Supplementary Information for

Redox-responsive cationic supramolecular polymer constructed from small molecules as a promising gene vector

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1. Materials

Tetraethylenepentamine (TEPA) (95%, Shanghai Aladdin Reagent Co. Ltd.), ferrocenecarboxaldehyde (98%, Shanghai Aladdin Reagent Co. Ltd.), sodium borohydride (NaBH₄) (96%, Shanghai Sinopharm Chemical Reagent Co. Ltd.), 1,1,4,7,7-pentamethyldiethylenetriamine (PMDETA) (98%, Alfa Aesar), 1,7-octadiyne (98%, Shanghai Aladdin Reagent Co. Ltd.), branched polyethyleneimine (PEI) (99%, Mₜ = 10 kDa, Shanghai Aladdin Reagent Co. Ltd.), glutathione (GSH) (≥98%, Sigma-Aldrich), hydrogen peroxide (H₂O₂) (30 wt%, Shanghai Sinopharm Chemical Reagent Co. Ltd.), sodium sulfate (Na₂SO₄) (≥99%, Shanghai Sinopharm Chemical Reagent Co. Ltd.), sodium hydroxide (NaOH) (99%, Shanghai Sinopharm Chemical Reagent Co. Ltd.), hydrochloric acid (HCl) (37%, Shanghai Sinopharm Chemical Reagent Co. Ltd.), were used as received without further purification. β-Cyclodextrin (β-CD) and its derivatives (Shanghai Sinopharm Chemical Reagent Co. Ltd.) were dried for 48 h at 60 °C in vacuum oven before use. Copper(I) bromide (CuBr) (98%, Aldrich) was firstly purified by stirring with acetic acid for several hours, filtrating, and then washing with acetic acid, ethanol and diethyl ether in succession until it turned into white, finally stored under vacuum before use. Methanol (MeOH) from Shanghai Sinopharm Chemical Reagent Co. Ltd. was treated with dry molecular sieve and distilled before use. Dimethyl formamide (DMF) from Shanghai Sinopharm Chemical Reagent Co. Ltd. was treated with calcium hydride and distilled before use. Acetic acid, ethanol, diethyl ether and dichloromethane (CH₂Cl₂) etc. were from Shanghai Sinopharm Chemical Reagent Co., Ltd, and distilled water was used as received.

2. Instruments and Measurements

Nuclear Magnetic Resonance (NMR)

¹H NMR spectra were recorded on a Varian Mercury plus 400 NMR spectrometer.
(400 MHz) with deuterated dimethyl sulfoxide (DMSO-$d_6$) and deuterated chloroform (CDCl$_3$) as solvents at 293 K. The chemical shifts were referenced to residual peaks of deuterated solvents: DMSO-$d_6$ (2.48 ppm), CDCl$_3$ (7.26 ppm).

**Ultra Performance Liquid & Quadrupole-Time-of-Flight Mass Spectrometer (UMS) (UPLC & Q-TOF-MS)**

UPLC & Q-TOF-MS measurement was performed on a Waters-ACQUITY™ UPLC & Q-TOF-MS Premier (Waters Corporation, USA) at room temperature with deionized water ($\beta$-CD dimer) and methanol (ferrocene dimer) as the solvents.

**Dynamic Light Scattering (DLS)**

DLS measurement was performed on a Malvern Zetasizer NanoS apparatus equipped with a 4.0 mW laser operating at $\lambda = 633$ nm. All samples were measured at a scattering angle of 90° at 25 °C. Before testing, the dust was firstly eliminated by filtering the sample solutions through some absorbent cotton. The concentrations of sample solutions were 0.5, 1.0, 2.5, 5.0, 10, 15 and 20 mM, respectively. The sample solution (10 mM) with the addition of H$_2$O$_2$ or GSH was placed in the cell for at least 15 min prior to the measurement to allow for thermal equilibration and chemical equilibration.

**UV-Vis Absorption Spectra**

The UV-Vis absorption of the sample solutions was measured at room temperature by using a Thermo Electron-EV300 UV-Vis spectrophotometer. The slit-width was set as 1 nm, and scan speed was set as 480 nm/min.

**Zeta Potential Measurement**

The zeta ($\zeta$) potential of CSP in PBS buffer was measured using a Malvern Zetasizer
NanoS at 25 °C. The cuvettes were filled with the CSP solution, and the measurement was performed in the ζ-model for a minimum of 10 cycles and a maximum of 100 cycles.

**Atomic Force Microscopy (AFM)**

The polyplex morphology was visualized using an atomic force microscopy (AFM) system with the Dimension 3100 model with a Nanoscope IIIa controller (Veeco, Santa Barbara, CA). 40 μL of CSP/pDNA complexes in deionized water (pH 7.4) containing approximate 0.08 μg of pDNA at various N/P ratios (5 and 20) were dropped onto freshly cleaved mica sheets for 5 min. The excess solution was removed with a piece of filter paper, and the samples were dried naturally in air at room temperature for 24 h. The samples were imaged using the tapping mode with setting of 256 × 256 pixels. Image analysis was performed using Nanoscope software after removing the background slope by flattening images.

**Agarose Gel Electrophoresis**

The TEPA/pDNA and CSP/pDNA complexes with various N/P ratios were prepared by adding different volumes of TEPA solutions or CSP solutions to pDNA solutions in PBS buffer containing 0.4 μg pDNA, followed by vortexing for 6 s and incubated for 30 min at room temperature. After mixing 5 μL of 0.5 × loading buffer with polyplex solutions, the resulting polyplex solution was analyzed on 1% agarose gel containing 0.5 μg/mL ethidium bromide. Gel electrophoresis was carried out in 0.5 × Tris-Borate-EDTA (TBE) buffer at 100 V for 1 h in a Sub-Cell system (Bio-Rad Laboratories, CA). DNA bands were visualized by a UV lamp using a Gel Doc system (Synoptics Ltd., UK).

**Cell Cultures**

COS-7 cells, HeLa cancer cells and MCF-7 breast cancer cells were cultured in
DMEM (4.5 g/L glucose) supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/mL), and streptomycin (100 μg/mL) at 37 °C under a 5% CO₂ humidified atmosphere. Confluent cells were subcultured every 3 days using standard procedure.

**Cell Viability**

For MTT assay, COS-7 cells were seeded into 96-well plates at a seeding density of 5000 cells/well in 200 μL medium. After 24 h incubation, the culture medium was removed and replaced with 200 μL of medium containing 50 μL of PEI solutions or CSP solutions with a series of concentrations (0.001, 0.005, 0.01, 0.05, 0.1, 0.5 and 1.0 mg/mL). The cells were grown for 48 h. Then, 20 μL of 5 mg/mL MTT assays stock solution in PBS buffer was added to each well. After the cells were incubated for 4 h, the medium containing unreacted dye was carefully removed. The obtained blue formazan crystals were dissolved in 200 μL of DMSO, and the absorbance was measured in a Perkin-Elmer 1420 Multi-label counter at a wavelength of 490 nm.

**In Vitro Transfection Assay**

For luciferase expression studies, a series of cells (COS-7 cells, HeLa cancer cells and MCF-7 cancer cells) were seeded at a density of 10⁴ cells per well in 96-well plates and incubated for 16-24 h until 60-70% confluent at 37 °C and 5% CO₂. Immediately prior to transfection, the medium was removed. Then, these cells were washed and replaced with fresh and prewarmed DMEM in the absence of 10% FBS. Polyplexes were added to each well, and these cells were incubated at 37 °C for 4 h. The medium was then replaced with fresh DMEM supplemented with 10% FBS, 10% FBS/0.05 mM H₂O₂ or 10% FBS/0.10 mM H₂O₂, and then incubated for an additional 48 h. The luciferase assay was carried out according to manufacturer’s protocol (Promega, Madison, WI). Relative light units (RLUs) were measured with GloMaxTM 96 microplate luminometer (Promega). The obtained RLUs were normalized with respect to protein concentration in the cell extract determined.
using the BCA protein assay kit (Beyotime, China).

3. Synthesis Details

3.1 Synthesis of β-CD dimer

Mono-(6-azido-6-desoxy)-β-cyclodextrin (β-CD-N$_3$) was prepared according to our previous literature.$^{[1]}$ The reaction between 1,7-octadiyne (0.106 g, 1.00 mmol) and β-CD-N$_3$ (2.9 g, 2.5 mmol) was conducted with 20 mL anhydrous $N,N$-dimethylformamide (DMF) by adding catalyst CuBr (0.287 g, 2.0 mmol) and PMDETA (420 μL, 2.0 mmol). Under the protection of nitrogen, the reaction was carried out at 70 °C with vigorous stirring for 48 h. The reaction was terminated by exposing the reaction solution to ambient air for 1 h with stirring. The resulting solution was concentrated to 20 mL and recrystallized in methanol twice to obtain the white solid. The crude product was dissolved in 30 mL deionized water, enclosed in dialysis membrane (MWCO = 2.0 kDa), and then purified by dialyzing in deionized water for 48 h to remove
the excess $\beta$-CD-N$_3$. After removal of the water by freeze drying, a white powder was obtained (1.0 g, yield: 41.2%).

$^1$H NMR (DMSO-$d_6$, 400 MHz) (Fig. S1): $\delta_H$ (ppm) = 1.64 (s, c-$H$ on alkyl, 4H), 2.60 (s, b-$H$ on alkyl, 4H), 2.89 (s, 4'-$H$ on $\beta$-CD, 2H), 3.07 (s, 2'-$H$ on $\beta$-CD, 2H), 3.30 (m, 2,4-$H$ on $\beta$-CD, 24H), 3.62 (m, 3,5,6-$H$ on $\beta$-CD, 52H), 3.96 (s, 6'-$H$ on $\beta$-CD, 4H), 4.50 (m, 6-OH on $\beta$-CD, 12H), 4.81 (m, 1-$H$ on $\beta$-CD, 12H), 5.02 (s, 1'-$H$ on $\beta$-CD, 2H), 5.71 (m, 2,3-OH on $\beta$-CD, 28H), 7.75 (s, $H$ on triazole, 2H). UPLC & Q-TOF-MS of $\beta$-CD dimer (Fig. S2): calculated for [M + H]$^{2+}$: 1213.58, found m/z: 1213.9163 [M + H]$^{2+}$.

Fig. S1. $^1$H NMR spectrum of $\beta$-CD dimer in DMSO-$d_6$. 
3.2 Synthesis of ferrocene dimer

![Diagram of ferrocene dimer synthesis](image)

**Scheme S2.** Synthesis route of ferrocene dimer.

The ferrocene dimer was prepared as follows. Tetraethylenepentamine (757 mg, 4.0...
mmol) and ferrocenecarboxaldehyde (2.568 g, 12 mmol) were dissolved in 60 mL of anhydrous methanol, and then degassed and filled with nitrogen. The reaction mixture was placed in an oil bath preheated at 70 °C for 24 h. After the reaction solution was cooled to 0 °C in an ice-water bath, 1.362 g (36 mmol) of NaBH₄ was slowly added to the above solution for about 30 min. Subsequently, the reaction mixture was left to react for another 12 h at room temperature under stirring. With the addition of 4 mL of 3 M HCl aqueous solution, the reaction system was continued to react for 2 h. After evaporation of methanol under reduced pressure, the crude product was purified with silica gel column chromatography (CH₂Cl₂-methanol). Finally, the product was collected as red-brown oil. Yield: 1.8 g (77%).

¹H NMR (CDCl₃, 400 MHz) (Fig. S3): δ_H (ppm) = 2.74 (m, -NHCH₂CH₂NH-, 16H), 3.48 (m, Ferrocene-CH₂-NH-, 4H), 4.14 (m, H on Ferrocene, 18H). UPLC & Q-TOF-MS of ferrocene dimer (Fig. S4): calculated for [M + H]⁺: 586.38, found m/z: 586.2312 [M + H]⁺.

![Fig. S3. ¹H NMR spectrum of ferrocene dimer in CDCl₃.](image-url)
4. Preparation of Cationic Supramolecular Polymer

Different amounts of equimolar β-CD dimer and ferrocene dimer were firstly dissolved in deionized water by ultrasonication for about 5 min and then stirred at room temperature for an additional 24 h. Subsequently, the supramolecular oligomer or polymer was obtained.

5. Supplemented Figures

5.1. Determination of association constant for Fc/β-CD system
**Fig. S5.** The UV absorption of ferrocenecarboxaldehyde upon stepwise addition of excess β-CD in DMF/H₂O (v/v, 1/1). The concentration of ferrocenecarboxaldehyde was 1 × 10⁻⁵ M.

As shown in **Fig. S5**, the association constant between ferrocenecarboxaldehyde and β-CD in DMF/H₂O (v/v, 1/1) solution was determined by following the UV absorptions at 314 nm. The concentration of ferrocenecarboxaldehyde was set at 1 × 10⁻⁵ M. With the addition of β-CD, the absorption of ferrocenecarboxaldehyde gradually enhanced. Since β-CD can exactly form a 1:1 inclusion complex with ferrocene,[²] the inclusion complexation of β-CD (H) with ferrocenecarboxaldehyde (G) is expressed by the following equation:

\[
H + G \rightleftharpoons \text{H} \cdot \text{G}
\]

We employed the usual double reciprocal plot according to the modified Hidebrand-Benesi equation:

\[
\frac{1}{\Delta A} = \frac{1}{K_a \Delta \varepsilon [H][G]} + \frac{1}{\Delta \varepsilon [G]}
\]

Where H, G, Kᵦ represent host (β-CD), guest (ferrocenecarboxaldehyde) and association constant, respectively. ΔA denotes the absorbance difference before and after host molecules are added. Δε, which denotes the difference of the molar extinction coefficient.
between the host and host-guest complex at the same wavelength, is $2.13 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$ for Fc/β-CD system. The association constant $K_a$ is calculated by the equation:

\[
K_a = \frac{b}{k} = 2.95 \times 10^4 \text{ M}^{-1}
\]

Where $k$ is the slope value of the line plot, and $b$ is the intercept of the line plot.

5.2. Supramolecular complexes formation between β-CD₂ and Fc₂

![Figure S6](#)

**Fig. S6.** UV-Vis spectra of β-CD₂ and Fc₂ at different molar ratios of 8:1, 5:1, 3:1, 1:1 and 0:1. The concentration of Fc₂ was 0.5 mM.

As shown in **Fig. S6**, the UV-Vis spectroscopy was utilized to confirm the monomeric association effect. The aqueous solution of Fc₂ (0.5 mM) displays a diagnostic absorption of ferrocenyln group at 428 nm. The addition of β-CD₂ induces a 3.55-fold increase of Fc₂ absorption, indicating the formation of Fc/β-CD inclusion complexes.

5.3. Determination of zeta potential of cationic supramolecular
Fig. S7. Zeta potential of cationic supramolecular polymer in PBS buffer as a function of molar concentration.

5.4. Determination of mobility of cationic supramolecular polymer

Fig. S8. Mobility of cationic supramolecular polymer in PBS buffer as a function of molar concentration.

5.5. Determination of conductivity of cationic supramolecular polymer
Fig. S9. Conductivity of cationic supramolecular polymers in PBS buffer as a function of molar concentration.

5.6. \( p \)DNA condensation capacity by TEPA

Fig. S10. Agarose gel electrophoresis retardation of \( p \)DNA by TEPA at various N/P ratios of 1, 2, 3, 5, 10, 15 and 20.

In Fig. S10, even though the N/P ratio of TEPA/\( p \)DNA polyplex is up to 20, \( p \)DNA can migrate through agarose gel at roughly the same rate as naked \( p \)DNA. This result indicates that \( p \)DNA cannot be condensed efficiently by the small molecule of TEPA.
5.7. Cell viability of cationic supramolecular polymer

![Cell Viability Chart](image)

**Fig. S11.** Cell viability assay of cationic supramolecular polymer as well as branched PEI (10 kDa) incubated in COS-7 cells for 48 h. Black bars represent the mean values ($n = 5$).

As shown in Fig. S11, when the sample concentration is lower than 0.01 mg/mL, the cell viability of branched PEI is 97.5% for 0.001 mg/mL as well as 98.1% for 0.005 mg/mL, while that of CSP is 101.4% for 0.001 mg/mL as well as 96.1% for 0.005 mg/mL. Obviously, there is no significant difference for the cell viability values of branched PEI and CSP at lower sample concentration. The results indicate that both branched PEI and CSP with lower sample concentration exhibits much lower cytotoxicity in COS-7 cells.

However, when the sample concentration is 0.05 mg/mL, the cell viability of branched PEI incubated in COS-7 cells for 48 h falls below 10%, whereas that of cationic supramolecular polymer is more than 70%. Furthermore, the cell viability of cationic supramolecular polymer is still more than 30% while the concentration is up to 1.0 mg/mL.
Therefore, compared to commercially available 10 kDa branched PEI, the cationic supramolecular polymer exhibits very low cytotoxicity in COS-7 cells.

6. References
