Supporting Information

A biodegradable adamantane polymer with ketal linkages in its backbone for gene therapy

Santanu Maity[a], Priya Choudhary[b], Manu Manjunath[b], Aditya Kulkarni[b], Niren Murthy[a]*

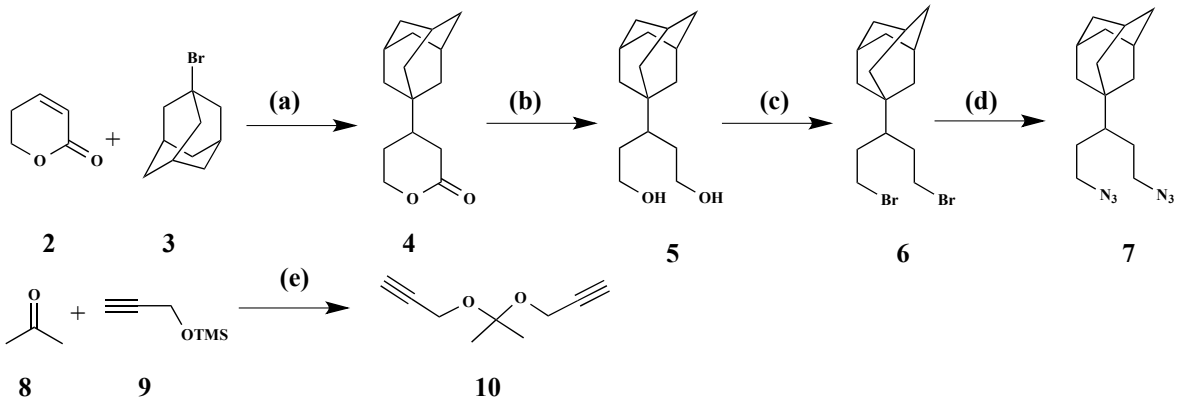
[a]University of California, Berkeley, 140 Hearst Memorial Mining Building, Berkeley, CA 94709
[b]Aten Biotherapeutics Pvt. Ltd., Bangalore, India

Table of content:

S1. Materials and methods:

All chemical reagents used for the synthesis of pADK were purchased from Sigma-Aldrich Chemical Co., Acros organics, Alfa Aesar, AK Scientific and were used without further purification. Anhydrous solvents were purchased from Sigma-Aldrich. Bottle grade solvents were purchased from VWR Internationals and used without further distillation. Silica-gel (mesh size 70-120) was used for purification. All ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were taken with a Bruker (400 MHz). Field emission scanning electron microscopy (SEM) measurements were performed using a Hitachi S 5000.

S2. Synthesis of pADK



Scheme S1: Synthesis of pADK (a) AIBN, Bu_3SnH, Toluene, 100°C, 61%; (b) $LiAlH_4$, THF, rt, 94%; (c) CBr_4, PPh_3, DCM, 0°C to rt, 84%; (d) NaN_3, DMF-water (6:1), 50°C, 97%; (e) TMS-OTf, DCM, -78°C,

S2.1 Synthesis of 4-adamantyl-tetrahydro-2H-pyran-2-one (**4**)

To a stirred solution of toluene (20 mL) was added **3** (4.50 g, 0.02 mol), **2** (19.06 g, 0.19 mol) and tributyltinhydride (6.30 g, 0.021 mol), the solution was heated to 100°C and AIBN (0.34 g, 10 mol%) was added, and refluxed for 2 h. The reaction mixture was cooled down to room temperature. A solution of 0.2 M potassium fluoride (100 mL) was added to the reaction mixture and vigorously stirred overnight under room temperature, and a white solid residue formed. The white solid residue was filtered off and the residue was washed with ethyl acetate (3 × 50 mL). The filtrate was diluted with ethyl acetate (50 mL) and water (50 mL). The resulting solution was extracted and the organic phase was washed with brine (2 × 50 mL). Ethyl acetate was removed under vacuo and the crude product was purified by silica gel column chromatography (hexane/ethyl acetate = 9/1) to afford 3.19 g of **4** as a white solid. (62% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 4.38-4.33 (m, 1H), 4.21-4.15 (m, 1H), 2.58-2.52 (dd, 1H, J = 8 Hz, 16 Hz), 2.35-2.28 (dd, 1H, J = 12 Hz, 16 Hz), 1.98 (br, 3H), 1.87-1.84 (m, 1H), 1.72-1.44 (m, 12 H). ^{13}C (150 MHz, $CDCl_3$)

δ: 68.86 (s), 41.80 (s), 38.82 (s), 37.02 (s), 34.24 (s), 30.37 (s), 28.29 (s), 22.92 (s). HRMS (70 eV, EI): calcd for $C_{15}H_{21}O_2$ $[M]^+$: calcd 233.1620, found: 233.1627.

S2.2 Synthesis of 4-adamantyl-pentane-1,5-di-ol (**5**)

To a stirred solution of dry THF (30 mL) was added **4** (1.02 g, 4.37 mmol) and the solution was cooled down to 0°C. The solution was stirred at 0°C for an additional 10 min, and lithiumaluminiumhydride (0.166 g, 4.37 mmol) was portion wise added. The reaction mixture was stirred for 2 h at 0°C under an inert atmosphere. A mixture of THF/water (5/1) was dropwise added to the reaction mixture at 0°C until gas production ceased. A 5% potassium hydroxide solution (10 mL) was added to the reaction mixture and was stirred vigorously at room temperature for 1 h. $MgSO_4$ (15 g) was added to the reaction mixture and was stirred for 30 min. The white cake was filtered through a celite bed and the filtrate was washed with acetone (5 × 30 mL). The solvent was removed under vacuum and the crude product was purified by flash silica gel column chromatography (hexane/acetone = 3/1) to afford 1.0 g of **5** as a white solid. (85% yield) ^{1}H NMR (400 MHz, $CDCl_3$) δ: 4.05 (s, 2H), 3.49-3.38 (m, 4H), 1.79 (bs, 3H), 1.68-1.67 (m, 2H), 1.55-1.43 (m, 6H), 1.35 (br, 6H), 1.13-1.01 (m, 3H), 0.83-0.80 (1H); ^{13}C (150 MHz, $CDCl_3$) δ: 58.00 (s), 37.40 (s), 35.32 (s), 33.09 (s), 31.22 (s), 27.44 (s), 24.48 (s); HRMS (70 eV, EI): calcd for $C_{15}H_{24}O$ $[M\text{-}H_2O]^+$: calcd 220.1933, found: 220.1920.

S2.3 Synthesis of 4-adamantyl-1,5-dibromo-pentane (**6**)

To a stirred solution of **5** (2.20 g, 9.34 mmol) in DCM (200 mL) was added tetrabromomethane (12.38 g, 37.36 mmol). The solution was stirred for 10 min at room temperature to achieve a clear solution. The reaction mixture was cooled down to 0°C and stirred for 10 min at 0°C. Triphenylphosphine (14.69 g, 56.04 mmol) was added to the reaction mixture at 0°C over a period of 30 min. The reaction mixture was allowed to warm to room temperature and was stirred for 20 h. The reaction mixture was quenched with distilled water (100 mL) and was diluted with DCM (100 mL). The reaction mixture was extracted with DCM and the combined organic layer was washed with brine (2 × 50 mL). The solvent was removed under vacuum and was purified by silica gel column chromatography (hexane/ethyl acetate = 99/1) to afford 3.06 g of **6** as a colourless oil. (yield 92%) ^{1}H NMR (400 MHz, $CDCl_3$) δ:3.44-3.36 (m, 4H), 1.76 (br, 3H), 1.67-1.65 (m, 2H), 1.56-1.44 (m, 6H), 1.33 (bs, 6H), 1.15-1.03 (m, 3H), 0.84-0.81 (m, 1H). ^{13}C (150 MHz, $CDCl_3$) δ: 62.00 (s), 36.42 (s), 34.30 (s), 32.12 (s), 31.22 (s), 26.47 (s), 24.40 (s). HRMS (70 eV, EI): calcd for $C_{15}H_{23}Br_2$ $[M]^+$: calcd 363.0224, found: 363.0237.

S2.4 Synthesis of 4-adamantyl-1,5-diazido-pentane (**7**)

To a stirred solution of **6** (506 mg, 1.38 mmol) in DMF (5 mL) and water (0.7 mL) was added sodium azide (361 mg, 5.55 mmol) and the resulting reaction mixture was heated to reflux for 14 h. The reaction mixture was quenched with water (20 mL) and extracted with ether (100 mL). The collective organic layer was washed with brine (3 × 30 mL) and dried over $MgSO_4$. The crude product was purified over silica gel column

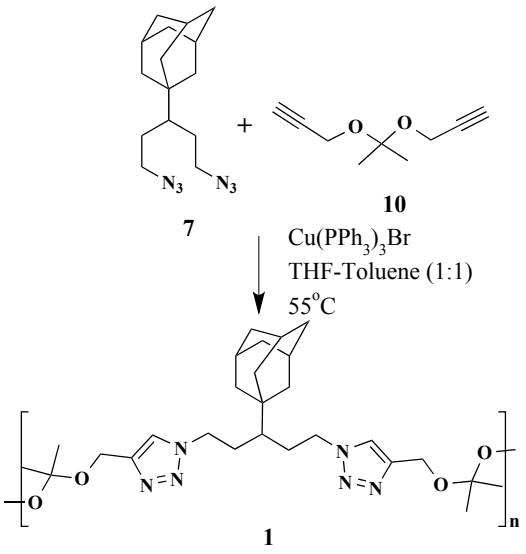
chromatography (hexane/ethyl acetate = 99/1) to afford **7** (286 mg, 84%) as a colourless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.37-3.21 (m, 4H), 1.99 (br, 3H), 1.92-1.84 (m, 2H), 1.72-1.50 (m, 12H), 1.28-1.24 (m, 2H), 0.84-0.80 (m, 1H). ^{13}C (150 MHz, $CDCl_3$) δ: 51.35 (s), 43.19 (s), 39.45 (s), 37.03 (s), 35.54 (s), 28.79 (s), 28.44 (s). HRMS (70 eV, EI): calcd for $C_{15}H_{27}N_4$ $[M\text{-}N_2]^+$: calcd 263.2157, found: 263.2168.

S2.5 Synthesis of 2,2-dipropergyloxy-propane (**10**)

A flame dried two-neck round bottom flask was equipped with stir bar and cooled down under an argon stream. Dry DCM (3 mL), **9** (4.4 g, 0.034 mol) and **8** (1.2 mL, 0.017 mol) were added under inert atmosphere at rt. The reaction mixture was cooled down to –78°C and trimethylsilyl trifluoromethanesulphonate (50 μL, 20 mol%) was added to the reaction mixture. The solution was stirred at -78 °C for 2.5 h. After completion of the reaction pyridine (0.6 mL) was added. The reaction mixture was poured in to saturated $NaHCO_3$ (20 mL) and extracted with ether (70 mL). The collective organic layer was washed with brine (2 × 25 mL). The crude product was purified by silica gel column chromatography (hexane/triethyl amine = 100/1) to afford **10** (1.5 g, 58%) as a colourless oil ^{1}NMR (400 MHz, $CDCl_3$) δ: 4.14 (d, 4H, J = 2 Hz), 2.39 (t, 2H, J = 2 Hz), 1.41 (s, 6H). ^{13}C (150 MHz, $CDCl_3$) d: 101.59 (s), 80.45 (s), 73.42 (s), 49.44 (s), 24.67 (s). HRMS (70 eV, EI): calcd for $C_9H_{13}O_2$ $[M]^+$: calcd 152.0837, found 152.084.

***S2.6 Synthesis of Mono-6-(p-toluenesulfonyl)-6-deoxy-β-cyclodextrin (β-CD-OTs)*:** This was prepared and purified as described in a protocol elsewhere[1], except that β-cyclodextrin (35 g, 30.8 mmol) was dissolved into 350 mL water along with 1-(p-toluenesulfonyl)imidazole (8.9 g, 40.0 mmol) and the reaction mixture was stirred at 20 °C for 4 h. The crude reaction mixture was recrystallized repeatedly from water to isolate the monotosylated CD of almost 90% purity. The product was further purified by HPLC (acetonitrile/water 15 ml/min) to afford a white solid (18% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ : 7.80-7.66 (d, 2H), 7.50-7.33 (d, 2H), 5.93-5.48 (b, 14H), 4.87-4.70 (s, 7H), 4.63-4.08 (b, 6H), 3.75-3.43 (m, 28H), 3.43-3.11 (m, 14H), 2.43-2.34 (s, 3H).

S2.7 Synthesis of 6-(bPEI 1800)-6-deoxy-b-cyclodextrin (β-CD-PEI1800): Polyethyleneimine (1.8 kDa, branched form, 561 mg, 0.312 mmol) was dissolved in 10 mL dry DMF at 75 °C under Ar. Once dissolved, β-CD-OTs (100 mg, 0.078 mmol) and 100 mg NaI were added and the reaction mixture was stirred overnight. The next day, the crude reaction mixture was distilled under vacuum to remove the DMF and the crude solid was precipitated in a 100 mL acetone thrice, followed by dialysis against DMSO and H_2O for 2 days each using a dialysis bag (Life Technologies, 2000 MWCO). The dialyzed product was isolated by lyophilization to afford a 240 mg pale yellow solid (35% yield). ^{1}H NMR (300 MHz, D_2O) δ : 5.00 (s, 2H), 3.79-3.12 (br, C2-6H, PEI-NH, β-CD-OH), 2.90-2.50 (m, 82H).



Scheme S2: Synthesis of pADK

S2.8 Synthesis of pADK (**1**)

General Procedure: To a degassed solution of **7** in 2 mL solvent was added **10** and $Cu(PPh_3)_3Br$ (5 mol%). The solution was stirred with heating at 55°C for 14 h. The viscous solution was poured into a large excess of ethyl acetate (30 mL). The resulting precipitate was removed by centrifugation. The solid product was redissolved and reprecipitated in DCM and ethyl acetate respectively. The same process was continued 3 times to achieve an off white powder. The molecular weight was determined with gel permeation chromatography in chloroform (**table 1**). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.76 (s, 2H), 4.66 (br, 4H), 4.31 (d, 4H, J = 7 Hz), 2.20 (br, 2H), 1.95 (br, 4H), 1.69-1.47 (m, 23H); GPC (polypore 300 X 7.5 mm, 1 mL/min, $CHCl_3$) M_n = 49472, M_w = 86315, PDI = 1.74.

Table 1: Synthesis of pADK

Entry	Condition	Mn	Mw	PDI
Polymer 1	0.2 μM monomers, Toluene-THF (1:1), $Cu(PPh_3)_3Br$ (5 mol%), 55 °C, 24 h	49472	86315	1.74
Polymer 11	1 μM monomers, DMF, CuBr (5 mol%), rt, 5 days	2359	4382	1.85
Polymer 12	1 μM monomers, DMF-DIPEA (4:1), CuI (5 mol%), 18 h, rt	1550	2263	1.46
Polymer 13	1 μM monomers, $CHCl_3$, $Cu(PPh_3)_3Br$ (5 mol%), 55 °C, 24 h	2018	2917	1.44

Polymer 14	0.2 μM monomers, $CHCl_3$, $Cu(PPh_3)_3Br$ (5 mol%), 55 °C, 24 h	3669	6248	1.70
Polymer 15	0.2 μM monomers, Toluene, $Cu(PPh_3)_3Br$ (5 mol%), 55 °C, 24 h	3373	24744	7.33
Polymer 16	0.2 μM monomers, 1,4-dioxane, $Cu(PPh_3)_3Br$ (5 mol%), 55 °C, 24 h	1983	2811	1.41
Polymer 17	0.2 μM monomers, THF, $Cu(PPh_3)_3Br$ (5 mol%), 55 °C, 24 h	6647	36218	5.45
Polymer 18	0.2 μM monomers, Toluene-THF (2:1), $Cu(PPh_3)_3Br$ (5 mol%), 55 °C, 24 h	10512	42615	4.05

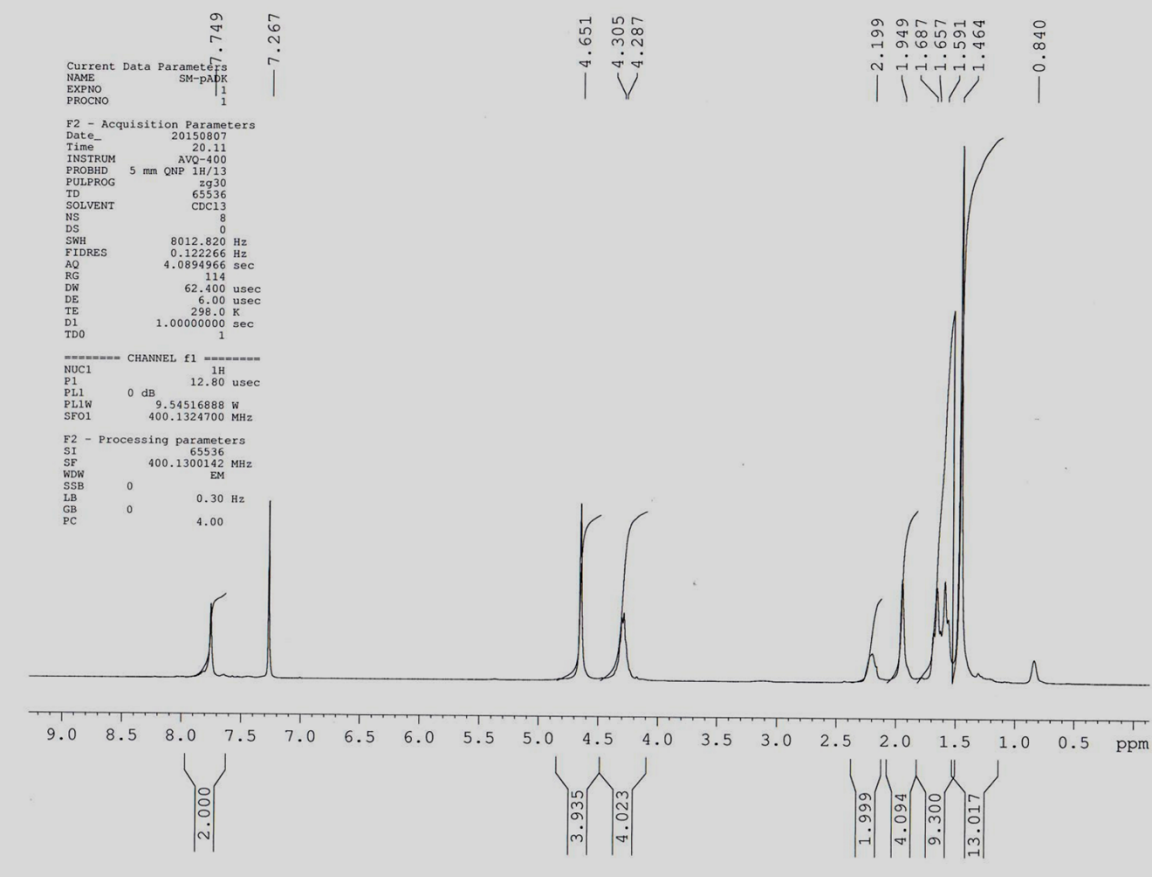
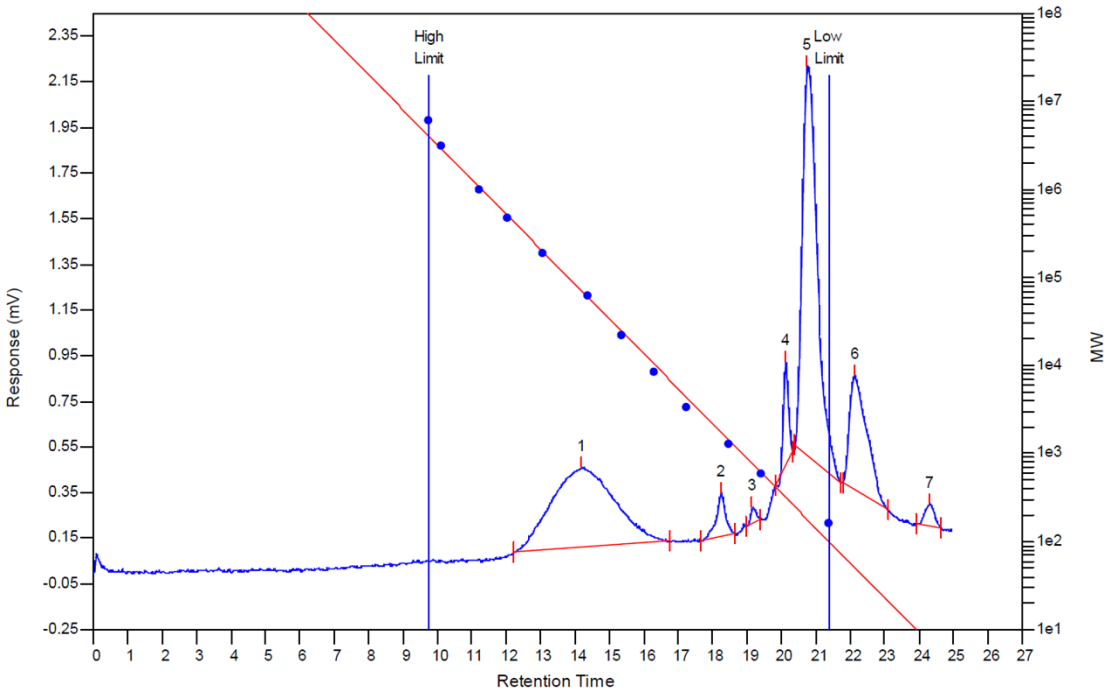


Figure S1: 1H NMR of pADK (polymer 1)

Analysis of polymer with gel permeation chromatography

Gel permeation chromatography (GPC) was carried out on a LC/MS Agilent 1260 Infinity set up with a guard and two Agilent Polypore 300 x 7.5 mm columns at 25 °C. All GPC analyses were performed on a 0.2 mg/mL solution of polymer in HPLC grade chloroform. An injection volume of 25 µL and a flow rate of 1 mL/min were used throughout the analysis. Calibration was performed with narrow polydispersity polystyrene standards ranging from Mw = 100 to 4,068,981.



MW Averages

Peak No	Mp	Mn	Mw	Mz	Mz+1	Mv	PD
1	70198	49472	86315	134498	183172	80029	1.74472
2	1753	1773	1806	1841	1878	1801	1.01861
3	776	753	757	760	763	756	1.00531
4	317	315	316	317	318	316	1.00317
5	178	158	165	171	177	164	1.0443
6	50	43	45	47	49	45	1.04651
7	6	7	7	7	7	7	1

Figure S2: Gel permeation chromatography of pADK (polymer 1).

S3. pADK-β cyclodextrin complexation and characterization

S3.1 pADK microparticle preparation and cyclodextrin response study

A solution of 5 mg of rhodamine B dissolved in 0.1 mL water was added to 1 mL of DCM containing pADK (15 mg). The resulting heterogeneous mixture was uniformly mixed with a homogenizer for 3 min. The turbid solution was added to 10 mL of a 1% PVA solution and stirred for 3 h. The microparticles were isolated by centrifugation at 4000 rpm for 10 min and washed 4 times with double distilled water and lyophilyzed. The particles were analysed with a Hitachi S 500 SEM.

0.2 mg of microparticles in 1 mL of DMSO were added into a 1 mL PBS solution containing 5 mM methyl-β-cyclodextrin or just plain PBS. The solutions were allowed to incubate for various time points and then centrifuged at 5000 rpm, the supernatant was isolated and analysed for rhodamine via fluorescence.

S3.2 MTS Cell Viability Assay:

The cytotoxicity of the pADK:CD1800 (molar ratio 1/1) complexes was evaluated using the MTS assay in HeLa cells using bPEI (25 kDa) as a benchmark. The relative cell viabilities were measured as a function of amine densities of the CD^+ and bPEI species. HeLa cells were cultured in complete DMEM medium supplemented with 10% FBS at 37 °C, 5% CO_2, and 95% relative humidity. The cells were seeded in a 96-well microtiter plates (Dow Corning) at densities of 7,500 cells/well. After 24 h, the culture media was replaced with serum-free culture media containing increasing amine concentrations of PEI1800, CD1800, pADK:CD1800 complexes and bPEI 25000. Cells were incubated for 24 h. After 24 h, 15μL of MTS reagent was added to each well and incubated for 2 h. Following the incubation period, the absorbance was measured using a microplate reader (Multiskan GO, Thermo) at a wavelength of 492 nm. The cell viability (%) relative to control cells cultured in media without polymers was calculated with $[A]_{test}/[A]_{control} \times 100\%$, where $[A]_{test}$ is the absorbance of the wells with polymers and $[A]_{control}$ is the absorbance of the control wells. All experiments were conducted for six samples and averaged. In this study, LD50 was the concentration of the carrier at which the relative cell viability decreased to 50%.

S3.3 pADK:CD1800:pDNA complexation study

The complextion ability of pADK:CD1800:pDNA was determined by 1% agarose (low melting point) gel electrophoresis. The agarose gel were precast in TBE buffer with ethidium bromide. A mixture of pADK:CD1800 (1/1) containing 0.2 μg of pDNA at different N/P (=10, 20, 30) ratios were loaded onto the gel and compared against EGFP-pDNA only. A 1:5 dilution of loading dye was added to each well and electrophoresis was carried out at a constant voltage of 55V for 1h in TBE buffer. The pDNA bands were then visualized under a UV transilluminator at a wavelength of 365 nm.

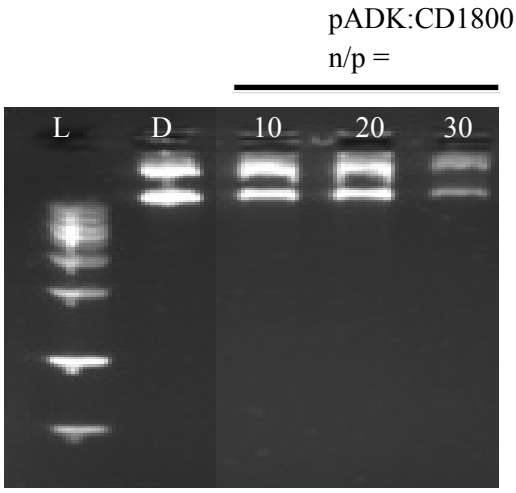


Figure S3: pADK:CD1800:pDNA complexation efficiency study measured by agarose gel electrophoresis: (L) DNA ladder, (D) EGFP-pDNA, (10) pADK:CD1800:pDNA (N/P=10), (20) pADK:CD1800:pDNA (N/P=20), (30) pADK:CD1800:pDNA (N/P=30).

S3.4 In Vitro Transfection Experiment: HeLa cells were cultured in complete DMEM medium supplemented with 10% FBS at 37°C, 5% CO_2, and 95% relative humidity respectively at a cell density of 7500 cells/well in 8-well chamber slides. After 24 h, the culture media was replaced with serum-supplemented media containing the pADK:CD1800 complexes containing 250 ng EGFP-pDNA at N/P ratios of 30. The cells were incubated with the complexes for 4 h, after which the spent media was aspirated and fresh serum-supplemented media was added. After a further 36 h incubation, the media was aspirated and the cells were washed with PBS. Bright field and dark field (green fluorescence) images were then taken using a Floid Imaging System (Life Technologies). %GFP mean fluorescence intensity was calculated relative to lipofectamine 2000, which was considered as a 100%. The experiment was performed in conditions that were optimized for lipofectamine 2000 use.

S4 Reference

1. G. Tripodo, C. Wischke, A. T. Neffe and A. Lendlein, *Carbohydr Res*, 2013, **381**, 59-63.