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# A Supramolecular Approach to Improve the Gene Transfection Efficacy of Dendrimers

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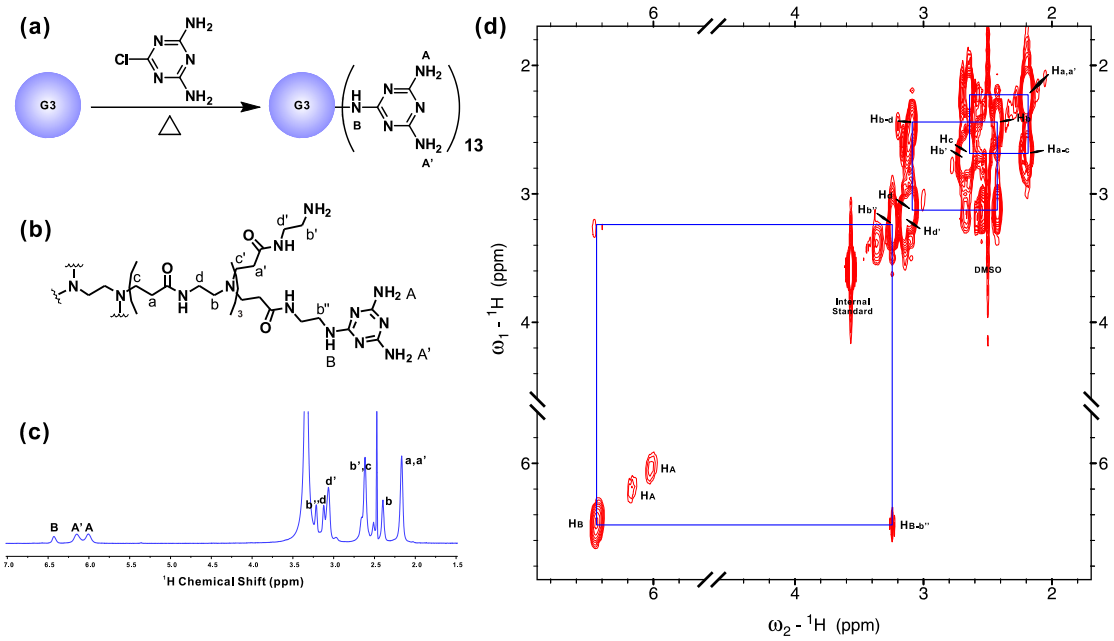



**Figure S1.** The synthesis route (a), molecular structure (b), $^1$H NMR (c) and 2D COSY spectrum (d) with proton assignments in d6-DMSO of G3-DAT$_{13}$ conjugates. The presence of cross-peak $H_{B\text{-}b''}$ indicates DAT moieties has been conjugated onto the primary amines of G3 PAMAM dendrimer. The average number of DAT moieties conjugated on the surface of each G3 PAMAM dendrimer was calculated according to the peak area ratio of dendrimer protons ($H_{a,a'}$) and DAT protons ($H_{A,\,A'}$ and $H_B$).

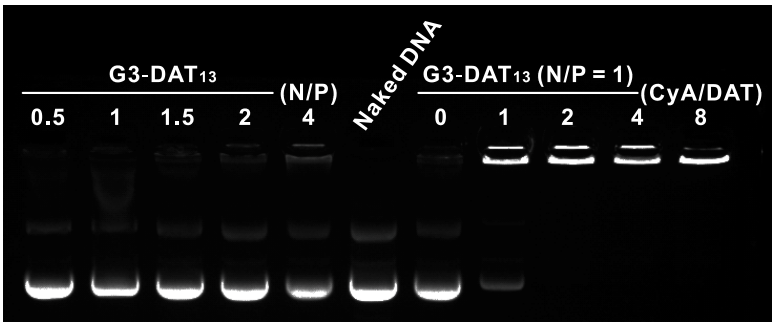
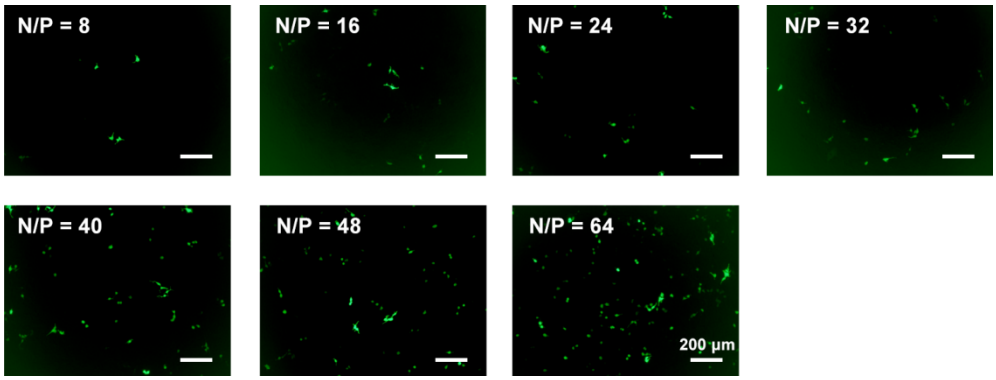
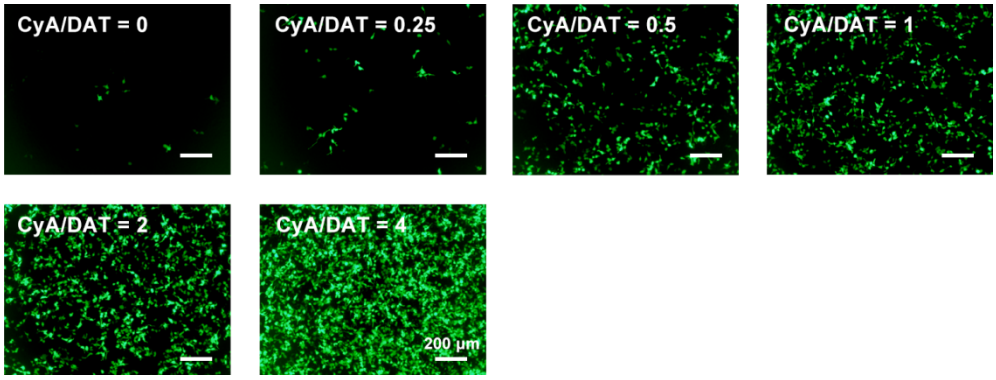



**Figure S2.** Agarose gel electrophoresis assay for DNA complexation ability of G3-$DAT_{13}$ at different N/P ratios (N/P ratio is 0.5, 1, 1.5, 2, and 4, respectively, Left) and at an N/P ratio of 1 in the presence of CyA (CyA/DAT molar ratio is 0, 1, 2, 4, and 8, respectively, Right).




(a)




（b）

**Figure S3.** EGFP expressions in HEK293 cells mediated by G3-$DAT_{13}$ at different N/P ratios (a) and G3-$DAT_{13}$/CyA at different CyA/DAT molar ratios (N/P = 64) (b) for 48 h.

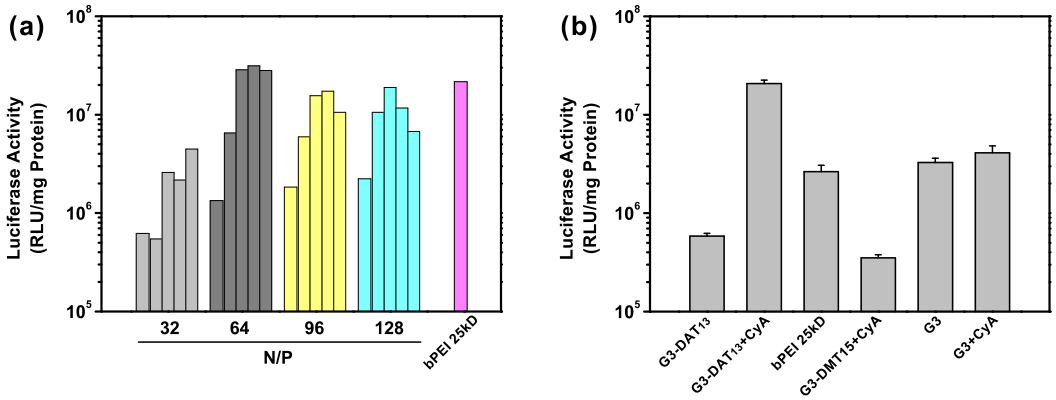
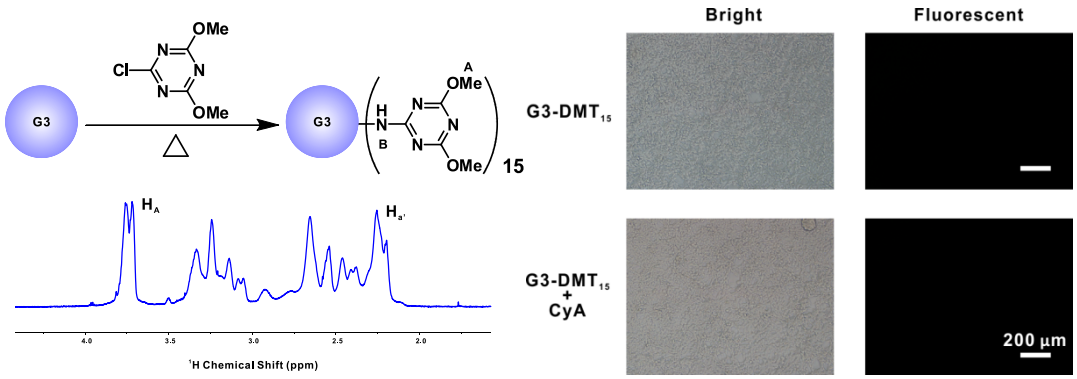
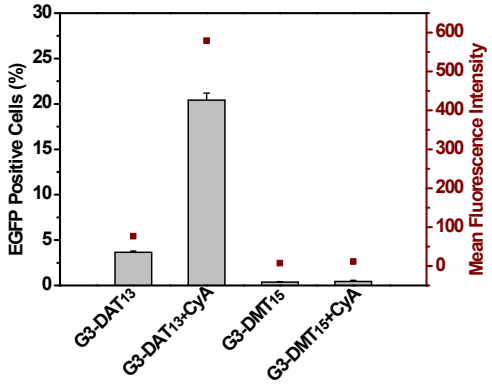



**Figure S4.** Quantitative analysis of luciferase expression in COS-7 cells mediated by (a) G3-DAT13/CyA at different N/P ratio of 32, 64, 96 and 128 and at different CyA/DAT molar ratio of 0, 2, 3, 4 and 5; (b) G3-$DAT_{13}$, G3-$DAT_{13}$/CyA, unmodified G3, G3-$DAT_{13}$/TE buffer, and bPEI 25KD. The N/P ratio of G3-$DAT_{13}$/DNA polyplex is 64 and the molar ratio of CyA to DAT is 4. For G3/DNA polyplex, the N/P ratio is 64 and CyA/DAT molar ratio is 4. The optimal N/P ratio of bPEI 25KD is 8.




(a) (b)




(c)

**Figure S5.** (a) Synthesis and $^{1}H$ Characterization of G3-$DMT_{15}$. (b) EGFP expressions in HEK293 cells mediated by G3-$DMT_{15}$/DNA and G3-$DMT_{15}$/DNA/CyA polyplexes for 48 h. The N/P ratio of the polyplex is 64 and the molar ratio of CyA to DMT is 4. (c) Quantitative analysis of EGFP expression in HEK293 cells mediated by G3-$DAT_{13}$ and G3-$DMT_{15}$ in the absence or presence of CyA for 48 h. The N/P ratio of G3-$DAT_{13}$/DNA and G3-$DMT_{15}$/DNA polyplexes is 64. And the molar ratio of CyA/DAT is 4.

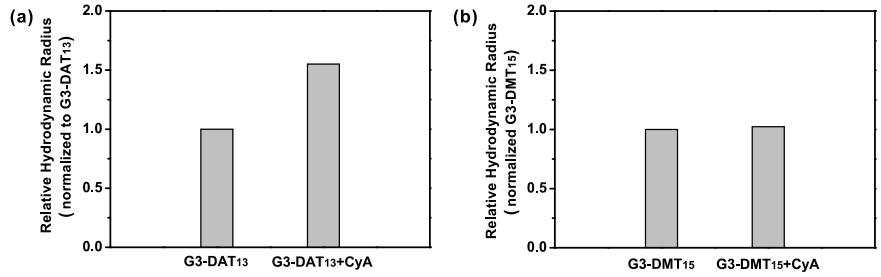



**Figure S6.** Relative hydrodynamic radius of G3-$DAT_{13}$ (a) and G3-$DMT_{15}$ (b) in the absence or presence of CyA in $D_2O$ analyzed by PGSE diffusion NMR. The molar ratio of CyA/DAT is 8.

An increase in hydrodynamic radius of G3-$DAT_{13}$ is observed after the addition of CyA, in comparison, CyA addition fails to increase the size of G3-$DMT_{15}$, suggesting the absence of assembled structures in the G3-$DMT_{15}$/CyA system.

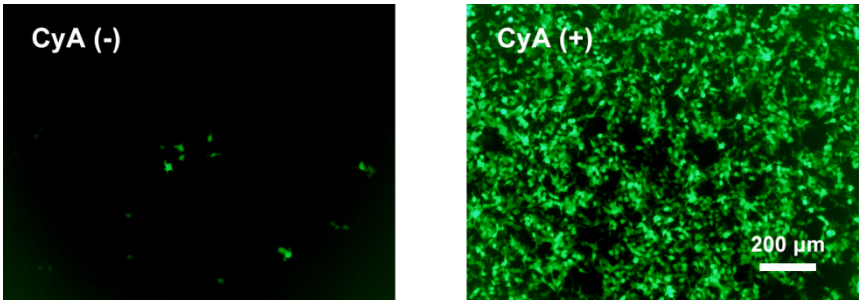



**Figure S7.** Transfection efficacy of G3-$DAT_{13}$ pre-incubated with CyA before DNA complexation. HEK293 cells were incubated with the polyplex for 48 h. The N/P ratio of the polyplex is 64 and the molar ratio of CyA to DAT is 4.

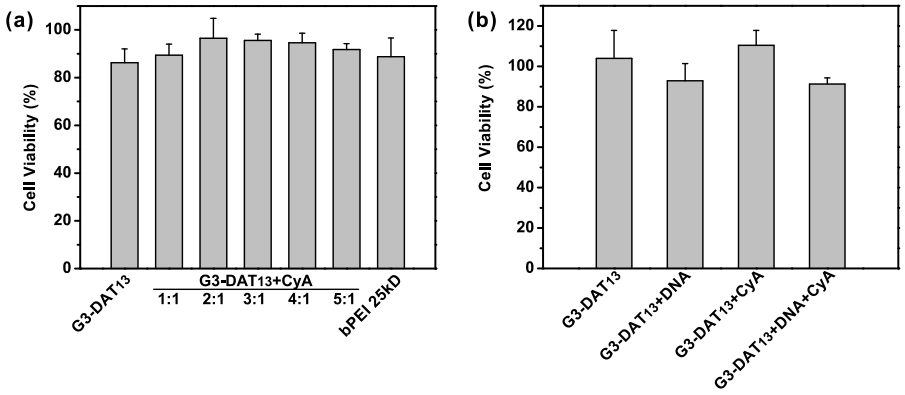



**Figure S8.** (a) Viability of HEK293 cells incubated with G3-$DAT_{13}$ in the presence of different amounts of CyA. (b) Viability of HEK293 cells incubated with G3-$DAT_{13}$/DNA polyplex in the presence or absence of CyA at a CyA/DAT molar ratio of 4. The N/P ratio of the polyplex is 64. The N/P ratio for the bPEI 25KD polyplex is 8.

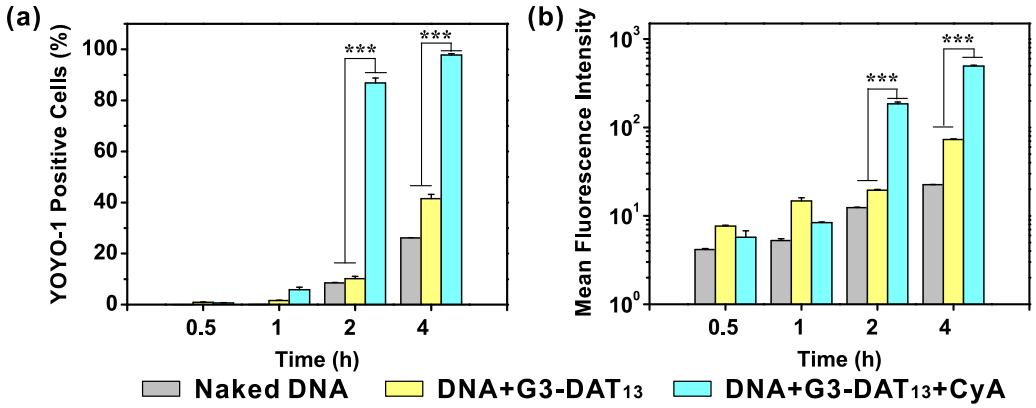



**Figure S9.** Cellular uptake of YOYO-1 labeled naked DNA and G3-$DAT_{13}$/DNA polyplexes in the absence or presence of CyA in COS-7 cells. (a) Percent of YOYO-1 positive cells, (b) Mean fluorescence intensity of the cells. The N/P ratio of the polyplex is 64 and the molar ratio of CyA to DAT is 4. Error bar represent the s.e. (n = 3). ***$p < 0.001$ analyzed by students' t-test.

## Materials and Methods

### Materials

Ethylenediamine-cored and amine-terminated G3 PAMAM dendrimers (6909 Da) were purchased from Dendritech, Inc. (Midland, MI). 2-chloro-4,6-dimethoxy-1,3,5-triazine, bPEI 25KD and cyanuric acid (CyA) were purchased from Sigma-Aldrich (St. Louis, MO). 2-chloro-4,6-diamino-1,3,5-triazine was obtained from Aladdin Co. (Shanghai, China). YOYO-1 iodidie was purchased from Invitrogen (Carlsbad, CA). G3 PAMAM dendrimer was received in water and distilled to remove the solvent before further experiment. All the other chemicals were used as received without further purification.

### Synthesis of DAT- and DMT-functionalized dendrimers

DAT- and DMT-functionalized G3 PAMAM dendrimer is synthesized by the following method. Briefly, to a 50% (v/v) ethanol solution, G3 PAMAM dendrimer and 2-chloro-4,6-diamino-1,3,5-triazine or 2-chloro-4,6-dimethoxy-1,3,5-triazine were dissolved at a molar ratio of 16. The solution was stirred at 80 $^{o}$C for 24 h. $NaHCO_3$ was added to remove the yielding acid. The products were dialyzed (MWCO, 3500 Da) against double-distilled water and lyophilized as white powders. The products were characterized by $^{1}$H and 2D COSY NMR in d6-DMSO (699.804 MHz).

The CyA titration and temperature variation experiments were conducted in $D_2O$ or d6-DMSO.

DOSY NMR was conducted by a standard PGSE sequence on the same NMR instrument at 298.2 $\pm$ 0.1 K. The heater and cooling unit was switched on to reach and stabilize the desired temperature, which avoids the influence of temperature variation on diffusion measurement. The time interval ($\Delta$) between gradient pulses is 100 ms and 300 ms for internal standard dioxane and dendrimer conjugate, respectively. The duration time of gradient pulses ($\delta$) is 3 ms. The recycle time is 10 s. The pulse gradients (g) linearly increased in 16 steps to attenuate the spin-echo signal,

and the maximum gradient strength is 70 G/cm. The gradient pulse was calibrated on a mixture of $D_2O$ and $H_2O$ (10% $D_2O$ and 90% $H_2O$) under the same experimental conditions. The diffusion coefficients (*D*) of the amino acids were obtained by fitting the spin-echo signal and gradient strength by the following equation:

$$I_n = I_0 \exp [-\gamma^2 D \delta^2 (\Delta - \delta/3) g^2]$$

where $I_n$ and $I_0$ are the intensities of spin-echo signal when the sine-shaped field gradient is present and absent, respectively, and γ represents the proton magnetogyric ratio ($2.68 \times 10^8$ $s^{-1}$ $T^{-1}$).

Dendrimers conjugates in the system can be regarded as spheres; we can describe their diffusion coefficients using an Einstein-Stokes equation.

$$D = kT / 6\pi\eta R_s$$

where *k* is the Boltzmann constant, *T* is the systematic temperature, $\eta$ is the viscosity of the solution, and $R_s$ is the hydrodynamic radius of the particle.

**Characterization of G3-$DAT_{13}$/DNA and G3-$DAT_{13}$/DNA/CyA polyplexes**

G3-$DAT_{13}$/DNA polyplexes were prepared by mixing 0.8 μg plasmid DNA (luciferase or EGFP) with G3-$DAT_{13}$ at different N/P ratios. N represents the number of surface primary amine groups. The two amine groups on DAT rings were not included because they are not protonated at pH 7.4 (pKa~5.1). P represents the number of phosphate anions in the DNA chain. The G3-$DAT_{13}$/DNA/CyA polyplexes were prepared as follows: CyA was dissolved in TE buffer (Tris buffer, 50 mM Tris-HCl, 10 mM EDTA, pH 8.0) by heating at a concentration of 5 mg/mL. Then different amounts of cyanuric acid were added to the G3-$DAT_{13}$/DNA polyplex solution. The prepared G3-$DAT_{13}$/DNA/CyA polyplexes were kept at room temperature for 20 min. The size and zeta potential of the prepared G3-$DAT_{13}$/DNA and G3-$DAT_{13}$/DNA/CyA polyplexes were measured by dynamic light scattering (Malvern Zetasizer Nano ZS90, Malvern, UK). The morphology of the prepared polyplexes was observed by transmission electron microscope (JEM-2010, JEOL, Japan).

**Ethidium bromide (EB) displacement assay**

2.5 μg EB was mixed with 10 μg luciferase plasmids and incubated at room temperature for 5 min in the dark. G3-$DAT_{13}$ was added into the DNA/EB solution at an N/P ratio of 64. Then, different molar ratios of CyA to DAT were added and the solutions were diluted to 1 mL by distilled water. Fluorescence intensity of the polyplex solution was measured using a fluorescence spectrophotometer (Hitachi, F-4500) with excitation and emission wavelengths at 540 and 600 nm, respectively.

**Agarose gel retardation assay**

0.8 μg luciferase plasmid was complexed with G3-$DAT_{13}$ at an experimental N/P ratio. CyA solution (5 mg/mL in TE buffer) was added to the polyplex. The molar ratio of CyA to DAT is set according to the need of experiment. Different concentrations of heparin (50, 100, 200, 300 μM) were added as a polyanionic competitor to release the bound plasmid DNA. The G3-$DAT_{13}$/DNA polyplexes in the absence of CyA was tested as controls.

**Cell culture**

HEK293 (a human embryonic kidney cell line, ATCC) and COS-7 (a fibroblast-like cell line derived from monkey kidney, ATCC) cells were used to evaluate in vitro transfection efficacy and cytotoxicity. The cells were grown in Dulbecco's modified Eagle's medium (DMEM, GIBCO Inc.) containing penicillin sulphate (100 units/mL), streptomycin (100 μg/mL) and 10% heat-inactivated fetal calf serum (FCS, GIBCO Inc.) at 37°C in a humidified 5% $CO_2$ and 95% air atmosphere.

**In vitro gene transfection**

Cells were cultured in 24-well plates for 24 h before gene transfection. 0.8 μg luciferase or EGFP plasmids were complexed with G3-$DAT_{13}$ at different N/P ratios. Then, different amounts of CyA were added into the G3-$DAT_{13}$/DNA polyplexes. The polyplexes were diluted to 250 μL using DMEM containing 10% serum and kept at room temperature for 20 min before incubation with cells. Then the cells were

incubated with the polyplexes for 6 h, followed by the addition of 500 μL fresh DMEM (containing 10% serum) to each well. bPEI 25KD and G3 PAMAM was tested as control at its optimal N/P ratio of 8. For EGFP transfection, the cells were digested with trypsin after 48 h of transfection. EGFP expressions in the cells were observed by a fluorescence microscopy (Olympus, Japan). Flow cytometry experiments were performed to quantitatively analyze the EGFP expressions. For luciferase transfection, the luciferase activity of transfected cells (relative luciferase light units/mg protein, RLU/mg protein) was performed according to the manufacture's protocols (Promega).

**Cytotoxicity assay**

MTT assay was performed to evaluate the cytotoxicity of CyA on G3-$DAT_{13}$ and G3-$DAT_{13}$/DNA polyplexes. HEK293 cells were seeded in 96-well plates in 100 μL DMEM overnight. The culture medium was replaced with DMEM containing polyplexes at concentrations equal to those used in gene transfection experiments. After incubation for 48 h, the medium were removed and 100 μL MTT solutions were added. The yielding formazan crystals were dissolved using DMSO. Absorbances of the solution in each well were detected at a wavelength of 490 nm. Five repeats were conducted for each sample.

**Cell uptake assay**

HEK293 or COS-7 cells were cultured in 24-well plates overnight. 20 μg luciferase plasmids were mixed with 4 μL YOYO-1 (100 μM) and incubated in the dark for 10 min. Then the labeled DNA was complexed with G3-$DAT_{13}$ conjugates. Then, CyA was added to the G3-$DAT_{13}$/DNA polyplex solution (CyA to DAT molar ratio of 4). The polyplex in the absence of CyA was tested as a control. The formed polyplexes were incubated with HEK293 cells for 0.5, 1, 2, and 4 h before the removal of polyplex containing medium. The cells were digested with trypsin and the cellular uptake of the YOYO-1 labeled polyplexes was analyzed by flow cytometry. Data were given as means ± S.E.M and analyzed by students' t-test.