Supplementary Information

Hydrogen-bond Dramatically Modulates Gene Transfection Efficacy of Surface-engineered Dendrimers

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Table S1. Characterization of the synthesized PAMAM dendrimer-DAT conjugates

Synthesized polymers	Feeding amount (per dendrimer)	Conjugated amount (per dendrimer)	Molecular Weight* (Da)
G5-DAT$_{29}$	32	29	31988
G5-DAT$_{46}$	51	46	33842
G5-DAT$_{55}$	64	55	34824
G5-DAT$_{66}$	77	66	36023
G5-DAT$_{80}$	96	80	37550
G4-DAT$_{30}$	32	30	17485

*Molecular weight of the synthesized dendrimer-DAT conjugates in Table S1 was calculated according to the theoretical molecular weight of dendrimer and number of DAT moieties conjugated to each dendrimer.

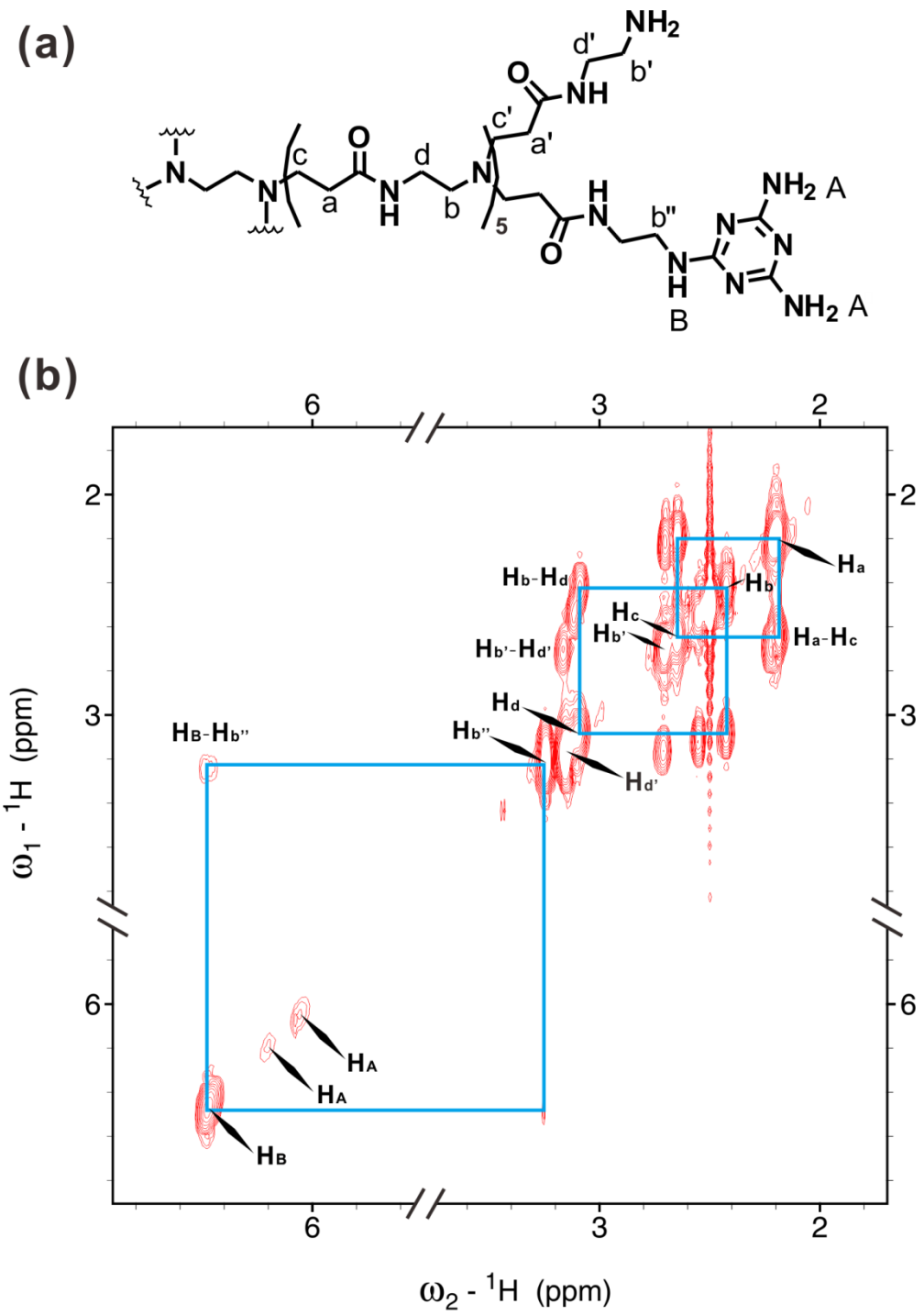

Fig. S1. (a) Chemical structure of the synthesized G5-DAT conjugates with proton labeling. (b) The COSY spectrum of G5-DAT_{66} confirmed the chemical shift assignments of H_B in G5-DAT_{66} due to the presence of cross-peaks between H_B and $H_{b''}$.

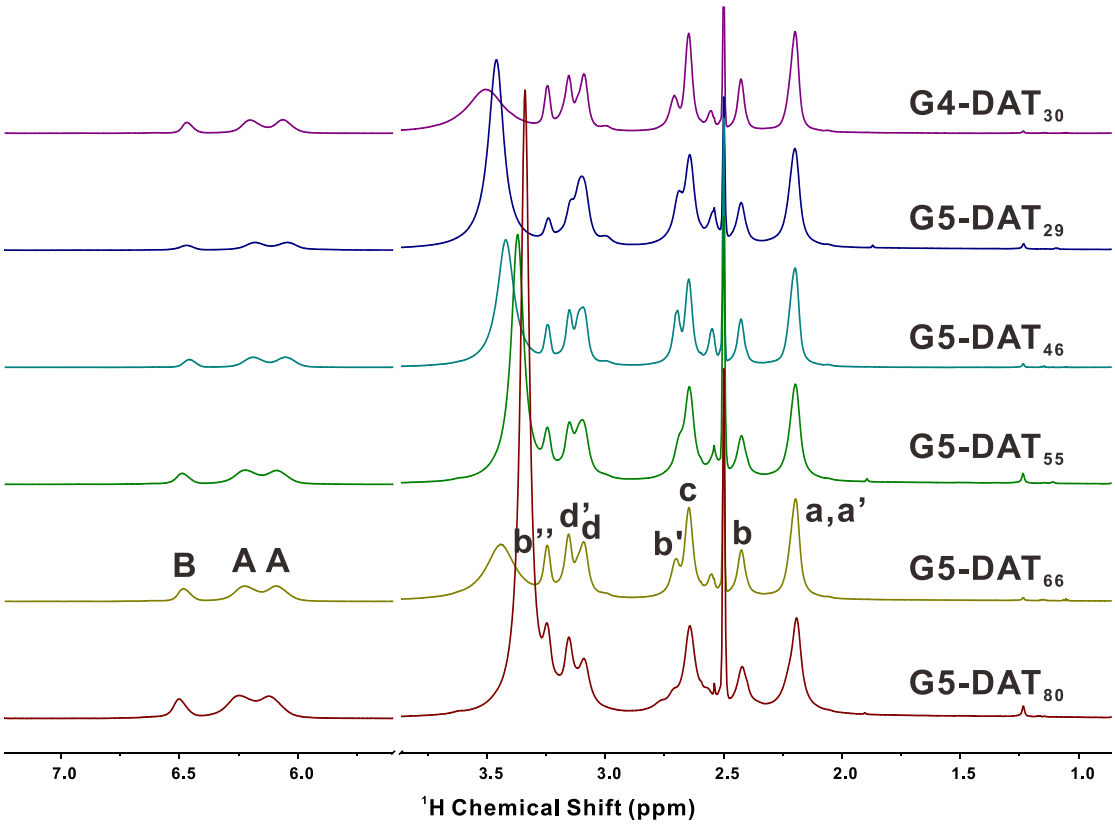

Fig. S2. ^{1}H NMR spectra of the synthesized dendrimer-DAT conjugates. The number of DAT moieties conjugated on the surface of each G4 or G5 PAMAM dendrimer was calculated according to the intensities of dendrimer protons ($H_{a,a'}$) and DAT protons (H_A, H_B).

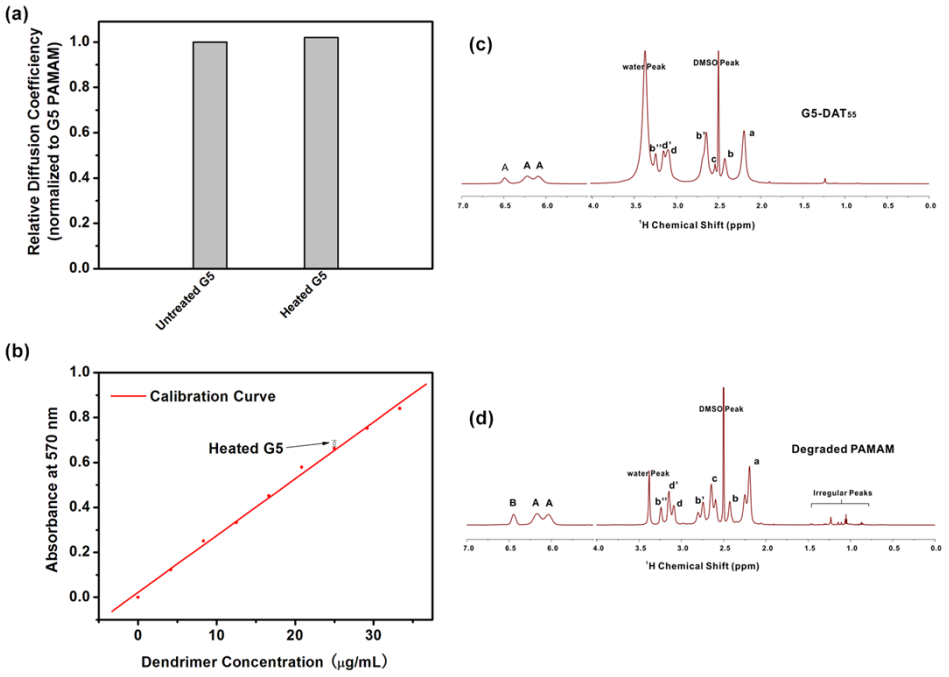

Fig. S3. (a) Relative diffusion coefficient of untreated G5 PAMAM dendrimer and heat-treated G5 PAMAM dendrimer at 80 °C. The diffusion coefficient of both dendrimers was relative to that of an internal standard-dioxane. (b) The density of primary amine groups on G5 PAMAM dendrimer before and after heat-treatment at 80 °C analyzed by a ninhydrin assay. The calibration curve is made by untreated G5 PAMAM dendrimer. The arrow indicates the point (black square) for heated G5 PAMAM dendrimer at 80 °C for 24 h. No significant change in primary amine density on G5 dendrimer before and after heat-treatment is observed. (c and d) The ^{1}H NMR spectra of G5-DAT_{55} synthesized at 80 °C (c) and 100 °C (d) in D6-DMSO. As shown in Fig. S3c, minimal degradation on dendrimer structure (0.8-1.5 ppm) is observed in the ^{1}H NMR spectrum of G5-DAT_{55}, while the material synthesized at a higher temperature such as 100 °C show obvious degradation on the dendrimer structure (0.8-1.5 ppm).

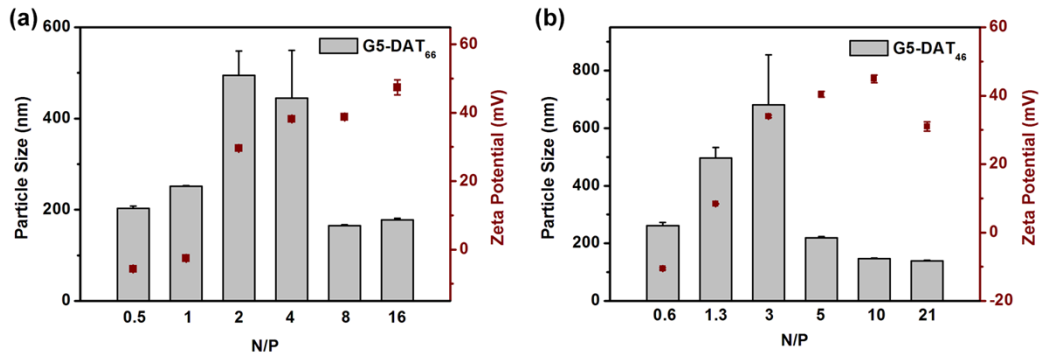

Fig. S4. Characterization of G5-DAT_{66}/DNA (a) and G5-DAT_{46}/DNA (b) polyplexes using dynamic light scattering. Error bars in (b) and (c) represent the s.e. (n = 3).

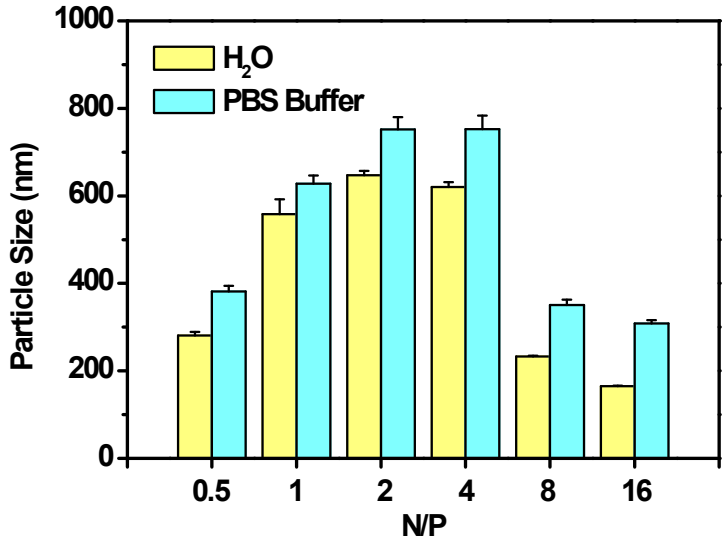

Figure S5. Characterization of size (a) and zeta-potential (b) of G5-DAT_{66}/DNA polyplexes at different N/P ratios.

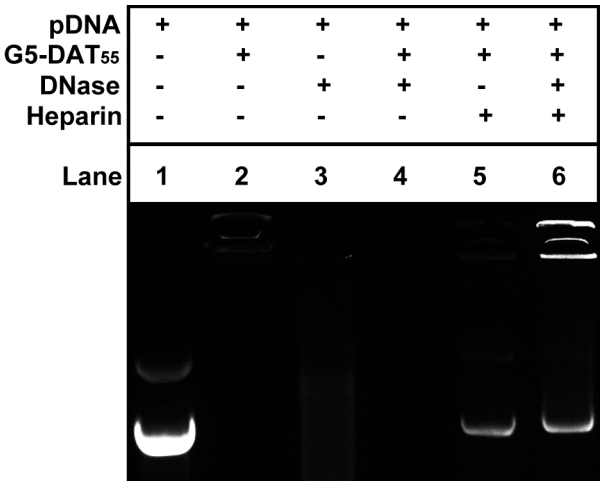

Fig. S6. DNase protection assay. Lane 1, luciferase plasmid DNA. Lane 2, G5-DAT_{55}/DNA polypexes at an N/P ratio of 9:1. Lane 3, luciferase plasmid DNA treated with DNase for 2 h. Lane 4, G5-DAT_{55}/DNA polyplexes at an N/P ratio of 9:1 treated with DNase for 2 h. Lane 5, G5-DAT_{55}/DNA polyplexes in the presence of heparin. Lane 6, G5-DAT_{55}/DNA polyplexes treated with DNase for 2h, followed by heat-inactivation of the DNase, and release of the bound DNA by heparin.

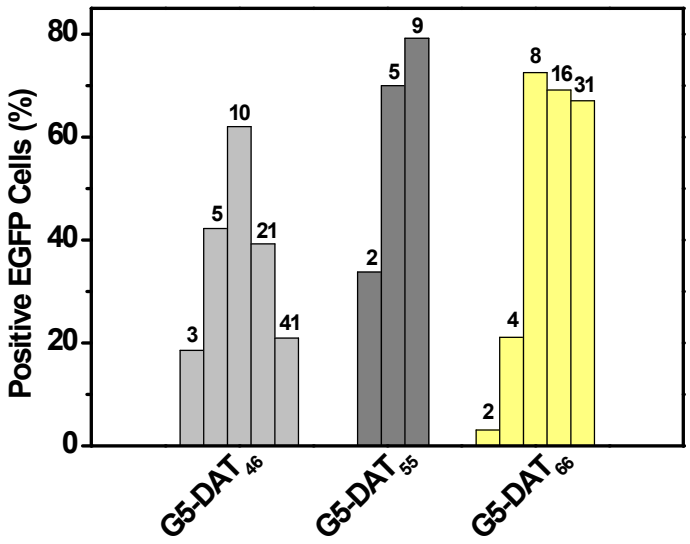

Fig. S7. Screening the optimal N/P ratios for G5-DAT_{46}, G5-DAT_{55} and G5-DAT_{66} in HEK293 cells. Cells were incubated with the polyplexes for 48 h. The positive EGFP cells after transfection were analyzed using flow cytometry. The corresponding N/P ratios were labeled on the top of each column. The optimized N/P ratio for each material is chosen at which the highest transfection efficacy is achieved and the polyplex is not cytotoxic on the transfected cells. The optimal N/P ratios are 10:1, 9:1 and 8:1 for G5-DAT_{46}, G5-DAT_{55} and G5-DAT_{66}, respectively.

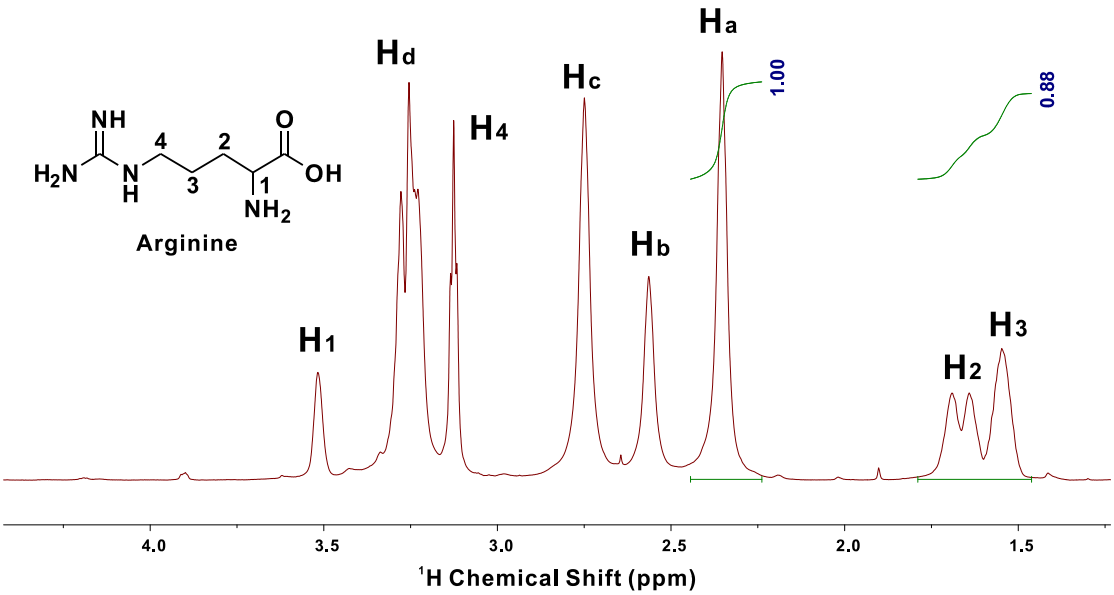

Fig. S8. ^{1}H NMR spectra of the synthesized G5-Arg_{110}. The number of arginine molecules conjugated to each G5 PAMAM dendrimer was calculated according to the peak areas of dendrimer protons (H_a) and arginine protons (H_2, H_3).

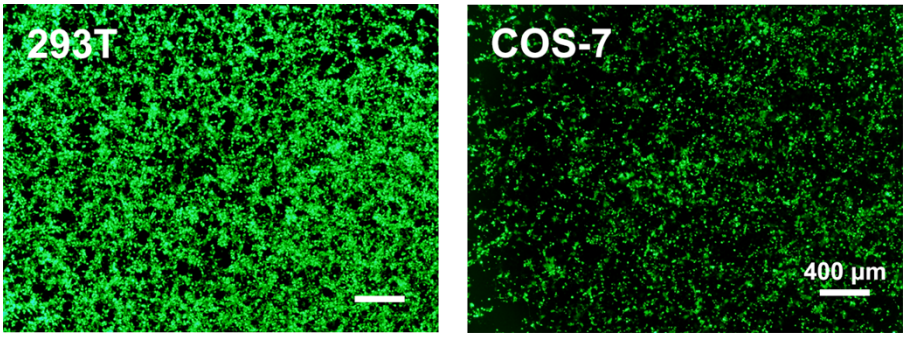

Fig. S9. EGFP expressions in HEK293 (a) and COS-7 (b) cells mediated by G5-DAT_{29} at optimal N/P ratios (6:1 on HEK293 cells and 12:1 on COS-7 cells). G5-DAT_{29} shows much lower efficacy than G5-DAT_{46}, G5-DAT_{55} and G5-DAT_{66} on delivering EGFP plasmids into both cells.

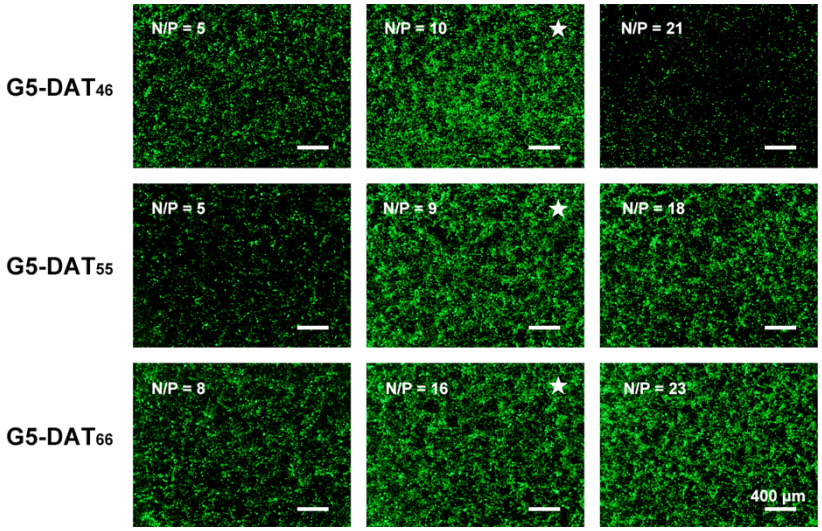

Fig. S10. Screening the optimal N/P ratios for G5-DAT_{46}, G5-DAT_{55} and G5-DAT_{66} in COS-7 cells. Cells were incubated with the polyplexes for 48 h. ⋆represents the optimal N/P ratio (10:1, 9:1 and 16:1 for G5-DAT_{46}, G5-DAT_{55} and G5-DAT_{66}, respectively). The optimized N/P ratio for each material is chosen at which the highest transfection efficacy is achieved and the polyplex is not cytotoxic on the transfected cells.

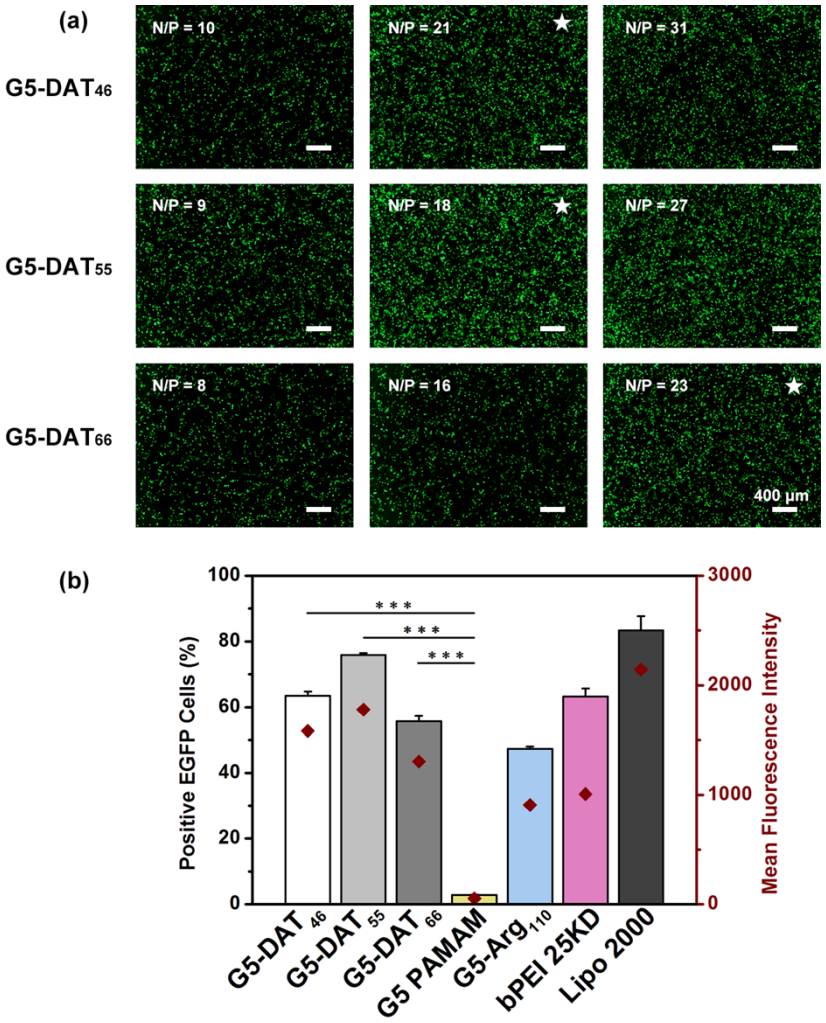

Fig. S11. (a) Screening the optimal N/P ratios for G5-DAT_{46}, G5-DAT_{55} and G5-DAT_{66} in CHO cells. The cells were incubated with the polyplexes for 48 h. ⋆represents the optimal N/P ratio (21:1, 18:1 and 23:1 for G5-DAT_{46}, G5-DAT_{55} and G5-DAT_{66}, respectively). The optimized N/P ratio for each material is chosen at which the highest transfection efficacy is achieved and the polyplex is not cytotoxic on the transfected cells. (b) EGFP expressions in CHO cells mediated by G5-DAT_{46}, G5-DAT_{55}, and G5-DAT_{66}. G5 PAMAM, G5-Arg_{110}, bPEI 25KD and Lipo 2000 were used as controls. Positive EGFP cells after transfection were analyzed using flow cytometry. Diamonds represent mean fluorescence intensity. Error bars represent the s.e. (n = 3). ***$p < 0.001$ by students' t-test.

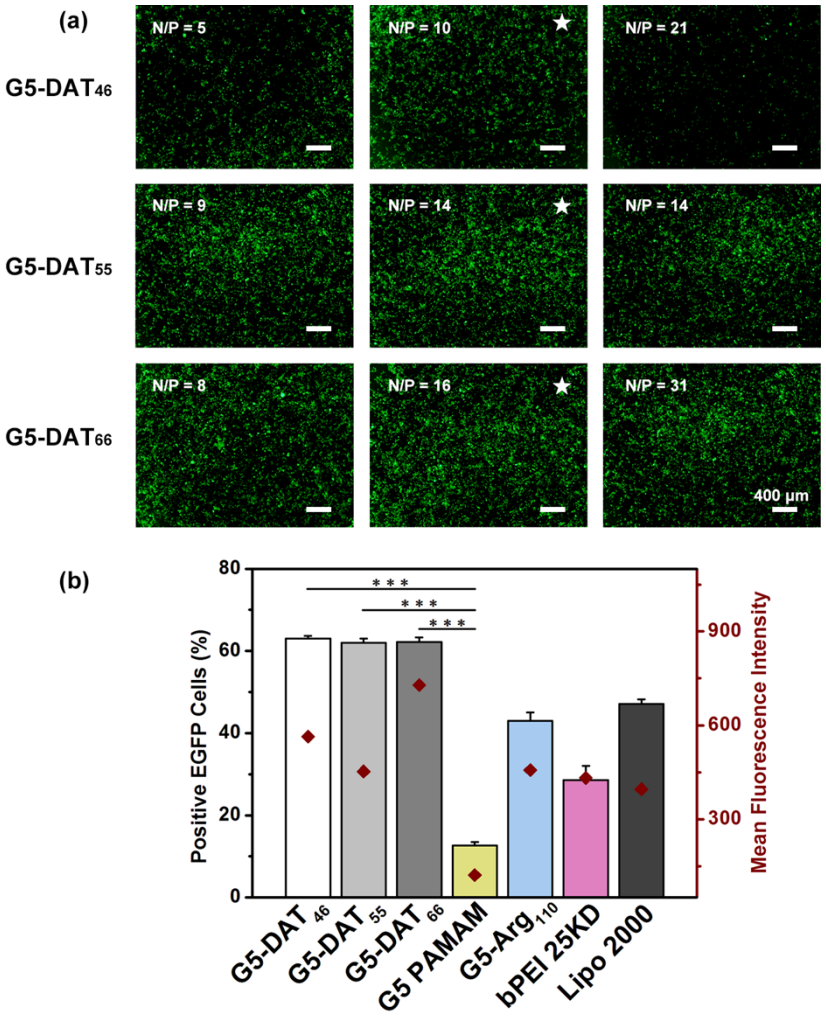

Fig. S12. (a) Screening the optimal N/P ratios for G5-DAT_{46}, G5-DAT_{55} and G5-DAT_{66} in HeLa cells. The cells were incubated with the polyplexes for 48 h. ⋆represents the optimal N/P ratio (10:1, 14:1 and 16:1 for G5-DAT_{46}, G5-DAT_{55} and G5-DAT_{66}, respectively). The optimized N/P ratio for each material is chosen at which the highest transfection efficacy is achieved and the polyplex is not cytotoxic on the transfected cells. (b) EGFP expressions in HeLa cells mediated by G5-DAT_{46}, G5-DAT_{55}, and G5-DAT_{66}. G5 PAMAM, G5-Arg_{110}, bPEI 25KD and Lipo 2000 were used as controls. Positive EGFP cells after transfection were analyzed using flow cytometry. Diamonds represent mean fluorescence intensity. Error bars represent the s.e. (n = 3). ***p < 0.001 by students' t-test.

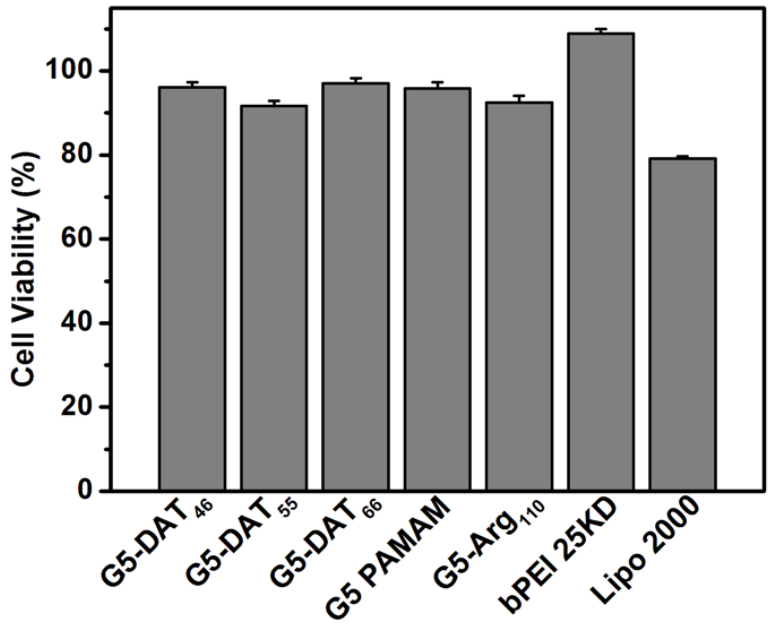

Fig. S13. Cell viabilities of the synthesized materials (G5-DAT_{46}, G5-DAT_{55} and G5-DAT_{66}) on COS-7 cells at their optimal N/P ratios determined by an MTT assay. G5-Arg_{110}, bPEI 25KD and Lipo 2000 were used as controls at their optimal tranfection conditions. Error bars represent the s.e. (n = 6).

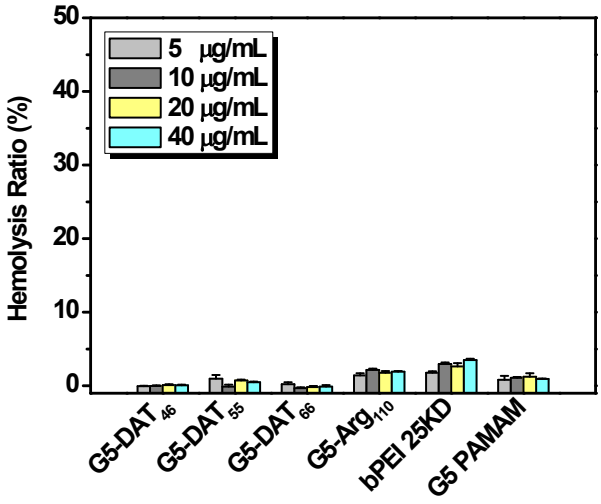

Fig. S14. Hemolytic activities of G5-DAT_{46}, G5-DAT_{55} and G5-DAT_{66} at concentrations of 5, 10, 20 and 40 μg/mL. Hemolytic activities of G5 PAMAM dendrimer, G5-Arg_{110} and bPEI 25KD were tested as controls.

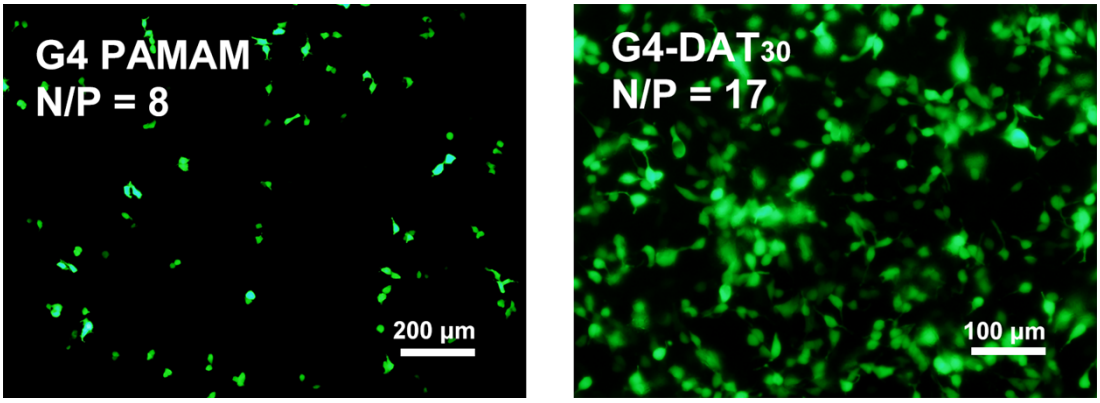

Fig. S15. EGFP expressions in HEK293 cells mediated by G4 PAMAM and G4-DAT_{30} at their optimal N/P ratios (8:1 and 17:1 for G4 and G4-DAT_{30}, respectively). DAT functionalization significantly improves the transfection efficacy of G4 PAMAM dendrimer.

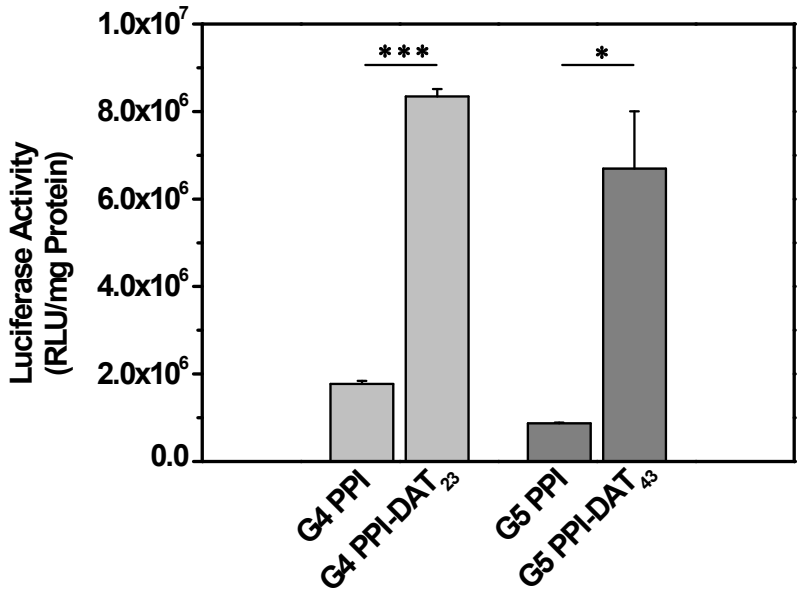

Fig. S16. Luciferase expressions in HEK293 cells transfected by G4, G5 PPI dendrimers and DAT-modified G4 and G5 PPI dendrimers (G4 PPI-DAT_{23} and G5 PPI-DAT_{43}) at their optimal N/P ratios (16:1, 3:1, 16:1 and 3:1 for G4 PPI, G4 PPI-DAT_{23}, G5 PPI and G5 PPI-DAT_{43}, respectively). Error bars represent the s.e. (n = 3). *$p < 0.05$, ***$p < 0.001$ by students' t-test.

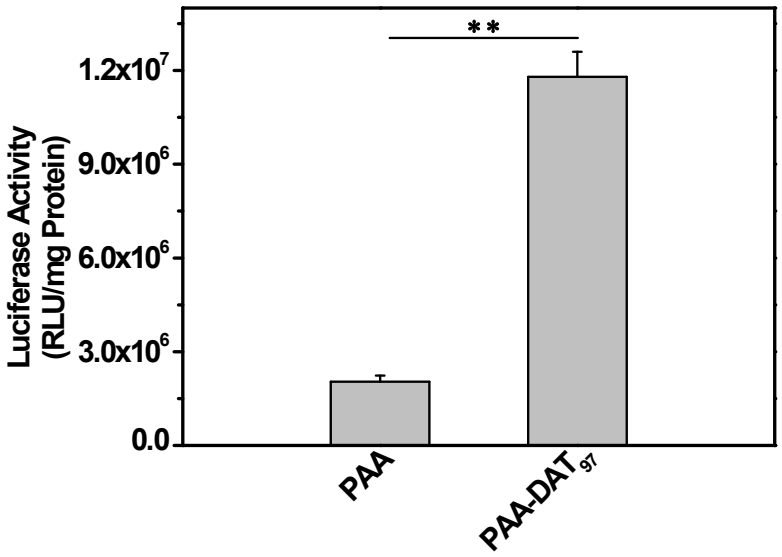

Fig. S17. Luciferase expressions in HEK293 cells transfected by poly(allylamine) (PAA) with a molecular weight of 17000 Da and PAA-DAT_{97} at their optimal N/P ratios (6:1 and 10:1 for PAA and PAA-DAT_{97}, respectively). Error bars represent the s.e. (n = 3). **p < 0.01 by students' t-test.

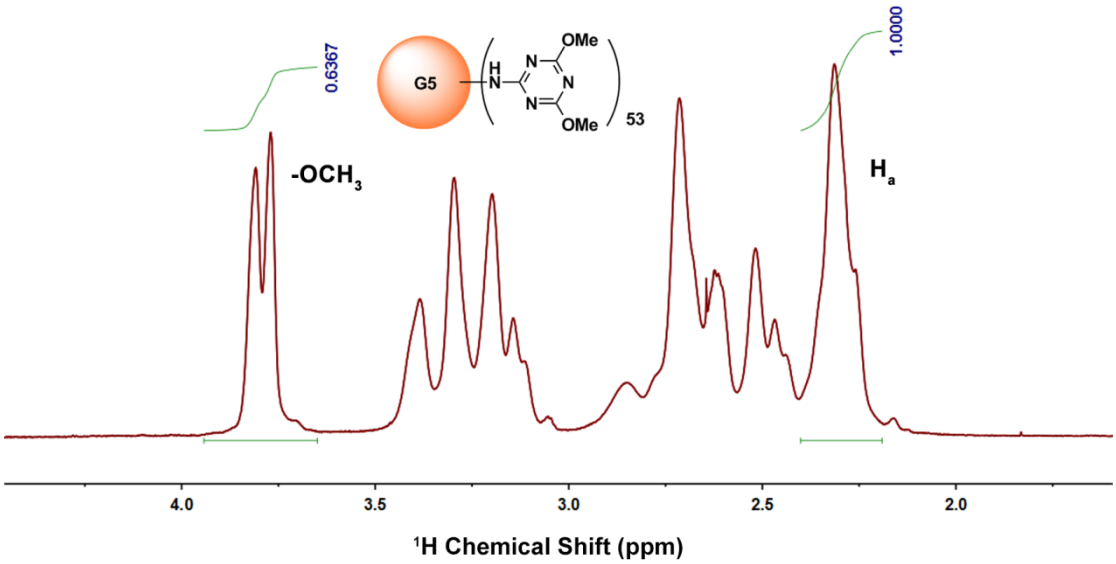

Fig. S18. ^{1}H NMR spectra of the synthesized G5-DMT$_{53}$. The number of DMT moieties conjugated to each G5 PAMAM dendrimer was calculated according to the peak areas of dendrimer protons (H_a) and DMT protons (-OCH_3).

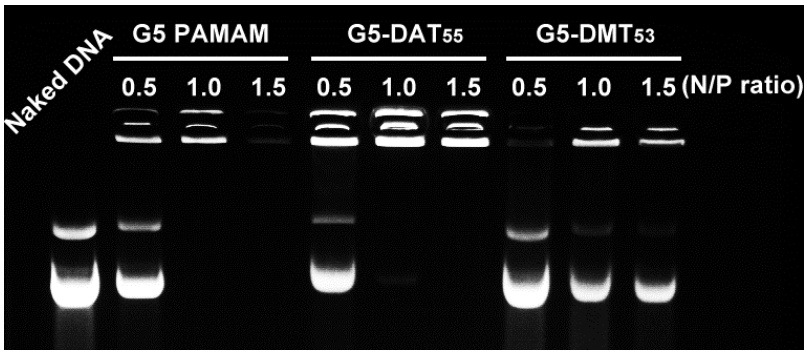

Figure S19. Comparison of DNA condensation ability among G5 PAMAM, G5-DAT_{55} and G5-DMT_{53} at an N/P ratio of 0.5:1, 1:1 and 1.5:1 using agarose gel electrophoresis. G5-DMT_{53} shows much lower DNA condensation ability as compared to G5-DAT_{55} with similar charge density and number of modified ligands. The result suggests that the hydrogen bonds between DAT and the DNA nucleobases are essential for DNA condensation.

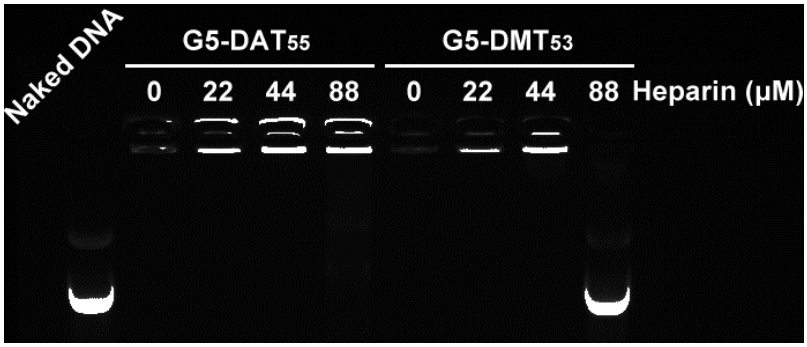

Figure S20. DNA release from the G5-DAT_{55}/DNA and G5-DMT_{53}/DNA polyplexes in the presence of different concentrations of heparin (0, 22, 44 and 88 μM, respectively). The polyplexes were prepared at an N/P ratio of 8:1. The G5-DAT_{55}/DNA polyplex is much more stable than the G5-DMT_{53}/DNA complex, which suggests that the hydrogen bonds between DAT and the DNA nucleobases are essential for polyplex stability.

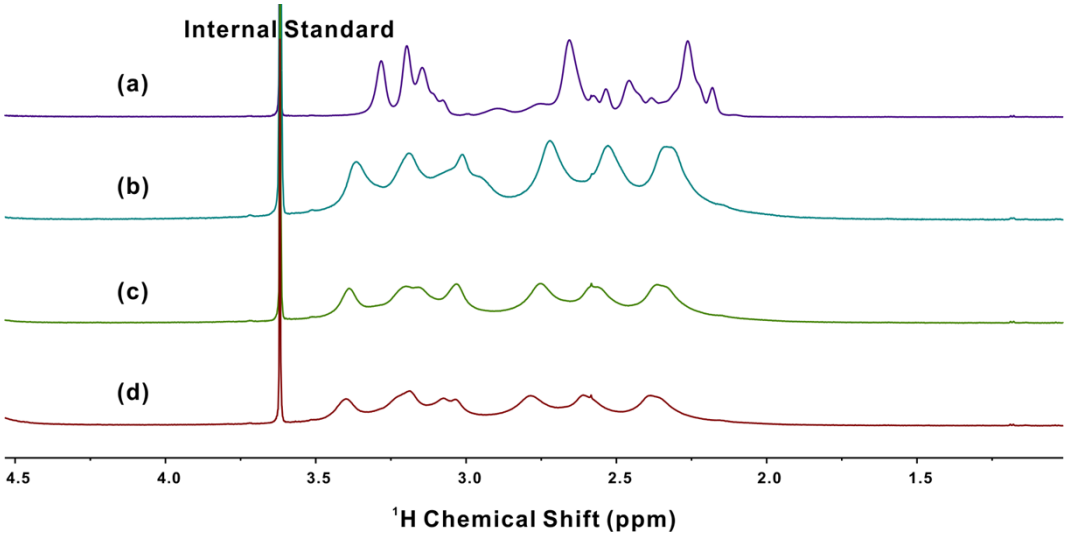

Figure S21. ^{1}H NMR spectra of G5-DAT$_{55}$ and G5-DAT$_{55}$/cyanuric acid complexes in D_2O at 25 ^{o}C. The molar equivalents of cyanuric acid to DAT are 0:1 (a), 2:1 (b), 4:1 (c) and 8:1 (d), respectively.

Note: The peaks of dendrimers become broaden after the addition of cyanuric, suggesting the formation of large G5-DAT$_{55}$/cyanuric acid aggregates. The formation of aggregates is driven by hydrogen bonds between cyanuric acids and the DAT moieties on G5-DAT$_{55}$.

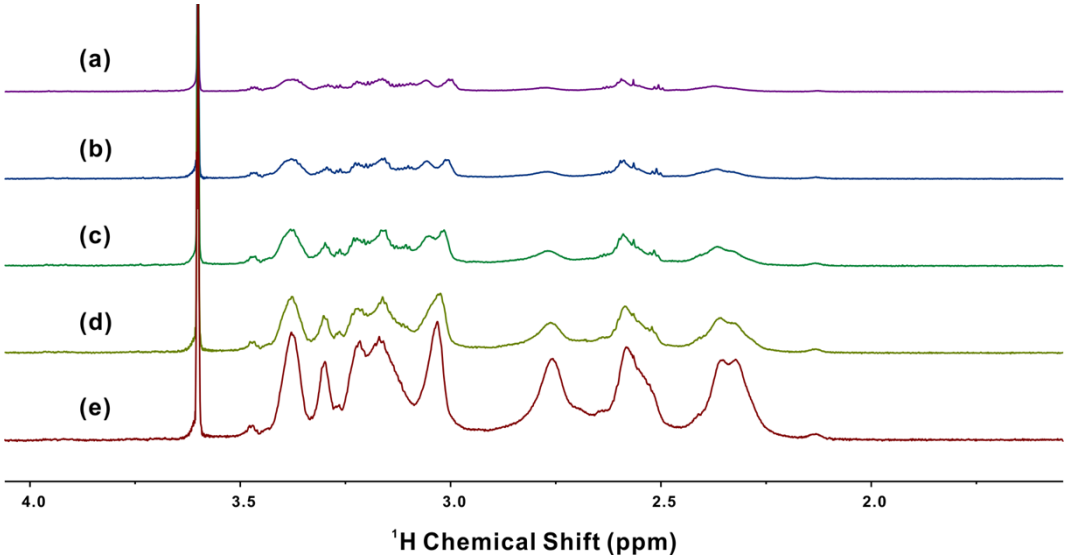

Figure S22. ^{1}H NMR spectra of G5-DAT$_{55}$/cyanuric acid complexes (the molar equivalents of cyanuric acid to DAT is 8:1) at different temperatures. (a) 25 ^{o}C, (b) 35 ^{o}C, (c) 45 ^{o}C, (d) 55 ^{o}C and (e) 65 ^{o}C.

Note: The peaks of G5-DAT$_{55}$/cyanuric acid complex become sharp at higher temperatures such as 65 ^{o}C, suggesting that the formation of G5-DAT$_{55}$/cyanuric acid complex is mainly driven by hydrogen bond interactions. Hydrogen bonds at high temperatures such as 65 ^{o}C are destroyed and therefore the sharp peaks are observed for the G5-DAT$_{55}$/cyanuric acid complex at 65 ^{o}C.

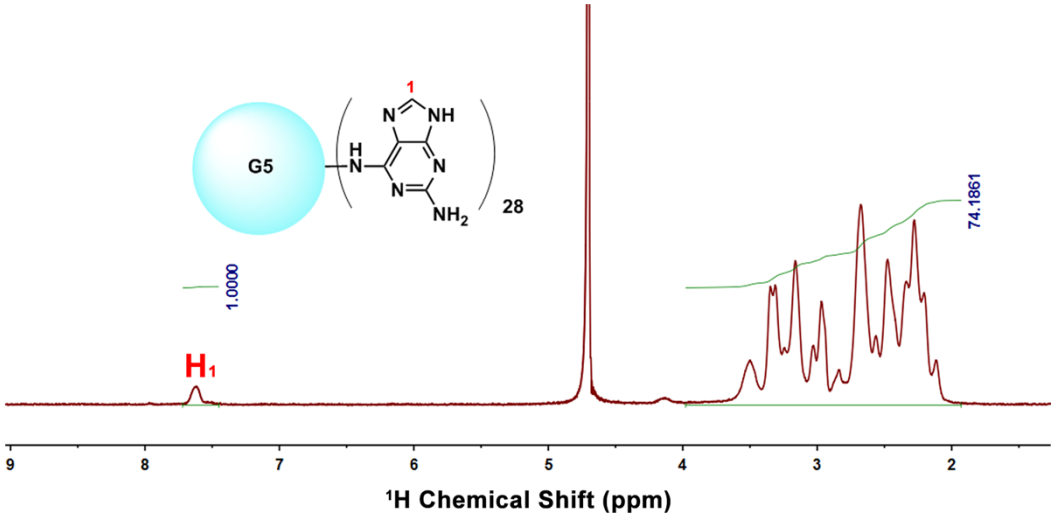

Fig. S23. ^{1}H NMR spectra of the synthesized G5-APu$_{28}$. The number of APu conjugated to each G5 PAMAM dendrimer was calculated according to the peak areas of dendrimer protons (H_{a-d}) and Apu protons (H_1).

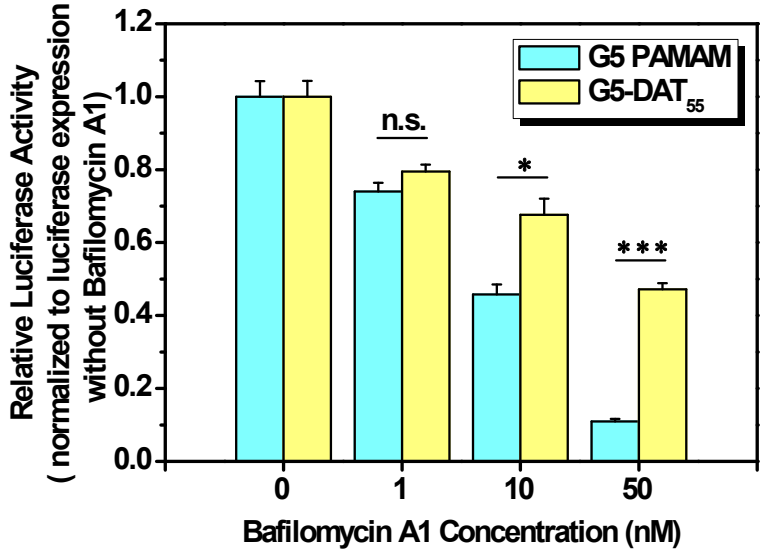

Fig. S24. Inhibition of luciferase gene expression in COS-7 cells transfected by G5-DAT_{55}/DNA and G5 /DNA polyplexes in the presence of different bafilomycin A1 concentrations (0, 1, 10 and 50 nM). The polyplexes were prepared at their optimal N/P ratios (9:1 and 8:1 for G5-DAT_{55} and G5 PAMAM, respectively). The data were normalized to luciferase activities of G5-DAT_{55}/DNA and G5 PAMAM/DNA polyplexes in the absence of bafilomycin A1, respectively. Error bars represent the s.e. (n = 3). *p < 0.05, ***p < 0.001 by students' t-test, n. s. represents not significant.

Note: Bafilomycin A1 inhibits the acidification in endosome and lysosome, which reduces endosomal escape of the polyplexes. As shown in Fig. S18, the addition of bafilomycin A1 significantly decreases the gene transfection efficacies of both materials. However, G5 PAMAM dendrimer is more sensitive to bafilomycin A1 than G5-DAT_{55}, suggesting that DAT modification on G5 PAMAM dendrimer surface does not increase the endosomal escape of G5 PAMAM/DNA polyplexes.

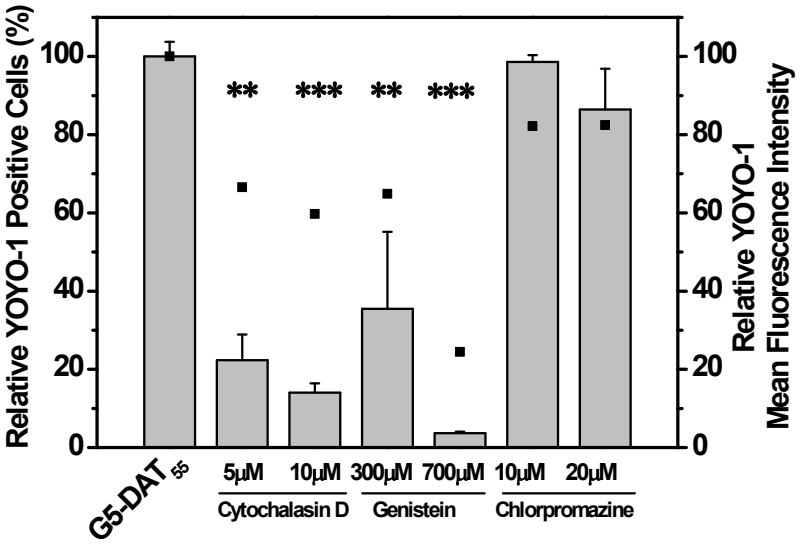

Fig. S25 Cellular uptake pathways of G5-DAT_{55}/DNA polyplexes. DNA was labeled with YOYO-1 before the formation of polyplexes. COS-7 cells were pretreated with genistein (350 and 700 μM), cytochalasin D (5 and 10 μM), and chlorpromazine (10 and 20 μM) for 1 h before the addition of polyplexes. Uptake efficacy of the polyplexes was evaluated by flow cytometry after 30 min incubation. (Column: relative positive YOYO-1 cells; Square: relative mean fluorescence intensity).

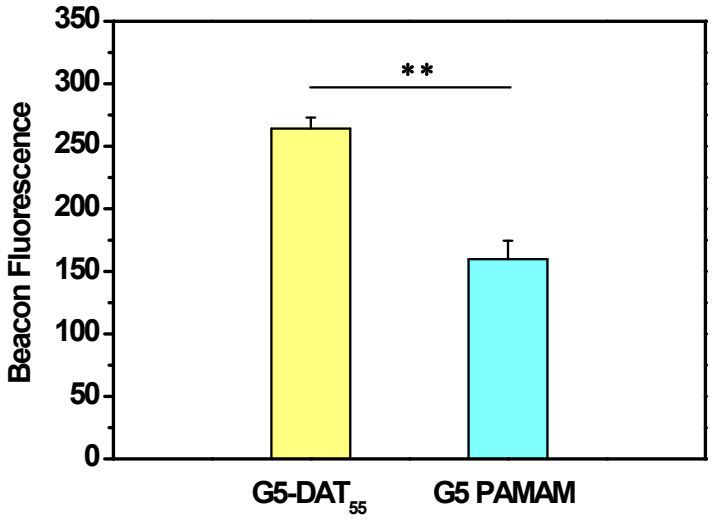

Fig. S26. The cytosolic nuclease activity in COS-7 cells transfected by G5-DAT_{55}/DNA and G5 PAMAM/DNA polyplexes at their optimal N/P ratios (9:1 and 8:1 for G5-DAT_{55} and G5 PAMAM, respectively) analyzed by an oligonucleotide molecular beacon assay. Higher beacon fluorensence of the cells indicates higher level of cytosolic nuclease activated. Error bars represent the s.e. (n = 3). **p < 0.01 by students' t-test.

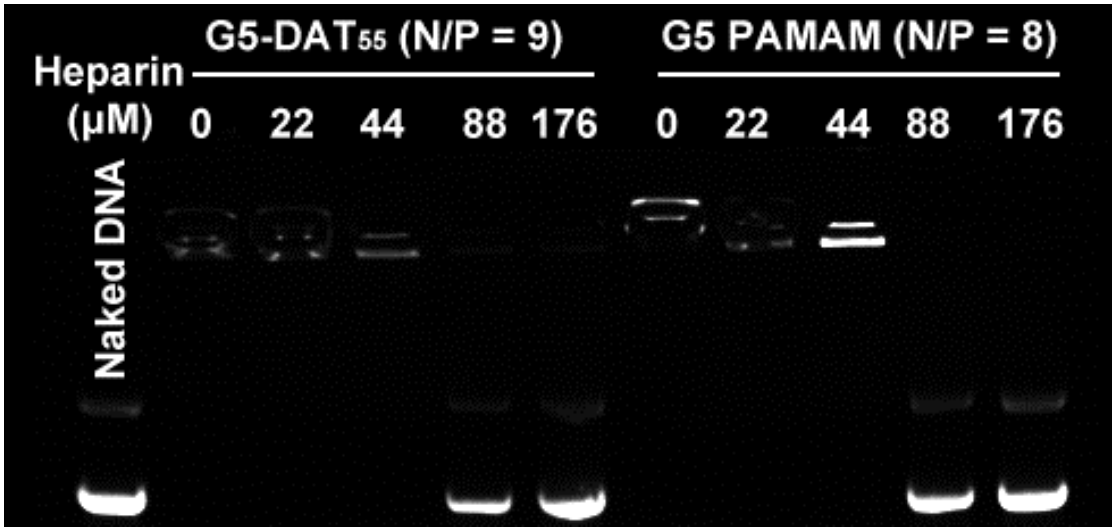

Fig. S27. DNA release from the G5-DAT_{55}/DNA and G5 PAMAM/DNA polyplexes in the presence of different concentrations of heparin (0, 22, 44, 88 and 176 μM, respectively). The polyplexes were prepared at their optimal N/P ratios (9:1 for G5-DAT_{55} and 8:1 for G5 PAMAM). No obvious difference in DNA release profile is observed for G5-DAT_{55}/DNA and G5 PAMAM/DNA polyplexes.

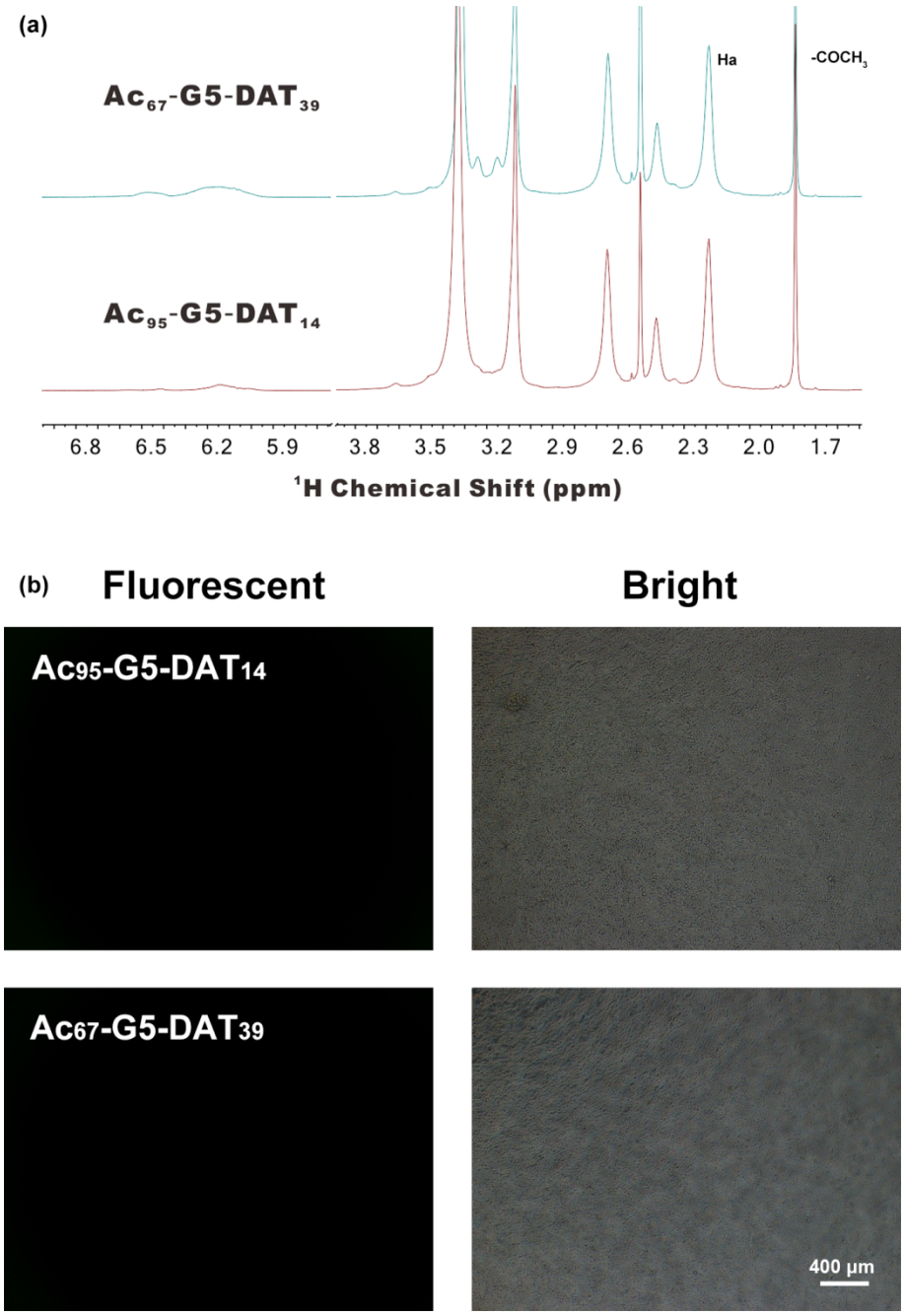

Fig. S28. (a) ^{1}H NMR spectra of the synthesized Ac_{67}-G5-DAT_{39} and Ac_{95}-G5-DAT_{14}. (b) EGFP expressions in HEK293 cells mediated by Ac_{67}-G5-DAT_{39} (w/w = 56.5:1) and Ac_{95}-G5-DAT_{14} (w/w = 100:1).

Note: To remove the residual amine groups on the surface of dendrimer-DAT conjugates, G5 PAMAM dendrimer was partially acetylated. The acetylated dendrimers (Ac_{67}-G5 and Ac_{95}-G5, average numbers of 67 and 95 acetyl groups were

modified on each G5 PAMAM dendrimer, respectively) were purified and followed by reacting with 2-chloro-4,6-diamino-1,3,5-triazine as described in the Methods Section. The products were characterized by ^{1}H NMR. As calculated from the ^{1}H NMR spectra in Fig. S21a, the final products are termed Ac_{67}-G5-DAT_{39} and Ac_{95}-G5-DAT_{14}, respectively.

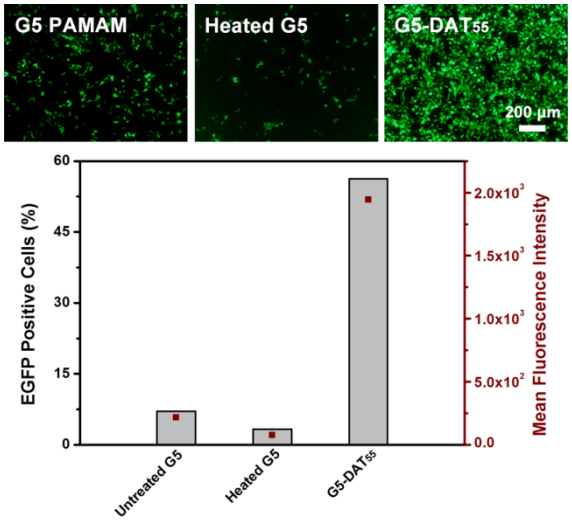

Fig. S29. Transfection efficacy of un-treated G5 PAMAM dendrimer, heat-treated G5 dendrimer and G5-DAT_{55} on HEK293 cells. The optimal N/P ratios for these materials are 8:1, 8:1 and 9:1, respectively. The EGFP positive cells are analyzed by flow cytometry.

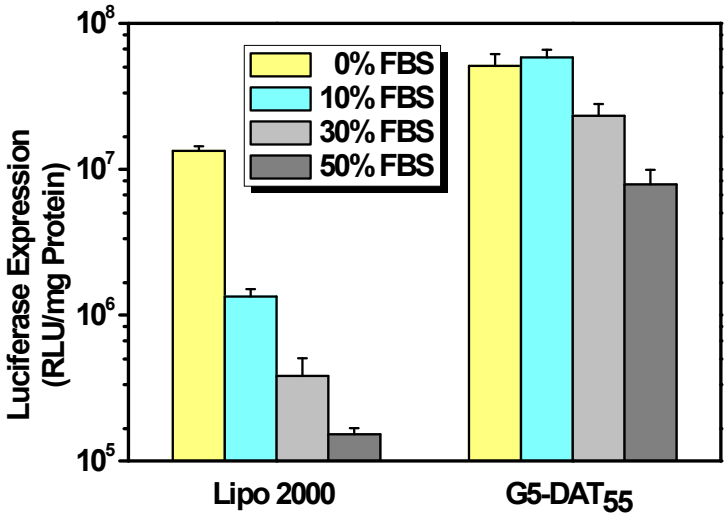

Fig. S30. Luciferase expressions in COS-7 cells transfected by G5-DAT_{55} (N/P = 9:1) and Lipo 2000 (1 μL/1.6 μg DNA) in mediums containing 0%, 10%, 30%, and 50% FBS, respectively. Error bars represent the s.e. (n = 3).