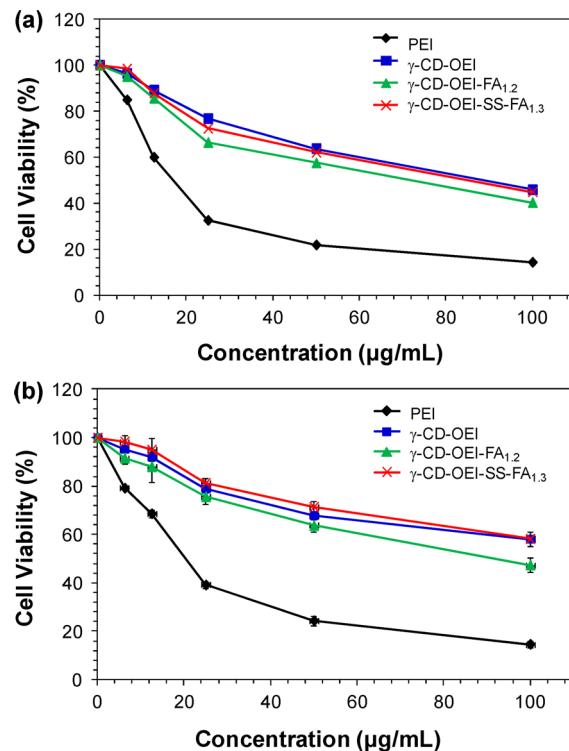


Figure 6. Cell viability assay in KB cell line. The cells were treated with PEI (25 kDa), γ -CD-OEI, γ -CD-OEI-FA_{1,2}, and γ -CD-OEI-SS-FA_{1,3} at various concentrations for 24 h in the absence (a) and presence (b) of FA (0.001 g/L) in RPMI 1640 medium. Data represent mean \pm standard deviation ($n = 5$).

positive KB cells and FR-negative A549 cells. We first investigated the influence of the grafting amount of the targeting group FA on the gene delivery ability of γ -CD-OEI-SS-FA in FR-positive KB cells in RPMI 1640 medium (Figure 7). The RPMI 1640 medium does not contain free FA, so the

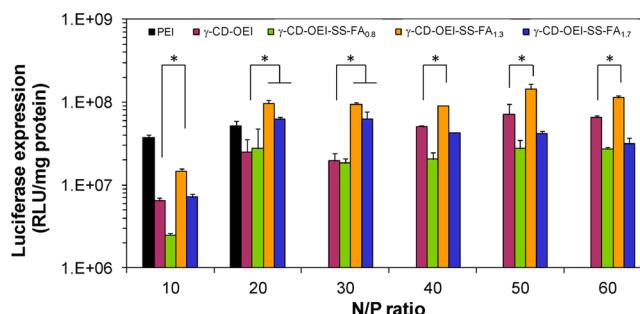


Figure 7. In vitro gene transfection efficiency of the pDNA polyplexes with PEI (25 kDa), γ -CD-OEI, γ -CD-OEI-SS-FA_{0,8}, γ -CD-OEI-SS-FA_{1,3}, and γ -CD-OEI-SS-FA_{1,7} in KB cells in RPMI 1640 medium in the absence of FA. Data represent mean \pm standard deviation (* $P < 0.05$, $n = 4$).

FA ligand attached to γ -CD-OEI-SS-FA can facilitate the FR-mediated endocytosis. γ -CD-OEI-SS-FA_{1,3} showed the highest gene transfection ability among all three γ -CD-OEI-SS-FA samples, namely, γ -CD-OEI-SS-FA_{0,8}, γ -CD-OEI-SS-FA_{1,3}, and γ -CD-OEI-SS-FA_{1,7}. The gene transfection efficiency of γ -CD-OEI-SS-FA_{1,3} was also higher than that of PEI (25 kDa) and γ -CD-OEI. Therefore, the grafting ratio of FA in γ -CD-OEI-SS-

FA_{1,3} is considered optimized, and γ -CD-OEI-SS-FA_{1,3} was used in all the other experiments in this work.

Figure 8 shows the gene transfection efficiency of γ -CD-OEI-SS-FA_{1,3} in KB and A549 cells in comparison with PEI (25 kDa).

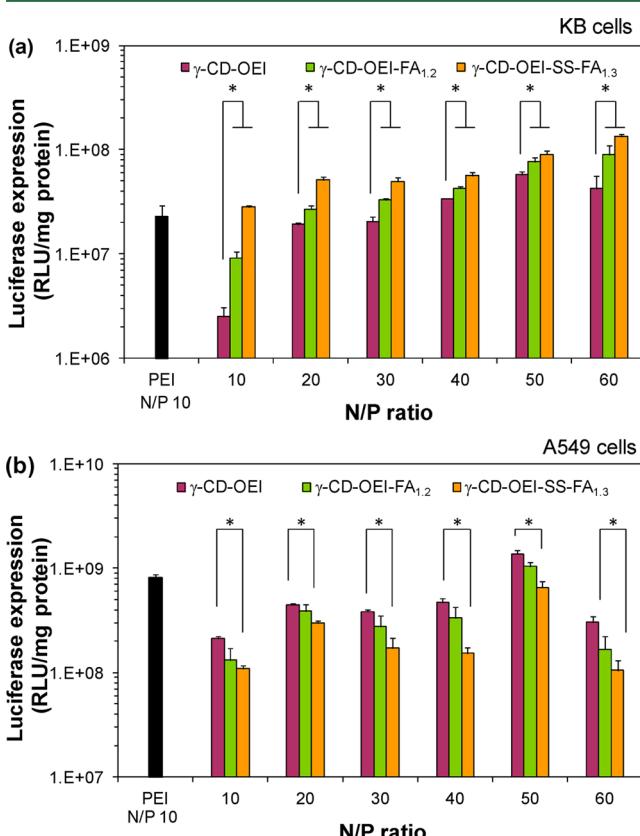


Figure 8. In vitro gene transfection efficiency of the pDNA polyplexes with PEI (25 kDa), γ -CD-OEI, γ -CD-OEI-FA_{1,2}, and γ -CD-OEI-SS-FA_{1,3} in KB cells (a) and A549 cells (b) in RPMI 1640 medium in the absence of FA. Data represent mean \pm standard deviation (* $P < 0.05$, $n = 4$).

kDa), γ -CD-OEI, and γ -CD-OEI-FA_{1,2} in RPMI 1640 medium (without FA in the medium). In FR-positive KB cells (Figure 8a), the gene transfection efficiency constantly followed the order γ -CD-OEI-SS-FA_{1,3} > γ -CD-OEI-FA_{1,2} > γ -CD-OEI. The gene transfection efficiency of γ -CD-OEI-SS-FA_{1,3} was also constantly higher than that of PEI (25 kDa). The results indicate that FA-grafted polymers significantly increased the gene transfection efficiency due to the FR-mediated cellular uptake. When comparing γ -CD-OEI-SS-FA_{1,3} and γ -CD-OEI-FA_{1,2}, the only difference is that the former had a disulfide link between FA and γ -CD-OEI, and the disulfide link could be cleaved by GSH within the cells and then FA could be released (Figure 3). Therefore, γ -CD-OEI-SS-FA_{1,3} could not only efficiently deliver pDNA into FR-positive cells through FR-mediated cellular uptake, but also cleave FA from the carrier to release FR within the cells, and then recover FR onto the cellular membrane to promote continuous FR-mediated cellular uptake of pDNA carried by γ -CD-OEI-SS-FA_{1,3}. This is the reason that the transfection efficiency of γ -CD-OEI-SS-FA_{1,3} was constantly higher than that of γ -CD-OEI-FA_{1,2}. At N/P ratio of 20 to 60, in general, the gene transfection efficiency of γ -CD-OEI-FA_{1,2} was 1–2-fold higher than that of γ -CD-OEI, while γ -CD-OEI-SS-FA_{1,3} was 2–4-fold higher than that of γ -

CD-OEI, indicating that the significant improvement of the gene delivery efficiency was caused by the FA-conjugation as well as the incorporation of the disulfide bond. However, in FR-negative A549 cells (Figure 8b), FR-mediated cellular uptake was impossible, and the FA-conjugation showed no positive effect on the gene transfection efficiency. Inversely, the gene transfection efficiency constantly followed the order γ -CD-OEI > γ -CD-OEI-FA_{1,2} > γ -CD-OEI-SS-FA_{1,3}, probably due to that the grafted FA interfered the normal endocytosis of the DNA polyplexes.

To further demonstrate the targeted effect of FA-grafted carriers, competition tests were carried out by addition of free FA at different concentrations (0.001, 0.010, and 0.100 g/L) to the cell culture medium during the gene transfection. As shown in Figure 9a, the gene transfection efficiency of both γ -CD-OEI-

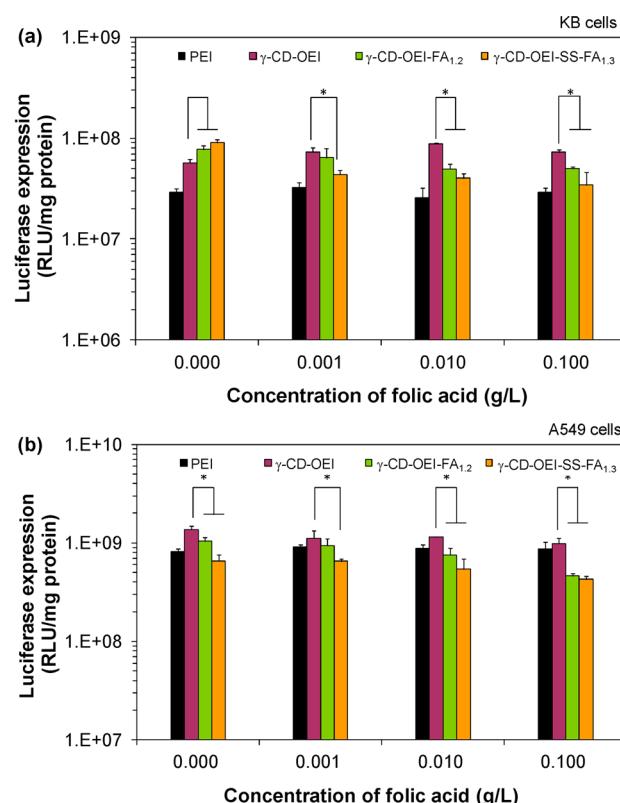


Figure 9. In vitro gene transfection efficiency of the pDNA polyplexes with γ -CD-OEI, γ -CD-OEI-FA_{1,2}, and γ -CD-OEI-SS-FA_{1,3} at a N/P ratio of 50 in comparison with pDNA polyplexes with PEI (25 kDa) at a N/P ratio of 10 in KB (a) and A549 (b) cells in RPMI 1640 medium treated with FA at different concentrations (0.000, 0.001, 0.010, and 0.100 g/L). Data represent mean \pm standard deviation (* $P < 0.05$, $n = 4$).

FA_{1,2} and γ -CD-OEI-SS-FA_{1,3} decreased with an increase in free FA concentration in FR-positive KB cells. In contrast, in Figure 9b, there were no significant changes in gene transfection efficiency for all carriers no matter whether there was FA conjugation or not in FR-negative A549 cells.

Sufficient FRs on the surface of cancer cells are a prerequisite to effect FR-mediated endocytosis.¹⁶ Therefore, the continuous recovering and recycling of FRs after cellular endocytosis is necessary for FA-targeted delivery. The bioreducible disulfide bond was used in our gene carrier to recover and recycle the FRs. Figure 10 demonstrates the results of the inhibitory test of disulfide bond using BSO, which can reduce the intracellular

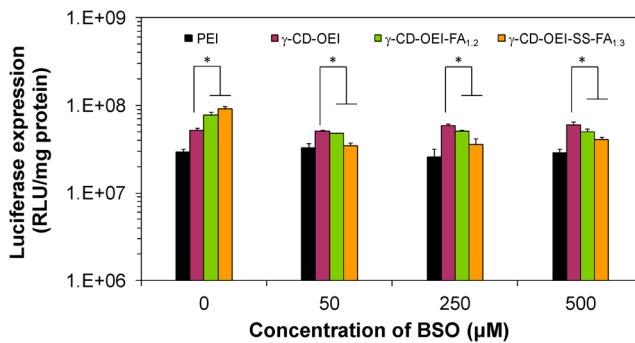


Figure 10. In vitro gene transfection efficiency of the pDNA polyplexes with PEI (25 kDa) at a N/P ratio of 10 and pDNA polyplex with γ -CD-OEI, γ -CD-OEI-FA_{1,2}, and γ -CD-OEI-SS-FA_{1,3} at a N/P ratio of 50 in KB cells in RPMI 1640 medium treated with BSO at different concentrations (0, 50, 250, and 500 μM). Data represent mean \pm standard deviation (* $P < 0.05$, $n = 4$).

glutathione concentration,^{35,36} resulting in inhibition of the cleavage of the disulfide bond. The transfection efficiency of γ -CD-OEI-SS-FA_{1,3} was significantly affected by the addition of BSO. It was 2-fold higher than that of γ -CD-OEI in the absence of BSO, but lower than that of γ -CD-OEI when BSO was used to inhibit the recovering and recycling of FRs in the FR-positive KB cells. The results further support our hypothesis that the disulfide bond plays an important role in this targeted delivery system. The FA-targeted delivery system with incorporation of disulfide bond is much superior to previous systems.¹¹

Finally, plasmid pEGFP-N1 encoding GFP was used to evaluate the GFP expression in KB cells. As shown in Figure 11, cells with strong green fluorescence were observed for EGFP polyplexes with γ -CD-OEI-SS-FA_{1,3}, which is significantly more than those of PEI (25 kDa), γ -CD-OEI, and γ -CD-OEI-FA_{1,2}. This result further confirms that the gene delivery was enhanced by the FA-targeting moiety as well as the disulfide bond that links FA with the star-shaped cationic polymer.

4. CONCLUSIONS

This work has designed and synthesized a new star-shaped cationic polymer containing a γ -cyclodextrin (γ -CD) core and multiple oligoethylenimine (OEI) arms with folic acid (FA) linked by a bioreducible disulfide bond for efficient targeted gene delivery. Three samples of the star-shaped cationic γ -CD-OEI-SS-FA polymers were prepared with ratios of FA to γ -CD-OEI of 0.8, 1.3, and 1.7. The γ -CD-OEI-SS-FA polymers could be cleaved efficiently and FA was readily released under reductive condition similar to intracellular environment. The γ -CD-OEI-SS-FA polymers was characterized and studied in terms of its gene delivery properties in FR-positive KB cells and FR-negative A549 cells under various conditions, in comparison with cationic polymers such as PEI (25 kDa), γ -CD-OEI star-shaped cationic polymer, γ -CD-OEI-FA polymer where FA was directly linked to the star polymer without disulfide linker. The γ -CD-OEI-SS-FA polymers showed similar cytotoxicity to γ -CD-OEI and γ -CD-OEI-FA polymers, and much lower cytotoxicity than PEI (25 kDa). The γ -CD-OEI-SS-FA polymers showed good DNA condensation ability and could retard pDNA at a N/P ratio of 2.5, which is similar to all comparison cationic polymers used in this study. The γ -CD-OEI-SS-FA polymers formed DNA nanoparticles of sizes ranging from 100 to 150 nm with positive zeta potential ranging from 20 to 40 mV at N/P ratios of 10 to 60.

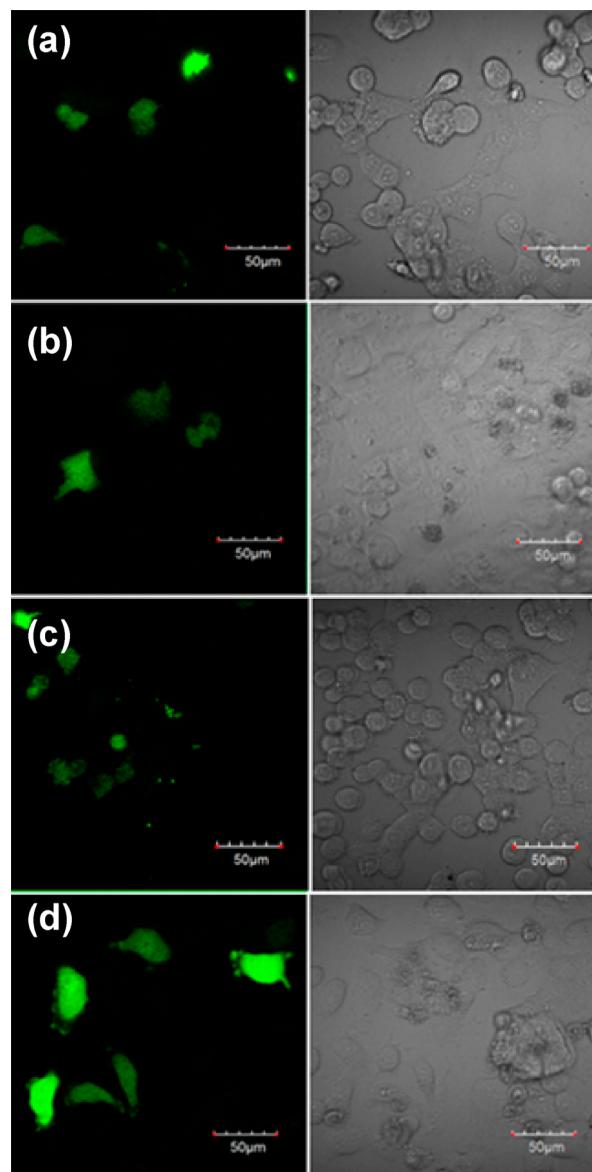


Figure 11. Confocal microscopy images of transfected KB cells by the EGFP polyplexes with PEI (25 kDa) (a), γ -CD-OEI (b), γ -CD-OEI-FA_{1,2} (c), and γ -CD-OEI-SS-FA_{1,3} (d) in RPMI 1640 medium in the absence of FA. N/P ratio was 10 (a) and 50 (b–d). For each experiment, the same field of cells was observed by fluorescent (left) and bright (right) fields to visualize GFP expression.

It was found that γ -CD-OEI-SS-FA_{1,3} with the FA to γ -CD-OEI ratio of 1.3 was the optimized composition for targeted gene delivery in FR-positive KB cells. The gene transfection efficiency of γ -CD-OEI-SS-FA_{1,3} was up to 6-fold higher than that of PEI (25 kDa), 2–4-fold higher than that of γ -CD-OEI, and 2-fold higher than that of γ -CD-OEI-FA_{1,2} in FR-positive KB cells. The disulfide linker of γ -CD-OEI-SS-FA_{1,3} could be cleaved by GSH within the cells. Therefore, γ -CD-OEI-SS-FA_{1,3} could not only efficiently deliver pDNA into FR-positive cells through FR-mediated cellular uptake, but also cleave FA from the carrier to release FR within the cells, and then recover FR onto cellular membrane to promote continuous FR-mediated cellular uptake of pDNA, to achieve very high levels of gene expression. FA competition and disulfide inhibitory tests demonstrated that the significantly enhanced gene expression was induced by the targeted effect of FA and the FR recovery

and recycling facilitated by disulfide linker in FR-positive KB cells. Moreover, in FR-negative A549 cells, all the effects due to FA-conjugation and disulfide bond were not observed.

This study has expanded the strategy of FA-targeted delivery by combining the smart FR-recycling function to achieve the significant enhancement of gene expression. The new FA-targeted and bioreducible carrier may be a promising efficient gene delivery system for potential cancer gene therapy.

■ ASSOCIATED CONTENT

Supporting Information

Additional ^1H and ^{13}C NMR spectra. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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