Construction of Traceable Cucurbit[7]uril-based Virus-Mimicking Quaternary Complexes with Aggregation-Induced Emission for Efficient Gene Transfection

*De-E Liu, Xiangjie Yan, Jinxia An, Jianbiao Ma, Hui Gao**

School of Material Science and Engineering, School of Chemistry and Chemical Engineering, Tianjin Key Laboratory of Organic Solar Cells and Photochemical Conversion, Tianjin University of Technology, Tianjin 300384 (P. R. China)

E-mail: ghhigher@hotmail.com, hgao@tjut.edu.cn (H. Gao)

Materials and instrumentals.

4,4'-Dimethoxybenzophenone and ethyl bromoacetate were purchased from Heowns Biochem Technologies LLC (Tianjin, China). Brorontribromide (BBr_3) was purchased from Chemical Technology Co. Ltd. (Shanghai, China). Glycidyle methacrylate was purchased from Polysciences Inc. (Oakville, ON, Canada). Polyethylenimine [M_w: 25 kDa, branched] and Cucurbit[7]uril (CB[7]) were purchased from Sigma-Aldrich (Shanghai, China). Luciferase assay kit was obtained from Promega (Madison, USA). BCA Protein Assay Reagent Kit was purchased from Pierce (Madison, USA). Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan) was employed in cytotoxicity assay. All other chemical reagents or solvents were purchased from Tianjin Chemical Reagent Co. Ltd. (Tianjin, China).

^{1}H-NMR spectra of TPE derivatives and the polymers were recorded by a Bruker AV-400 spectrometer (400 MHz, Bruker, Freemont, CA). $CDCl_3$, CD_3OD, DMSO-d_6 or D_2O was used as solvents. Chemical shifts were evaluated as parts per million, ppm (δ). Gel permeation chromatography (GPC) measurements were performed using the ViscoGEL C-Series columns (C-MBLMW-3078) from Malvern and 5% aqueous acetic acid medium as a mobile phase at a flow rate of 0.7 mL min^{-1} at 35 °C. Molecular weights were calibrated with PolyCALTM Dextran Std-T68K (Worcestershire ER14 1XZ, UK) as standards. The nitrogen content of PG was determined using an elemental analyzer (Vario EL cube CHNOS, Germany).

Synthesis of TPE.

Synthetic routes of TPE-$(COOH)_4$ are shown in Scheme S1 according to our previous literature report.[S1] Briefly, Zn dust (21.24 g, 324.8 mmol) was added to 80 mL of dry THF under nitrogen (N_2) atmosphere to form a suspension. $TiCl_4$ (5.4 mL, 49.8 mmol) was slowly added to the above suspension at -10 °C. The mixture was stirred for 30 min at room temperature and heated to 74 °C to continue stirring 2 h. After the reaction solution cooled down to -10 °C, 2.4 mL of anhydrous pyridine was added for another 10 min reaction. Then, 4,4'-dimethoxybenzophenone (8.04 g, 33.2 mmol) in 80 mL anhydrous THF was added to the solution. After refluxing for 16 h, the reaction mixture was cooled down to room temperature and filtered. The filtrate was evaporated under vacuum and the crude product was purified by column chromatography. Finally, compound 1 was obtained as a white solid in 89% yield (6.7 g). Furthermore, compound 1 (3 g, 6.6 mmol) dissolved in 40 mL DCM was cooled to -78 °C. BBr_3 (6.0 mL, 63.6 mmol) was then added under magnetic stirring. The reaction mixture was allowed to warm to room temperature and stirred overnight. The reaction product was hydrolyzed by shaking in 10 mL of water. The crude product was purified by recrystallization in THF/methanol. The compound 2 was obtained as a purple solid (6.7 g, yield 89.0%). Then, compound 2 (0.5 g, 1.26 mmol) and K_2CO_3 (1.4 g, 10.1 mmol) were suspended in 40 mL THF, followed by addition of ethyl bromoacetate (2.11 g, 12.62 mmol). After stirring 72 h at 65 °C under N_2 atmosphere, the crude product was collected by extraction and purified by chromatography. The compound

3 was obtained as a yellow solid (0.64 g, 68%). Finally, a mixture of compound 3 (0.5 g, 0.46 mmol) and NaOH (0.54 g, 13.5 mmol) was dissolved in 40 mL of THF/H_2O (1:1) mixture and then stirred for 4 h under reflux. After that, the mixture was rotary evaporated and mixed with 20 mL of H_2O, acidified pH to 2 *via* gradual addition of 37% HCl, and followed with filtration. The TPE-$(COOH)_4$ was obtained (0.34 g, 82%) and confirmed by ^{1}H-NMR (Figure S1). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 6.77-6.87 (d, 8H; Ar H), 6.61-6.68 (d, 8H; Ar H), 4.5 (s, 8H; -CH_2-). We will use TPE to represent TPE-$(COOH)_4$ in the following text.

Synthesis of PG.

According to our previous literature reports,[S2-S3] glycidyl methacrylate (GMA) was polymerized in THF using 2-bromoisobutyryl bromide as atom transfer radical polymerization initiator. In a typical procedure, a 1000-mL round-bottom flask was loaded with ATRP initiator (1 equ), bipyridyl (1.5 equ) and GMA (105 equ). THF was then added ([monomer] = 30 M), followed by Cu(I)Br (1 equ). The mixture was degassed and kept under inert atmosphere throughout the reaction. After 10 min at room temperature, it was heated to 90 ^{o}C, and the reaction was run for 24 h. After cooling down, the mixture was passed through a silica gel column with THF as an eluent to remove copper. After the solvent was evaporated, the product was dissolved in a small volume of THF and precipitated twice in diethyl ether. The polymer PGMA was further purified by Soxhlet extraction using diethyl ether, and dried. PGMA was then dissolved in acetonitrile at a

concentration of 12.5 g/L, followed by addition of excess amine (amine/epoxy group 20:1 molar ratio) to ensure the completion of the reaction. The reaction mixture was refluxed at 90 °C overnight under argon atmosphere. The solution was then cooled down, and the products were purified by dialysis (Spectra/Por RC, cut-off 15000) against water for 48 h. The ultimate product PG was obtained by freeze-dried (yields: 90%).

Preparation of quaternary polyplex PG/CB/TPE/DNA.

Typically, PG (1 mg/mL) and CB[7] (3 mg/mL) were respectively dissolved in PBS (pH 7.4) and sterilized by filtration with a 0.22 μm membrane. One molar equivalent of CB[7] was added to the PG solution, vortexed for 30 seconds, and allowed to stand for an hour to obtain the host-guest complex PG/CB with a molar ratio of 1:1 (ethylenediamine group in PG: CB[7]). Then, TPE (0.5 mg/mL, PBS 7.4) was added into the PG/CB solution with a carboxyl group molar ratio varying from 0.1 to 0.6 (the ethylenediamine group in PG was regarded as 1), vortex for 30 seconds to obtain the ternary complex PG/CB/TPE. After incubation for 30 minutes, pDNA (50 μg/mL) was added with different N/P ratio under stirring, and allowed to stand for 30 min to obtain the quaternary PG/CB/TPE/DNA complexes.

Confirmation of aggregation induced emission (AIE) characters of TPE

TPE powder was dissolved in PBS solution (0.5 mg/mL, pH 7.4) as stock solution. Then, proportional of TPE solution was mixed with PG solution (1 mg/mL in PBS, pH 7.4), or preformed complexed PG/CB solution with three different molar ratios (defined as:

ethylenediamine group in PG/CB/carboxyl in TPE; PG:CB:TPE = 1:0.5:0.4 or 0.5:0.25:0.4 or 0.25:0.13:0.4) with TPE final concentration of 0.1 mg/mL; and the host-guest molar ratio of PG:CB was fixed as 1:0.5. The fluorescence intensity of TPE under excitation at 330 nm was recorded using a fluorescence spectrophotometer (HITACHI F-4600).

Agarose gel electrophoresis

Gel retardation assay was conducted to estimate the pDNA compression ability of different nanocomposites (PG/DNA, PG/CB/DNA, PG/TPE/DNA, PG/CB/TPE/DNA). 5 μL of polyplex solution in PBS (pH 7.4) at N/P ratios of 1, 2, 3, 5, 8 and 10 was mixed with 2 μL of 40% glycerol and 1 μL of loading-buffer. The mixture was dripped into the channel of agarose gel containing 0.8% wt ethidium bromide (EtBr). Electrophoresis was performed in TBE buffer under 80 V for 90 min. pDNA was visualized by UV illuminator (Gel Documentation Systems, Bio-Rad, Hercules, CA). Heparin replacement gel electrophoresis was carried out to study the stability of different complexes by pretreating the complexes at a N/P ratio of 10 using heparin solution (concentration varying from 0-200 μg/mL) for 6 h before gel retardation assay. In order to investigate the stimuli-responsive characteristic of PG/CB/TPE/DNA complexes under acidic conditions, the agarose gel electrophoresis and heparin binding experiments at pH 5.0 were also performed following above experimental methods.

Particle size analysis and morphology examination

The mean particle size, size distribution and zeta potential of PG/DNA and

PG/CB/TPE/DNA complexes were investigated at 25 °C by dynamic light scattering measurements (DLS) on a Zetasizer Nano ZS90 (Malvern, Worcestershire, U.K.). Scanning electron microscope (SEM, JEOL, Japan) was conducted on a JSM-6700F type instrument with an accelerated voltage of 10 kV. Samples were purified by dialysis (Spectra/Por RC, cutoff 7000) against water for 2 h to remove the salts from PBS before dropping onto the SiO_2 slice. Then, 5 μL of complex solution with suitable concentration were dropped onto a double-polished SiO_2 slice, followed by air-drying for 48 h.

Cytotoxicity assays

Cell Counting Kit-8 (CCK-8, Dojindo Laborotories, Kumamoto, Japan) was employed to evaluate the cytotoxicity of the polyplex, where PEI_{25K}/DNA was included as a control. HeLa and HCT 116 cells were cultivated in a humidified 5% carbon dioxide atmosphere at 37 °C on a 96-well microplate, with 5000 cells immersed in complete growth medium per well. After incubation of the cells for 24 h, different polyplexes with pDNA were added to 96-well plates at 10 μL per well, and incubated for 48 h, respectively. The solution was then removed and replaced with 100 μL of RPMI-1640. Finally, CCK-8 solution was added to 96-well plates at 10 μL per well and incubated at 37 °C for 30 min. Absorbance was measured at 450 nm by a Microplate Reader (Epoch, Bio Tek, Winooski, VT). The cell viability was calculated according to the following equation:

$$\text{Cell viability (\%)} = OD_{450\ (\text{sample})}/OD_{450\ (\text{blank control})} \times 100$$

where the sample stands for the cells treated by polyplexes, and the blank control stands

for the untreated cells. All data were represented by a mean and standard error from eight samples.

Confocal Laser Scanning Microscopy.

Confocal laser scanning microscopy (CLSM, Zeiss LSM510, Oberkochen, Germany) was performed to observe the internalization and subcellular localization of polyplex. HeLa cells were seeded onto a 35 mm cell culture dish at a density of 10^5 cells and incubated for 24 h in 1 mL of RPMI 1640 medium supplemented with 10% FBS in humidified atmosphere of 5% CO_2 at 37 °C. The medium was exchanged with 1 mL of fresh medium with 10% FBS, followed by addition of 100 μL polyplex (labeled with Cy5). After 3 h of incubation, the cells were washed with PBS, followed by 3 h post-incubation in 1 mL of fresh media with 10% FBS. Then, the medium was removed and the cells were washed three times with PBS and treated with Lyso Tracker Green (YEASEN, Shanghai, China) to stain lysosome. The observation was carried out by a LSM510 (Nikon 108, Japan) at the excitation wavelength of 402 nm for TPE, 488 nm for Lyso Tracker Green, and 633 nm for Cy5-DNA.

***In vitro* gene transfection.**

Transfection assays were performed using the plasmid CAG-Luc as the reporter gene in HeLa and HCT116 cell lines. In brief, cells were seeded in a 24-well plate at a density of 4×10^4 cells per well in 400 μL RPMI 1640 medium supplemented with 10% FBS in humidified atmosphere of 5% CO_2 at 37 °C. After 24 h incubation, the culture medium was

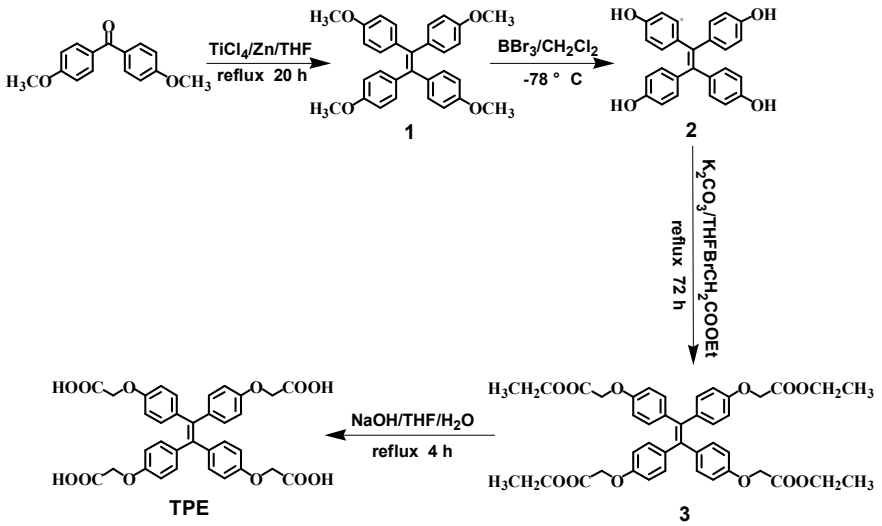
replaced with fresh complete RPMI 1640 medium, followed by addition of 40 μL polyplex solution containing 1 μg of pDNA per well, where PEI_{25K}/DNA was included as a control. After 48 h incubation, the cells were washed thrice with PBS and harvested by incubation with 100 μL cell lysis reagent. To test the luciferase activity, a commercial kit (Promega Co., Cergy Pontoise, France) and a luminometer (FLX800, Bio Tek, Winooski, VT) was applied to detect the light emission of a luciferin substrate (Promega) according to the standard protocol provided by the manufacturer. Protein concentration in the cell lysate was quantified by BCA protein assay kit (Pierce, Thermo Fisher Scientific, Waltham, MA). The transfection efficiency was expressed as relative light units per milligram protein (RLU/mg protein). All data were represented by a mean and standard error from four samples.

Flow cytometric assay.

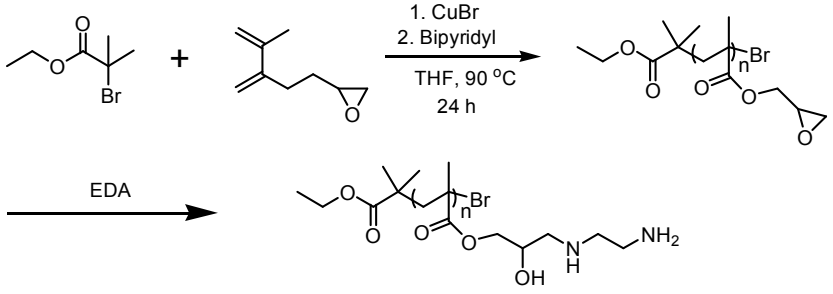
Cy5-labled DNA (50 μg/mL) was used to form PG/DNA and PG/CB/TPE/DNA complexes at the N/P ratio of 10. HeLa cells were seeded into 24-well culture plates at a density of 4×10^4 cells per well in 10% FBS containing culture medium (400 μL of medium per well) and cultured for 24 h. Then the medium was replaced with fresh whole culture medium and 40 μL of complex solution (N/P = 10) was added to each well (n = 3). After 12 h of incubation, the medium was removed and the cells were washed with 0.5 mL of PBS. The cells were treated with a trypsin-EDTA solution for 2 min and suspended in PBS, then analyzed using a flow cytometer (BDLSR II, BD, Franklin Lakes, NJ).

Statistical analysis.

Significant differences in cell viability and transfection efficiency between any two groups were evaluated using Student's *t*-test.

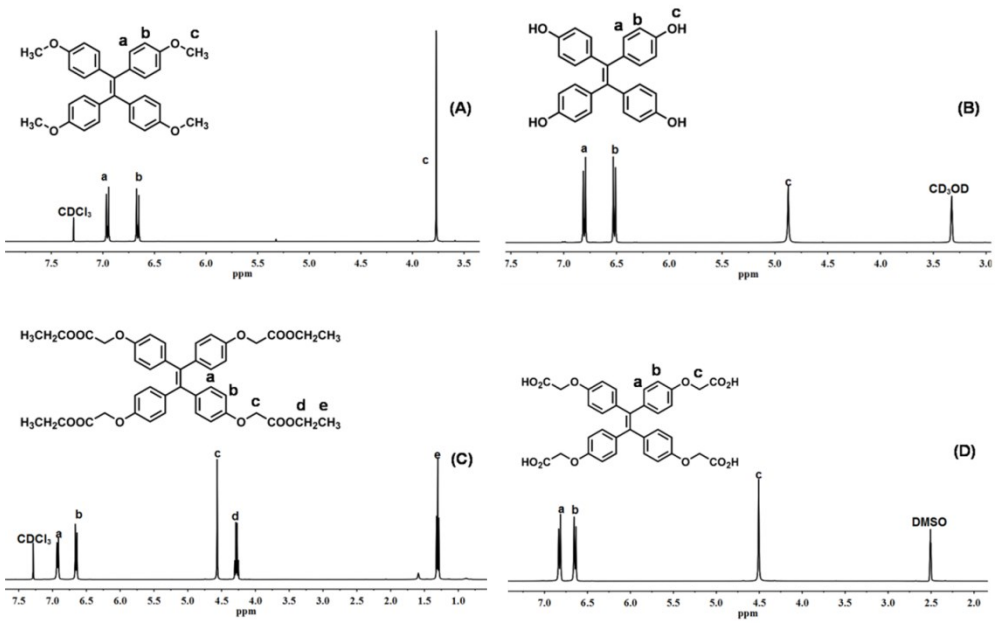


Scheme S1. Synthetic routes of TPE.

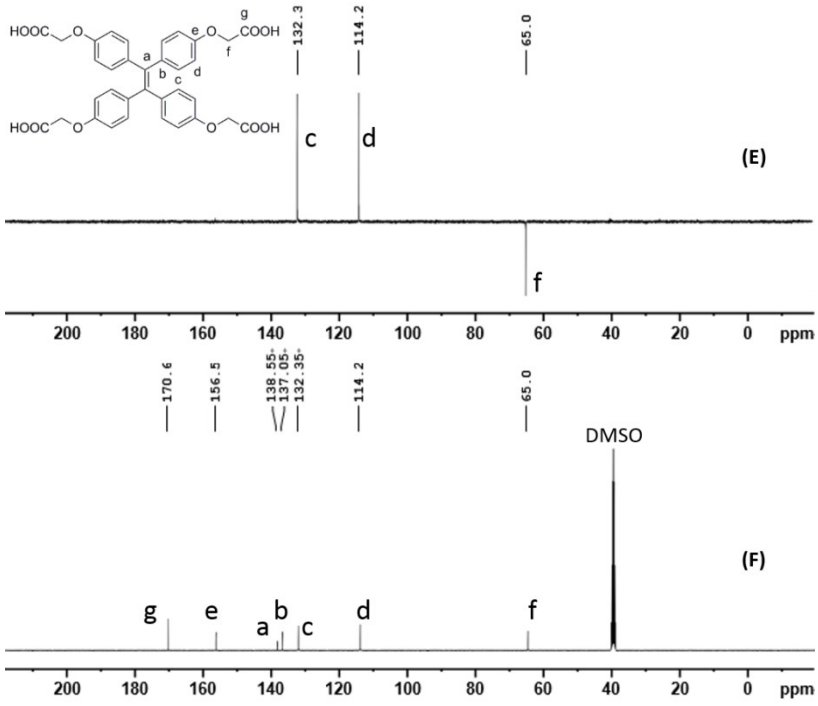


Scheme S2. Synthetic routes of PG.

1

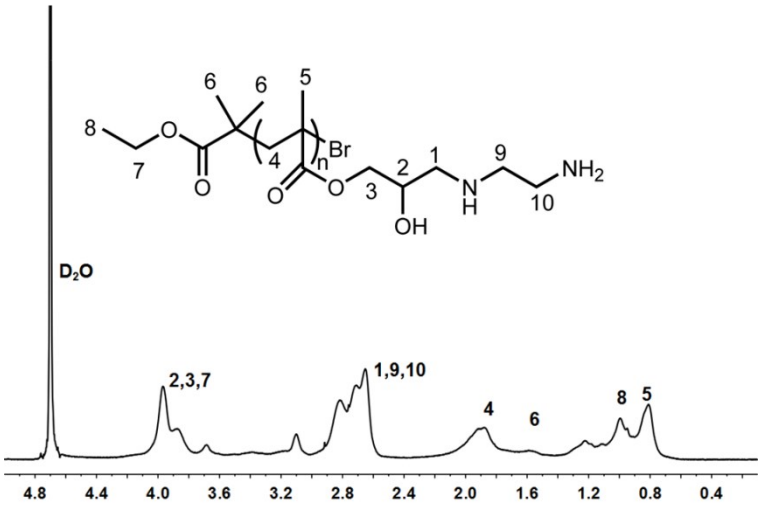


2



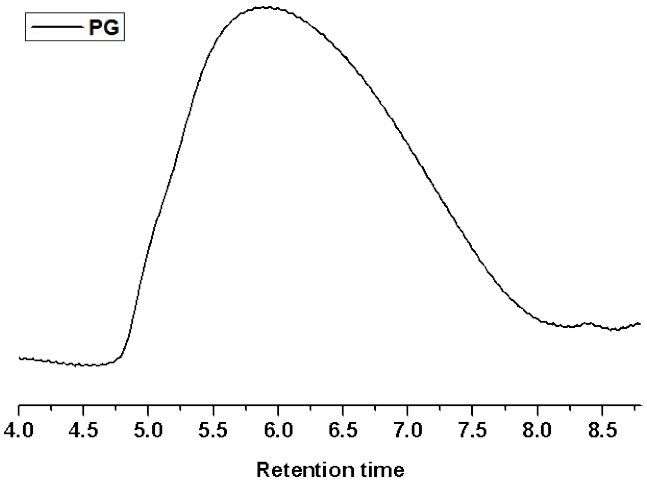
3 **Figure S1.** ^{1}H-NMR spectra of TPE derivatives (A-D) and $DEPT_{135}$ (E) and ^{13}C spectra (F) of

4 $TPE(COOH)_4$.



1

2 **Figure S2.** [1]H-NMR spectrum of PGMA-EDA.



$\overline{Mn}$	$\overline{Mw}$	PDI
14500	29100	2.0

3

4 **Figure S3**. GPC results of PG.

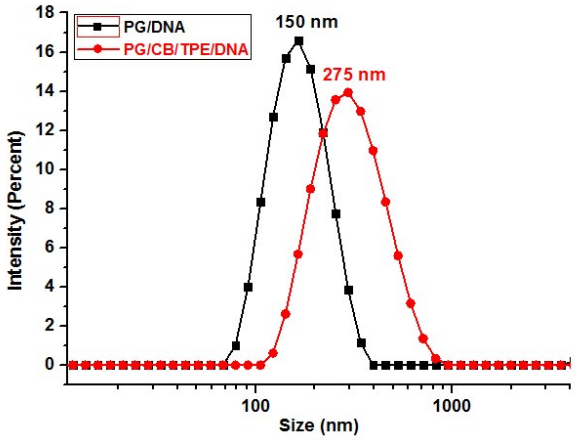


Figure S4. Intensity size distributions of PG/DNA (black) and PG/CB/TPE/DNA (red) complexes determined by DLS.

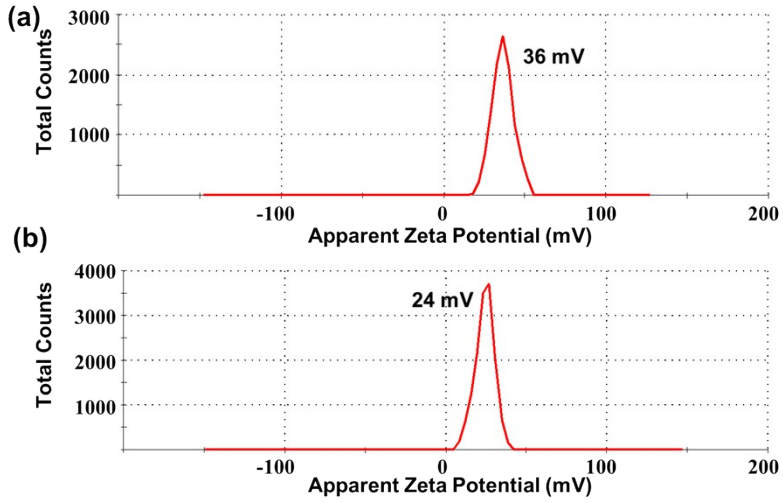


Figure S5. Zeta potential of PG/DNA (a) and PG/CB/TPE/DNA (b) complexes determined by DLS.

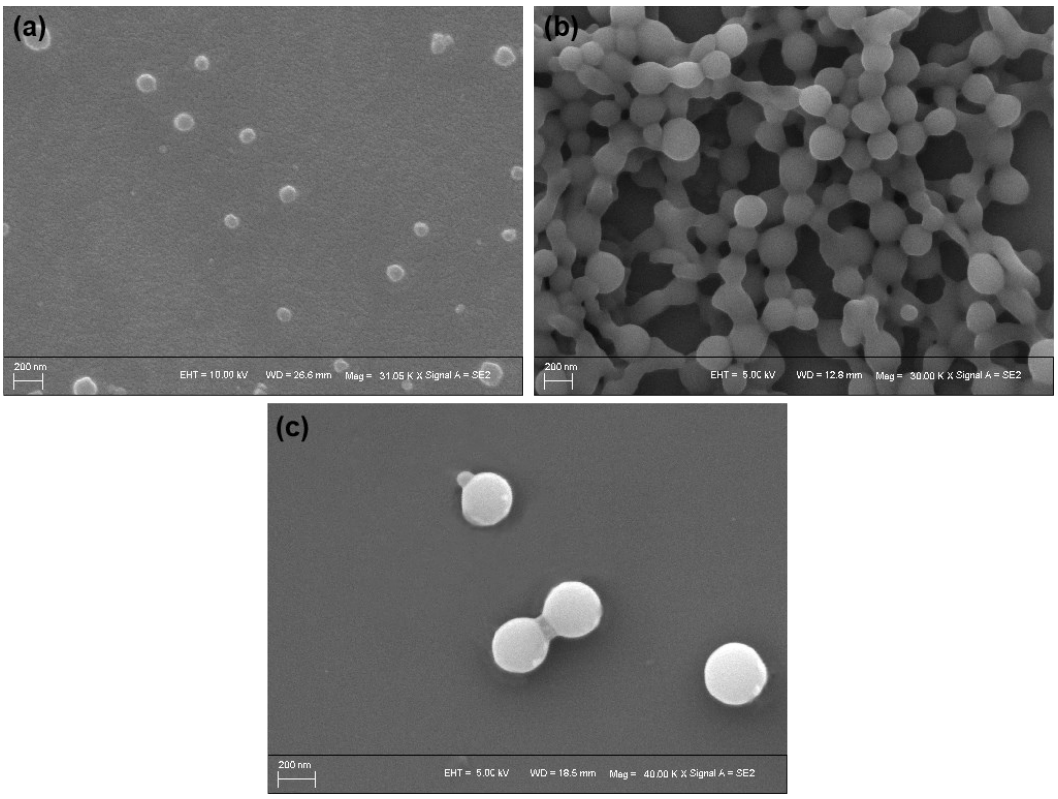


Figure S6. SEM images of (a) PG/DNA; (b) PG/CB/TPE; (c) PG/CB/TPE/DNA complexes.

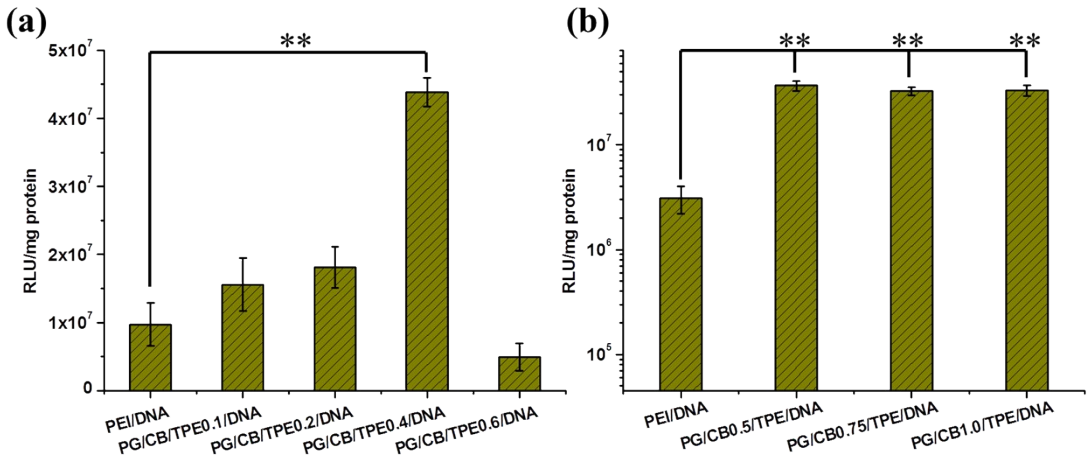


Figure S7. Transfection efficiencies of PEI/pDNA and quaternary complex with different ratios of TPE (0.1, 0.2, 0.4 and 0.6) (a) and CB[7] (0.5, 0.75 and 1.0) (b) at an N/P ratio of 10 to HeLa cells. PEI (M_w: 25 kDa, branched) was used as the control. Data are presented as the mean ± SD (n = 4). *p

< 0.05 and **p < 0.01).

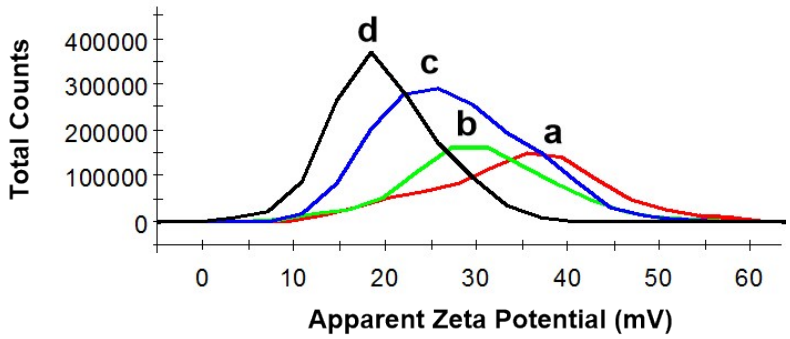


Figure S8. Zeta potential of PG/DNA (a), PG/TPE0.1/DNA (b), PG/TPE0.2/DNA (c) and PG/TPE0.4/DNA (d) complexes determined by DLS.

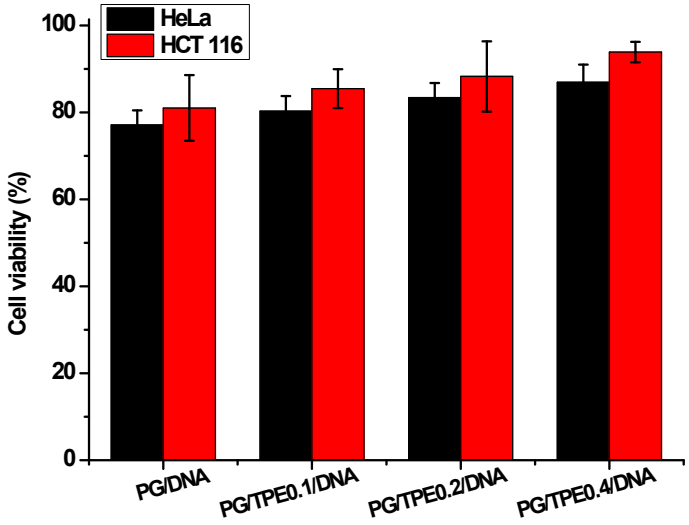


Figure S9. Cytotoxicity of PG/TPE/DNA complexes at various TPE ratios to HeLa and HCT 116 cells. Data are presented as the mean ± SD (n = 8).

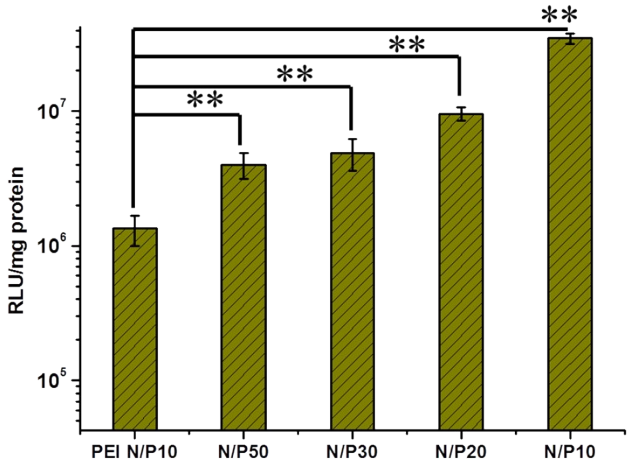


Figure S10. Transfection efficiencies of quaternary PG/TPE/CB/DNA complexes with different N/P ratio to HeLa cells. PEI (M_w: 25 kDa, branched) was used as the control. Data are presented as the mean ± SD (n = 4). *p < 0.05 and **p < 0.01).

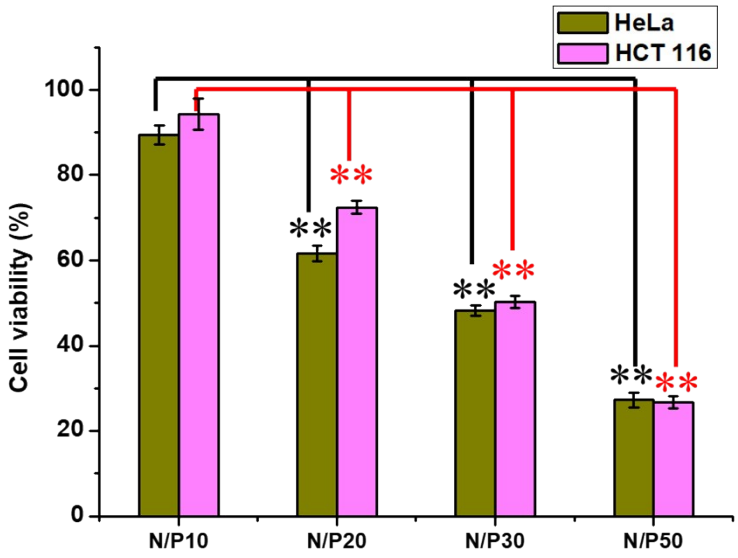


Figure S11. Cytotoxicity of PG/TPE/CB/DNA complexes at various N/P ratios to HeLa and HCT 116 cells. Data are presented as the mean ± SD (n = 8). *p < 0.05 and **p < 0.01).

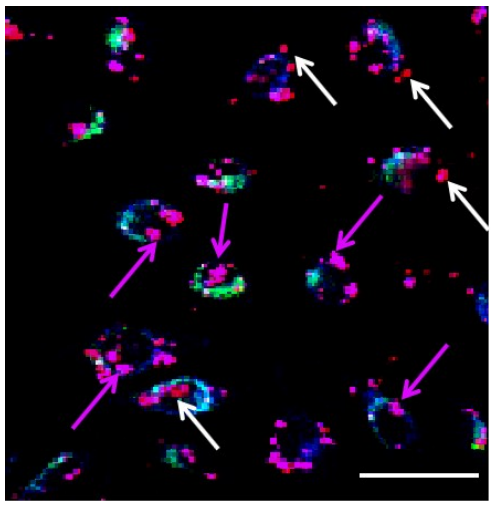


Figure S12. CLSM image of intracellular distribution of PG/CB/TPE/DNA complexes after 6 h incubation. The white arrows indicate the released free pDNA; pink arrows indicate the merged Cy5-pDNA (red) and TPE (blue) signals. Blue, TPE; red, Cy5- pDNA; green, Lyso Tracker Green. Scale bar = 100 μm.

[S1] Han, X.; Liu, D. E.; Wang, T.; Lu, H.; Ma, J.; Chen, Q.; Gao, H. Aggregation-induced-emissive molecule incorporated into polymeric nanoparticulate as FRET donor for observing doxorubicin delivery. *ACS Appl. Mater. Inter.* **2015**, *7*, 23760-23766.

[S2] Gao, H.; Jones, M.-C.; Tewari, P.; Ranger, M.; Leroux, J.-C. Star-Shaped alkylated poly(glycerol methacrylate) reverse micelles: synthesis and evaluation of their solubilizing properties in dichloromethane. *J. Polym. Sci. Part A: Polym. Chem.* **2007**, *45*, 2425-2435.

[S3] Gao, H.; Jones, M.-C.; Chen, J.; Prud'homme, R. E.; Leroux, J.-C. Core cross-linked reverse polymeric micelles. *Chem. Mater.* **2008**, *20*, 3063-3067.