Electronic Supplementary Information (ESI)

for

Incorporation of Aggregation-Induced-Emissive Tetraphenylethene Derivative into Cationic Gene Delivery Vehicles Manifested Nucleus Translocation of DNA as Uncomplexed Form

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Materials and methods

Materials

4-Bromobenzophenone was purchased from Shanghai Adamas Reagent Co., Ltd (Shanghai, China). Polyethylenimine [M_w: 25,000 by LS, branched] was purchased from Sigma-Aldrich (Shanghai, China). *β*-CD was recrystallized twice before use. A plasmid vector coding for luciferase with a CAG promoter was used in the DLS measurement, the *in vitro* cell viability and transfection experiments (RIKEN, Japan). Cell Counting Kit-8 (CCK-8) was used in the cell cytotoxicity assay (Dojindo, Japan). The Micro BCA Protein Assay Reagent Kit was purchased from Pierce Co., Inc. (Rockford, IL) and the luciferase assay kit was a product of Promega (USA). All other reagents were obtained from Tianjin Chemical Reagent Co. (Tianjin, China). 6-monotosyl-*β*-Cyclodextrin (*β*-CD-OTs) was synthesized according to previous reports.[1,2]

1. Synthesis of tetraphenylene (TPE) Derivatives

1.1 Synthesis and characterization of l, 2-Bis(4-bromophenyl)-l, 2-diphenylethene (TPEDBr)

As shown in Scheme 1, the bromized TPE was prepared via McMurry Olefination reaction from 4-bromobenzophenone.[3,4] Zinc (3.92 g, 60 mmol) was added into a 250 mL three-necked round-bottomed flask. The flask was degassed and flushed with dry nitrogen for three times, newly evaporated THF (60 mL) was subsequently injected. Under an ice-salt bath, the mixture was cooled down to -5-0 °C. Titanium tetrachloride (3.32 mL, 30 mmol) was added slowly, then warmed the suspension to 25 °C and kept for 30 min, and refluxed at 74 °C for 2 h. Afterwards the mixture was cooled to -5-0 °C again, and pyridine (0.5 mL) was injected under stirring for 10 min. Then a THF solution (20 mL) of 4-bromophenone (1, 5.22 g, 20

mmol) was added slowly. After refluxing overnight, the reaction mixture was cooled to room temperature, then the reaction was quenched with a 10% potassium carbonate aqueous solution and extracted by DCM for three times. The organic layer was washed with saturated brine and dried with anhydrous sodium sulfate for 4 h. After filtration and solvent evaporation, the residue was purified by silica gel column chromatography, using petroleum ether/DCM (10:1 by volume) as eluent. TPEDBr (2.94 g) was obtained as a white solid in 60.0 % yield. ^{1}H NMR (400 MHz, $CDCl_3$, δ): 7.25-7.19 (m, 4H; Ar H), 7.13-7.07 (m, 6H; Ar H), 7.03-6.96 (m, 4H; Ar H), 6.89-6.85 (m, 4H; Ar H).

1.2 Synthesis of 4,4'-(l,2-Diphenylethene-l,2-diyl)bis(l, 4-phenylene)diboronic acid (TPEDB)

In a 100 mL four-necked round-bottomed flask, 1,2-Bis(4-bromophenyl) -l,2-diphenylethene (0.4 g, 0.82 mmol) was dissolved in 20 mL of newly evaporated THF.[5] The flask was cooled to -78 °C with an acetone-dry ice bath, and then 1.0 mL (2.6 mmol) of n-butyllithium (2.5 M in hexane) was injected carefully under nitrogen. After stirring for 1 h, 0.46 mL (4.0 mmol) of tri-methyl borate was added and the mixture was allowed to react for 45 min. Then the mixture was warmed to room temperature. After being stirring overnight, the reaction was quenched with 1mL of dilute HCl solution. Following filtration and solvent evaporation, the product was purified by silica gel column chromatography using ethyl acetate/DCM (1:10, by volume) as eluent. The product was obtained as yellow solid (yield: 42 %). ^{1}H NMR (400 MHz, DMSO-d_6, δ): 7.96 [d, $B(OH)_2$], 7.54 (d, 4H; Ar H), 7.08-7.16 (m, 6H; Ar H), 6.92-6.99 (m, 8H; Ar H).

2. Syntheses of PEI- Cyclodextrin (PEI-CD) cationic polymer

PEI -CD polymer was synthesized as reported previously. [6-8] Potassium carbonate (0.56 g, 4 mmol) was added to a solution of branched PEI (560 mg, 56 μmol) and *β*-CD-OTs (2.6 g, 585μmol) in 30mL dimethyl sulfoxide (DMSO). After reaction at 80 °C for 48 h, the resulting crude product of PEI-CD was purified by dialysis (Spectra/Por RC, cut off 7000) against deionized water for 5 days before freeze-dried. The final product PEI-CD was obtained as a light yellow floppy solid (yield: 85%).

Characterization of TPE Derivatives and polymers

The ^{1}H-NMR spectra of TPE derivatives and the polymer were acquired on a 400 MHz Bruker Avance-400 spectrometer (400 MHz, Bruker, Freemont, CA) using DMSO, $CDCl_3$ and D_2O as solvents, respectively. The chemical shifts were referred to the solvent peaks, δ=2.50 ppm for DMSO, δ=7.26 ppm for $CDCl_3$ and δ=4.80 ppm for D_2O, respectively. FT-IR spectra were recorded on a Bio-Rod 6000 spectrometer (Thermo Electron, USA) to identify the chemical functional groups of the samples. The substance was finely grounded and dispersed into KBr pellets using a ratio of approximately 1 mg sample/100 mg KBr. Molecular weights and polydispersity of polymers were characterized by gel permeation chromatography (GPC, Viscotek) equipped with a refractive index detector, using 5% (volume) acetic acid solution as the mobile phase at a flow rate of 1.0 mL min^{-1} at 35 °C.

Sample Preparations

Preparation of Stock Solution of TPEDB in DMSO.[9,10] A stock solution of TPEDB at a concentration of 2 mM / 10 mM was prepared by dissolving respectively 21.0 mg (50 μmol) / 0.105 g (250 μmol) of TPEDB in 25 mL DMSO. The solution was stored in a refrigerator under 4 °C prior to use.

Preparation of stock solution of water-soluble TPEDB in Carbonate Buffer. Into a 100 mL volumetric flask were added 4.5 g of K_2CO_3, 760.9 mg of $KHCO_3$ and 80 mL of deionized water. 1 mL of the stock solution of TPEDB in DMSO (2.0 mM) was added under stirring. The volume of the solution was adjusted to 100 mL by adding appropriate amount of deionized water. The resultant solution of TPEDB (20 μmol) in the carbonate buffer (0.4 M, pH 10.5) containing 1.0 vol% DMSO was stirring for 15 min before use.

Preparation of PEI-CD-TPEDB. 1.0 mL of the stock solution of TPEDB (20 μmol) in the carbonate buffer containing 1.0 vol % DMSO and 1 mL of PEI-CD solution with different concentration (0-3 mg/mL) were added into a 10 mL volumetric flask. After shaking overnight, a solution of mixture in the carbonate buffer (0.2 M, pH=10.5) containing 0.5 vol % DMSO was obtained. The solution was allowed to stand for 1 min before its fluorescence (FL) was measured.

Preparation of polyplexes

The charge ratio (N/P) of polymers and pDNA was calculated as the moles ratio of the amino (N) from polymer to the phosphate groups (P) from pDNA. Briefly, polymers were dissolved in distilled water. 29.4 μL or 16.3μL TPEDB (10 mM) solution was respectively added to the PEI-CD at CD: TPEDB molar ratio of 1. Afterwards the mixture was vortexed for 30 s, standing for 30 min. All the solutions were sterilized by passing through 0.22 μm filter prior to complexation. Polymer/pDNA complexes were formed by adding the polymer solutions of prescribed concentrations to an equal volume of a defined pDNA solution (25 μg/mL) to obtain the N/P ratio of 1-10. The two solutions were mixed and vortexed for 30 s, followed by incubation at room temperature for 30 min to obtain the polymer/pDNA complexes.

Particle size and morphology measurement of complexes

The hydrodynamic sizes of complexes formulated by PEI-CD and PEI-CD-TPEDB were measured by a Zetasizer Nano ZS90 instrument (Malvern Instruments, Southborough, MA) at 25 °C, at N/P ratio of 10. The morphology of complexes was observed using a scanning electron microscope (SEM) on a JSM-6700F type field emission scanning electron microscope (JEOL, South Korea). SEM samples were prepared by depositing solutions of complexes (PEI-CD/pDNA, PEI-CD/TPEDB/pDNA at N/P = 10.0) on a silicon slide, separately. After evaporation of water, the samples were coated with a thin gold layer.

Agarose gel electrophoresis

The agarose gel retardation assay was performed to assess the mobility of polymer/pDNA complex under electric field. Routinely, diverse formulations of polymer/pDNA complexes were prepared at varying N/P ratios ranging from 1 to 10. Then, complexes with several complexation ratios were loaded onto a 0.8% agarose gel containing ethidium bromide (EtBr), and a constant voltage (80 V) was applied to the complex-loaded gel in 0.5 × TBE buffer for 90 min. DNA retardation was analyzed using a UV illuminator (Gel Documentation Systems, Bio-Rad, Hercules, CA).

Determination of Buffering Capacity

The buffering capacity of cationic polymers in the pH range 2-10 was determined by acid-base titration.[11] Polymers were dissolved into 5 mL of saline (0.9% NaCl solution) with a 10 mM amino group concentration, which was subsequently adjusted by 0.1 M NaOH to an initial pH 10. The solutions were titrated with a 0.1 M HCl solution with various volume increments. The pH of all the solutions was measured using a pH meter (INESA PHS-3C).

Pure water was used as the blank solution.

Cell viability assay

The *in vitro* cytotoxicity of the polymer vectors was determined by the evaluation of the viability of A-549 cells using Cell Counting Kit-8 (CCK-8) assay.[12,13] CCK-8 interacts with mitochondria in cells to generate water-soluble orange formazan product. The number of living cells is in direct proportion to the amount of formazan product. A-549 cells were suspended in complete medium and seeded in a 96-well plate at a concentration of 10,000/100 μL per well, and incubated in a humidified 5% CO_2 atmosphere at 37 °C. After 24 h incubation, the culture medium was replaced with fresh medium, and 10 μL solution of complexes at pDNA concentration of 50 μg/mL was added to each well. Following incubation for another 48 h, each well was treated with CCK-8 solution (10 μL) and the plate was cultured in CO_2 incubator for 2 h. Afterwards, the absorbance of the solution was monitored at 450 nm using an automatic BIO-TEK (Epoch, Gene company Limited) microplate reader. And the cell viability (%) relative to control cells cultured in medium without polymers was calculated from $[A]_{test}/[A]_{control}\times100\%$, where $[A]_{test}$ and $[A]_{control}$ are the absorbance values of the wells (with the polymers) and control wells (without the polymers), respectively. For each sample, the final absorbance was the average of those measured from eight wells in parallel.

***In vitro* transfection**

Transfection assays were performed using plasmid Cag-luc as the reporter gene in A549 cell lines. In brief, cells were seeded in 24-well plates at a density of 5×10^4 cells in 400 μl of medium/well for 24 h. After 24 h, the medium in each well was replaced with 400 μL fresh

medium. 40 μL of the polymer/pDNA complexes containing 1.0 μg pDNA at different N/P ratios were added into each well, followed by a further incubation for 48 h to express the luciferase reporter gene. The cultured cells were washed with PBS twice and lysed in 100 μL of the cell culture lysis reagent. Luciferase gene expression was quantified by a commercial kit (Promega Co. Cergy Pontoise, France) and a luminometer (FLX800, BioTek, Gene company Limited). The protein concentration of each well was analyzed using a protein assay kits (Pierce). Gene expression results were expressed as relative light units (RLUs) per milligram of cell protein (RLU/mg protein). The results were presented as a mean and standard deviation obtained from four samples.

Confocal laser scanning microscopy (CLSM)

For intracellular translocation, A549 cells were plated in a 35 mm glass-bottomed cell culture dish at an initial density of 1×10^5 cells and incubated overnight in 1 mL of culture media (RPMI 1640, 10% FBS). 150 μL of polyplex solution PEI-CD-TPEDB (containing 3.75 μg pDNA and corresponding amount of TPEDB) were added in serum-free medium. Following 2 h incubation at 37 °C in a humidified atmosphere with 5% (v/v) CO_2, the cells were washed twice with PBS and the serum-free medium was replaced with fresh complete RPMI 1640 medium. After another 22 h post-incubation, the medium was discarded and the cells were washed twice with PBS. Then the intracellular distribution of the complexes was observed by CLSM. CLSM observation was performed using an LSM510 (Nikon 108, Japan) confocal fluorescent microscope. For TPEDB, the excitation was 405 nm, and the emission filter was 425-475 nm; For Cy5-DNA, the excitation was 633 nm, and the emission filter was 662-737 nm.

Quantification of polymer and pDNA in cell nuclei and cytoplasm

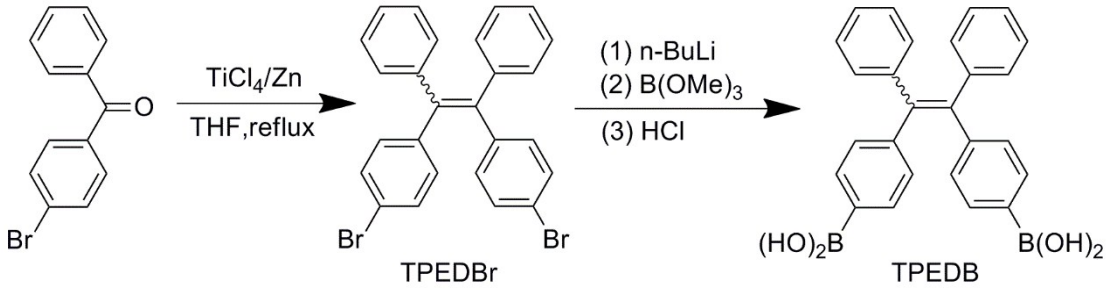
A549 cells were plated in a 35 mm glass-bottomed cell culture dish at an initial density of 1 × 10^5 cells and incubated overnight in 1 mL of culture media (RPMI 1640, 10% FBS). 150 μL of polyplex solution PEI-CD-TPEDB (containing 3.75 μg pDNA and corresponding amount of TPEDB) were added into the serum-free medium. Following 2 h incubation at 37 °C in a humidified atmosphere with 5% (v/v) CO_2, the cells were washed twice with PBS and the serum-free medium was replaced with fresh complete RPMI 1640 medium. After another 22 h post-incubation, the medium was discarded and the cells were washed twice with PBS, trypsinized and pelleted by centrifugation and resuspended in 0.5 mL of the cell lysis buffer [10 mM HEPES; pH 7.5, 10 mM KCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 0.5% Nonidet-40 and 0.5 mM phenylmethanesulfonyl fluoride (PMSF) along with the protease inhibitor cocktail (Sigma-Aldrich)], followed by 15 min incubation in ice with intermittent mixing. The reaction solution was then transferred for centrifugation at 12,000 g at 4 °C for 10 min. The pelleted nuclei was washed five times with the cell lysis buffer and resuspended in 0.5 mL of the nuclear extraction buffer containing 20 mM HEPES (pH 7.5), 400 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF with protease inhibitor cocktail and incubated in ice for 30 min. Nuclear extract was collected by centrifugation at 12,000 g for 15 min at 4 °C.

The quantity of polymer and pDNA was quantified based on fluorescence measurement for TPEDB and Cy5 in the cell fraction solutions of nuclei and cytoplasm. 0.1 mL of each fraction solution was transferred to a 96-well plate for fluorescence intensity measurement (TPEDB: ex 330 nm, em 400 – 475 nm; Cy5: ex 630, em 660 – 735 nm) by Multi-Mode

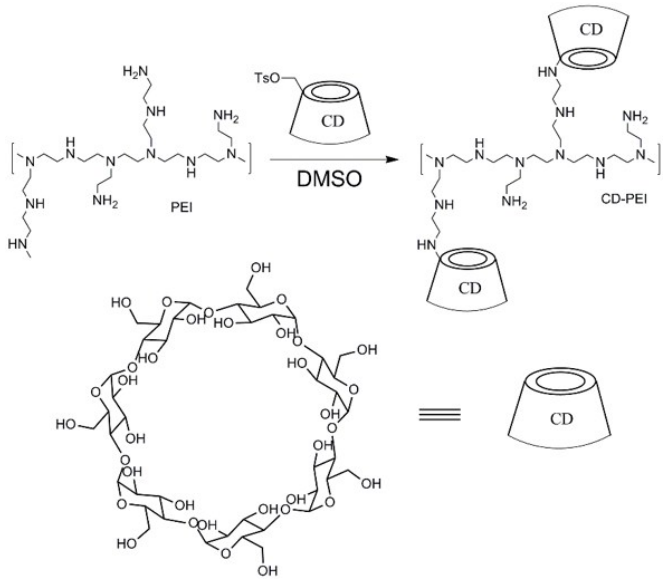
Microplate Readers (BioTek, VT). Of note, the background was subtracted based on fluorescence measurement for each cell fraction solutions of the cells without addition of the polyplexes. The ultimate result was expressed as the fluorescence intensity ratio of TPEDB:Cy5 for clarification of gene translocation into nucleus in the form of complexed form or uncomplexed form.

Statistical analysis

Significant differences in cell viability and transfection efficiency between the obtained polymers and PEI were evaluated using Student's t-test.



Scheme S1. Synthetic route for preparation of TPEDB.



Scheme S2. Synthetic route for preparation of PEI-CD.

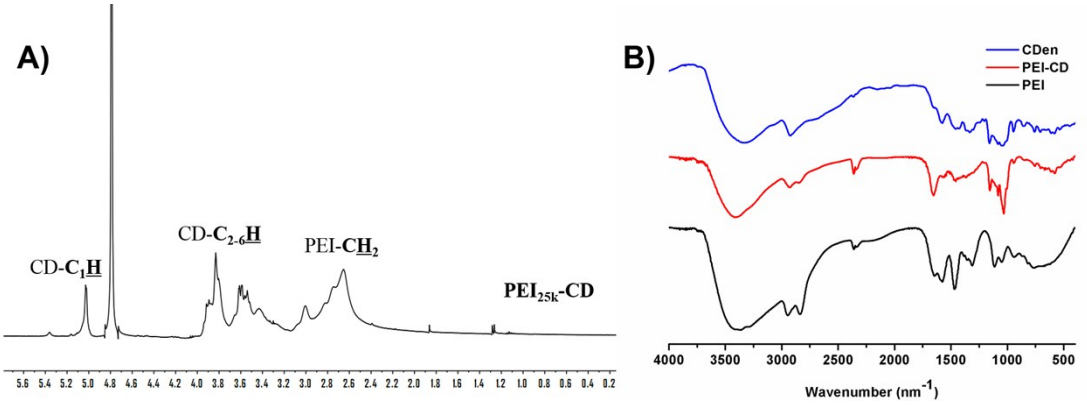


Figure S1. A) ^{1}H NMR of PEI-CDs in D_2O; B) FT-IR of CD, PEI and PEI-CD.

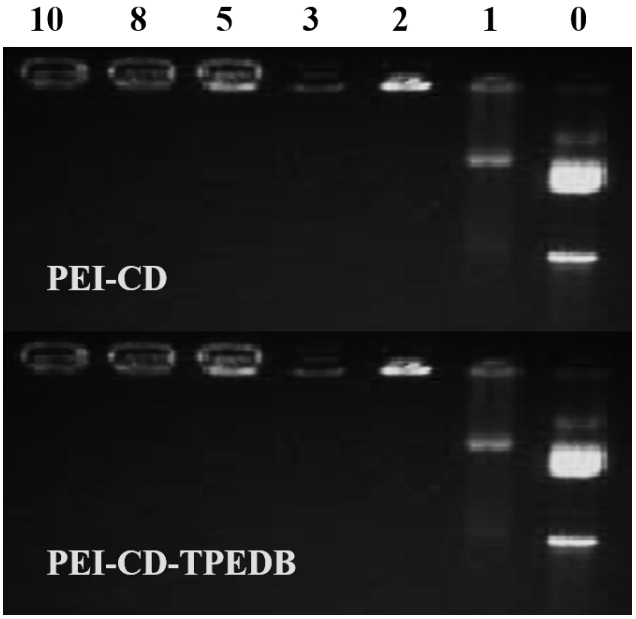


Figure S2. Gel electrophoresis of complexes. Note: pDNA (lane 1); polymer/pDNA complexes with N/P ratio of 1, 2, 3, 5, 8, and 10, respectively (lanes 2-7).

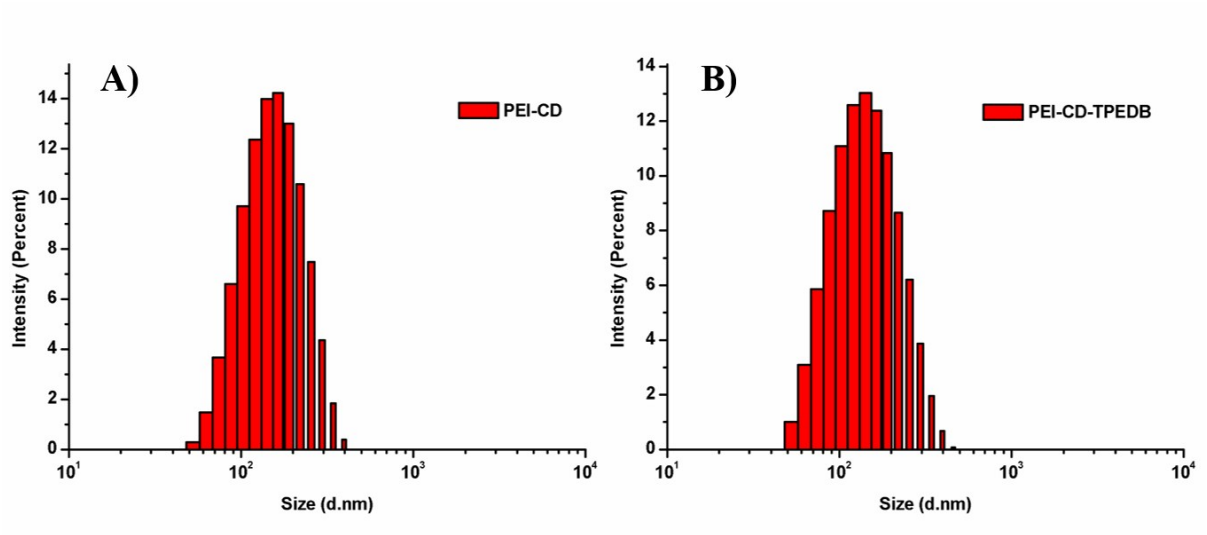


Figure S3. Hydrodynamic diameters of PEI-CD/DNA (A) and PEI-CD-TPEDB/DNA (B) polyplexes at N/P ratio of 10, using dynamic light scattering (DLS) measurements.

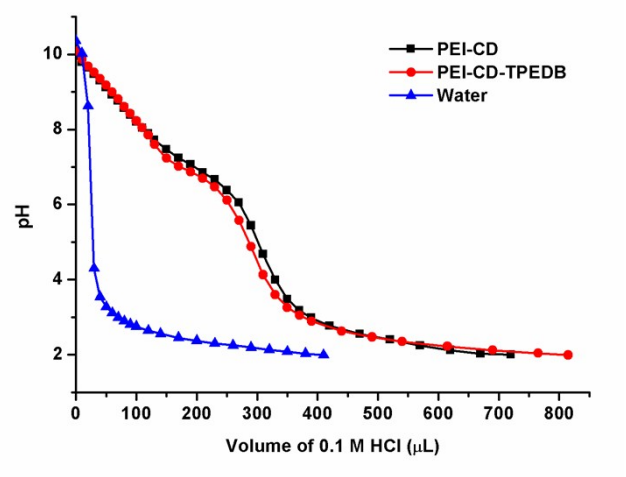


Figure S4. Determination of the buffer capacity of PEI-CD, PEI-CD-TPEDB, PEI, and water by acid−base titration. The cationic polymer solutions containing 10 mM amino groups were titrated with 0.1 M HCl solution.

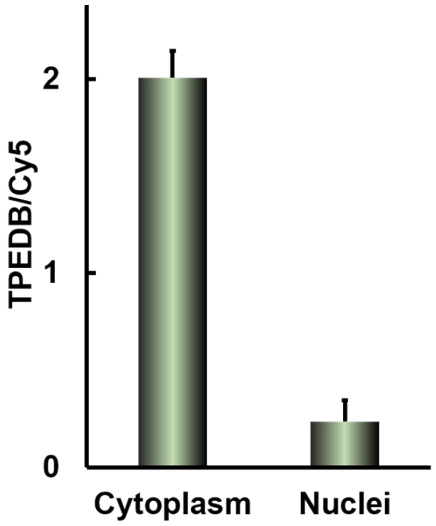


Figure S5. Determination the fluorescence intensity ratios of TPEDB:Cy5 for isolated cell fractions of nucleus and cytoplasm.

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