

Supporting Information for:

Supramolecularly Engineered Circular Bivalent Aptamer for Enhanced Functional Protein Delivery

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Experimental section

General. All reagents used were purchased from commercial suppliers, unless otherwise stated. All oligonucleotide synthesis reagents were purchased from Glen Research Corp. and ChemGenes Corp. Dibenzocyclooctyne-sulfo-N-hydroxysuccinimidyl ester and saporin were purchased from Sigma-Aldrich. Mono-6-azido-6-deoxy-beta-cyclodextrin was obtained from TCI Chemical. Water used in all experiments was DNA grade from Fisher Scientific. Phosphate buffered saline (10X PBS) was purchased from Fisher Scientific.

Synthesis of oligonucleotides. All oligonucleotides were synthesized on an ABI 3400 DNA synthesizer (Applied Biosystems, Inc., Foster City, CA) based on solid-state phosphoramidite chemistry at a 1 μmol scale. Synthesis started from controlled-pore glass (CPG) columns. All DNA sequences were deprotected according to the instructions from the manufacturer. Deprotected DNA was purified by reversed-phase high pressure liquid chromatography (HPLC) (ProStar, Varian, Walnut Creek, CA, USA), using a C18 column. The collected sequences were vacuum dried, desalted, and quantified by measuring the absorbance at 260 nm using a Varian Cary 100 UV-Vis spectrometer (Agilent Technologies, Santa Clara, CA, USA).

Mono-apt-DBCO conjugate. The purified amine-modified oligonucleotides (10 nmol) were dissolved in 100 μL of 0.1 M sodium carbonate buffer (pH 8.2). To this solution were added dibenzocyclooctyne-sulfo-N-hydroxysuccinimidyl ester (1.5 mg) dissolved in 50 μL DMF and 50 μL acetonitrile. The resultant suspension was stirred at room temperature for 4 hours. The solution was diluted to 400 μL with water and purified by RP-HPLC under the following conditions: C18 column, room temperature; flow rate: 1.0 mL min^{-1} ; eluent A: 0.1 M TEAA (pH 7.0); eluent B: acetonitrile; linear gradient, 5-30% B in 30 min; detection wavelength: 260 nm.

Mono-apt- β CD conjugate. Mono-apt-DBCO conjugate (10 nmol) was dissolved in 100 μL of 0.1 M triethylammonium acetate (pH 7.4). To this solution, was added Mono-6-azido-6-deoxy-beta-cyclodextrin (50 nmol) dissolved in 100 μL DMSO. The resulting

suspension was stirred at ambient temperature overnight. The solution was diluted to 400 μL with water and purified by RP-HPLC under the following conditions: C18 column, room temperature; flow rate: 1.0 mL min^{-1} ; eluent A: 0.1 M TEAA (pH 7.0); eluent B: acetonitrile; linear gradient, 5-30 % B in 30 min; detection wavelength: 260 nm.

Gel electrophoresis assay. Eight molar denaturing urea polyacrylamide gel electrophoresis was used to evaluate the formation of cb-aptamers. A 5 pmol DNA sample was mixed with loading buffer and then loaded into 8% polyacrylamide gel in electrophoresis buffer (9 mM Tris, pH 8.0, containing 9 mM boric acid and 1 mM EDTA). After electrophoresis, the gels were stained with cyber gold reagent according to manufacturer's instructions, and the images were captured using the FITC scanning channel on Typhoon.

Synthesis of AdA ligand for protein modification (Scheme S1). To a solution of 1-adamantanecarboxylic acid chloride (1, 2.1g, 10.81 mmol) and triethylamine (TEA, 0.4 mL, 3.31 mmol) in CH_2Cl_2 (20 mL) was added bis[2-(2-hydroxyethoxy)ethyl] ether (2.1g, 10.81 mmol) dropwise with stirring at room temperature. The mixture was stirred for 5 h and then extracted with CH_2Cl_2 . The combined organic layers were dried over anhydrous MgSO_4 and concentrated under reduced pressure. The crude product was purified using flash chromatography with petroleum ether /EtOAc (3/1) as the eluent, and compound **2** (3 g, 81 % yield) was obtained as a colorless oil and used directly for the next reaction.

To a solution of compound **2** (2g, 5.83 mmol) in CH_2Cl_2 (10 mL) was added triethylamine (0.3 mL, 0.217 g) dropwise with stirring. After stirring for 30 min, 4-nitrophenyl chloridocarbonate (1.78g, 8.83 mmol) was added dropwise at 0 $^\circ\text{C}$. The mixture was stirred for 4 h and extracted with CH_2Cl_2 . The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. Flash chromatography of the residue on silica gel using petroleum ether /EtOAc (9/1) as the eluent gave compound **3** (1.2 g, 80%). ^1H NMR (300 MHz, CDCl_3) δ 8.30 (d, J = 9 HZ, 2H), 7.41(d, J = 9 HZ, 2H), 4.46 (t, 2H), 4.21 (t, 2H), 3.82 (t, 2H), 3.70-3.67 (m, 10H), 2.17 (s, 4H), 2.01 (s,3H), 1.89 (d, J = 3 HZ, 6H), 1.74 (s, 2H).

Protein modification using AdA ligand. GFP protein (1 mg) was dissolved in 0.1 M NaHCO_3 buffer solution (pH = 9.5) at a protein concentration of 10 mg/mL, and compound **3** (0.125mg) dissolved in DMSO was added to the GFP solution. The reaction mixture was stirred for 2 h at room temperature, followed by ultrafiltration purification using Amicon Ultra Centrifugal Filters (MWCO=10k). Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) was used to characterize the chemically modified protein, and the quantum yield of GFP before and after AdA conjugation was determined to be 0.6 according to a previously reported method.¹

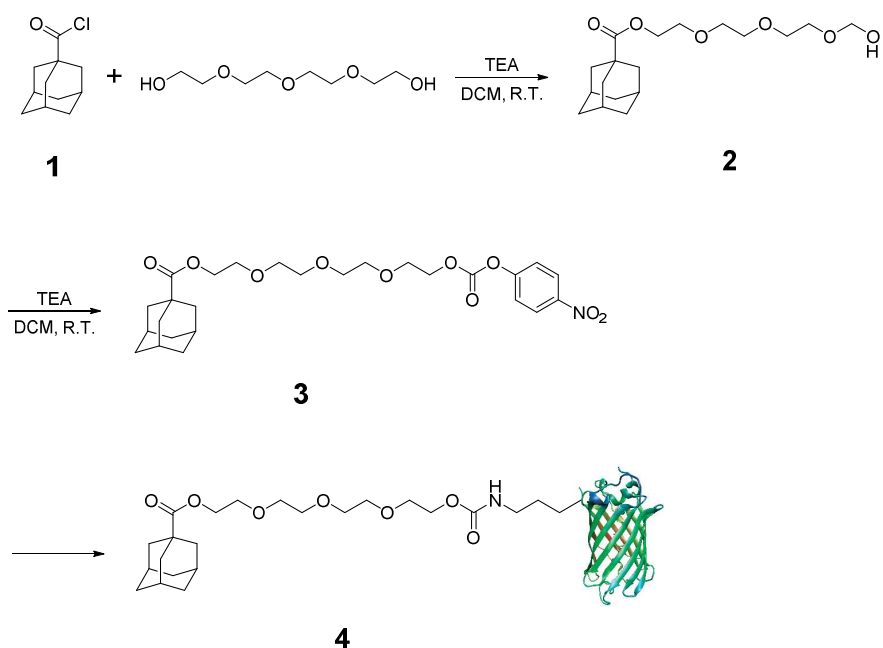
Cell culture. HeLa, CEM, and Ramos cells were purchased from ATCC (Manassas VA), cultured in a humidified atmosphere containing 5% CO₂ at 37 °C, and subcultured every four days. Low-glucose Dulbecco's modified Eagle's medium (DMEM, 1.0 g/L glucose, for HeLa cells) and high-glucose RPMI (4.5g/L, for CEM, and Ramos cells) both containing 10% fetal bovine serum (FBS) and 1% antibiotic (100 U/mL penicillin and 100 µg/mL streptomycin) were used to culture cells.

Flow cytometry experiments. HeLa cells (25,000) were seeded in a 24-well plate for 24 h prior to delivery. On the day of delivery, cells were washed three times with 1X PBS, followed by incubation with the desired concentration of each sample in 400 µL of full culture medium for 16 h. Then the cells were washed with 1X PBS, harvested and resuspended in PBS for flow cytometry analysis on an Accuri C6 flow cytometer. GFP fluorescence was measured by counting 10,000 events using the GFP channel under the same parameter setting, and the data were analyzed with FlowJo software.

Confocal microscopy study. For confocal laser scanning microscopy (CLSM) imaging of the cellular uptake of mono-apt-βCD/GFP-AdA and cb-apt-βCD/GFP-AdA, HeLa cells (200,000 cells) were seeded in a 35 mm glass bottom culture dish (MatTek, MA) one day before the experiment. On the day of delivery, the culture medium was removed and replaced with full culture medium containing 600 nM of either mono-apt-βCD/GFP-AdA or cb-apt-βCD/GFP-AdA, followed by 16 h of incubation at 37 °C. After removing medium, the cells were washed three times with cold phosphate buffered saline (PBS) for fluorescence imaging under a Leica TCS SP5 confocal microscope. The images were finally analyzed using LAS AF.

Cell viability study. All cell cytotoxicity results were evaluated by MTS assay (Promega, Madison, WI, USA). For intracellular delivery of AuNHC or saporin, CEM cells, Ramos cells (20,000 cells), or HeLa cells (5,000) were incubated with each sample at the desired concentration in a 96-well plate at 37 °C in 5% CO₂ for 24 h (AuNHC) or 36 h (saporin) in full culture medium, or for 4 hours in Opti-MEM followed by an additional 20 h incubation (AuNHC) with fresh culture medium. Then the supernatant was removed, followed by washing cells three times with 1X PBS. After washing, MTS reagent (20 µL CellTiter reagent) diluted in fresh medium (100 µL) was added to each well for 1 h incubation. Finally, the 96-well plate was scanned by a VersaMax tunable microplate reader (Molecular Devices, Inc., Sunnyvale, CA, USA), and the 490 nm absorbance was recorded and normalized to the signals from untreated cells.

Supplementary Figures



Scheme S1. Detailed synthesis route of adamantane ligand for chemical modification of protein.

Table S1: Oligonucleotide sequences used in this study.

Name	Detailed sequence information
Mono-apt-NH ₂	5'-P-TGA CTG ATT TAC GGT CTA ACT GCT GCG CCG CCG GGA AAA TAC TGT ACG GTT(NH ₂) AGA C-3'
Mono-apt _c	5'-P-CGT AAA TCA GTC AGT CTA ACT GCT GCG CCG CCG GGA AAA TAC TGT ACG GTT AGA C-3'
TMR-apt	5'-TGA CTG ATT TAC GGT CTA ACT GCT GCG CCG CCG GGA AAA

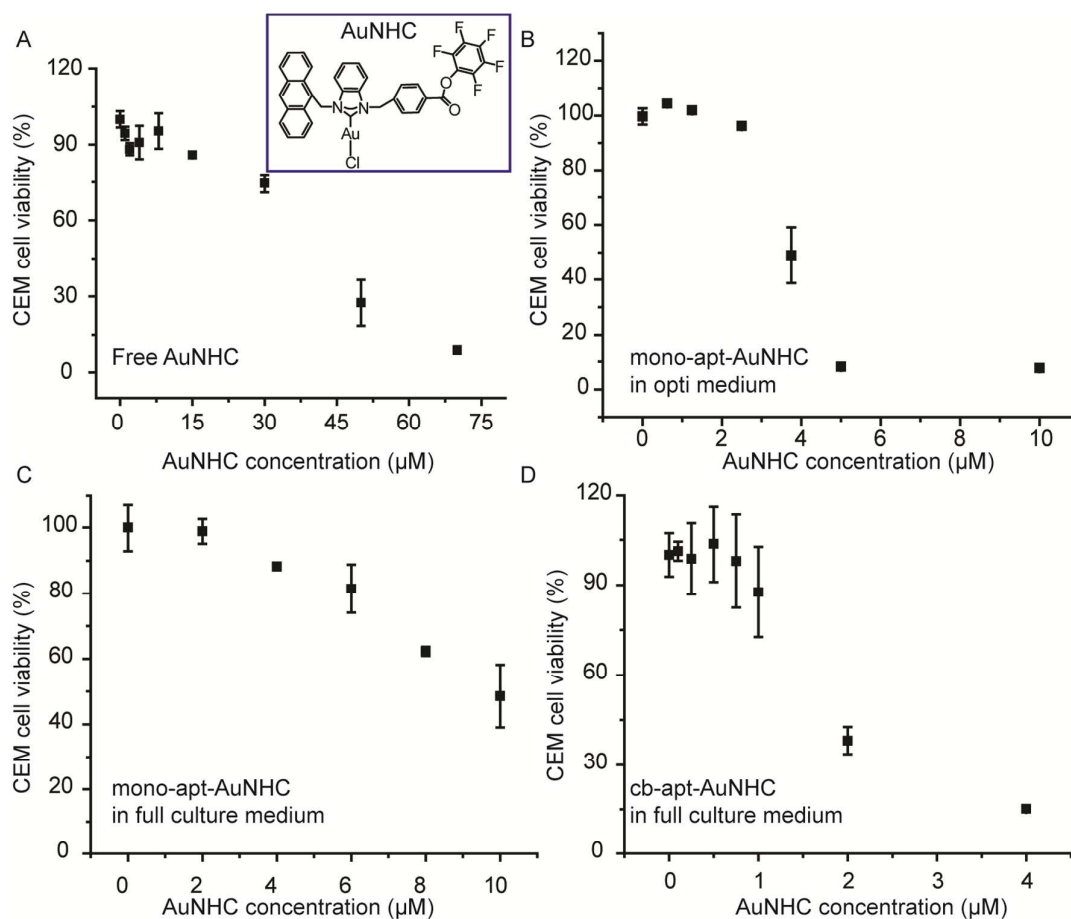


Figure S1. Molecular structure of AuNHC (inset image) and the viability of CEM cells treated with various concentrations of (A) free AuNHC in full culture medium, (B) mono-apt-AuNHC in Opti medium, (C) mono-apt-AuNHC in full culture medium, and (D) cb-apt-AuNHC in full culture medium. Cell viability was determined using MTS assay 24 h post-delivery. The error bars represent the standard deviations of three parallel measurements.

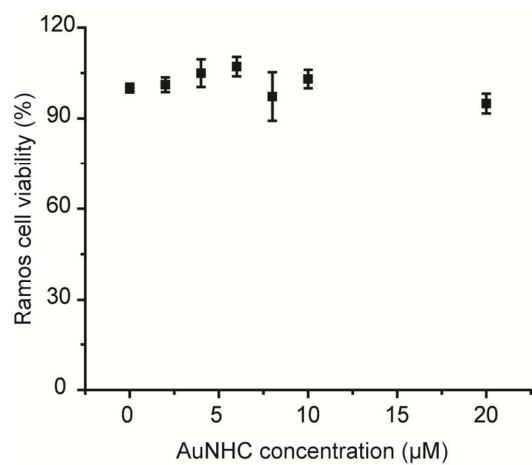


Figure S2. Cell viability of Ramos cells treated with various concentrations of cb-apt-AuNHC in full culture medium. Cell viability was determined using MTS assay 24 h post-delivery. The error bars represent the standard deviations of three parallel measurements.

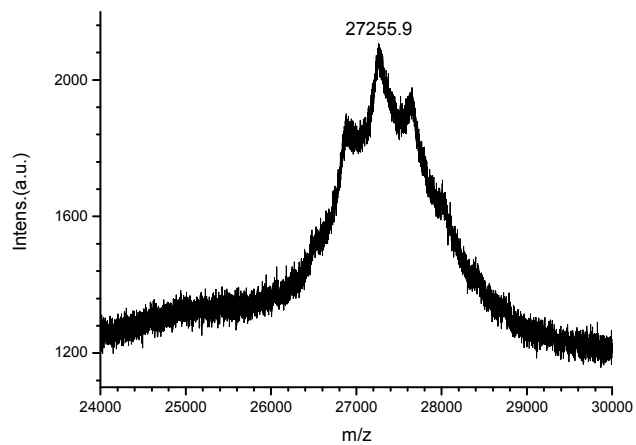


Figure S3. MALDI-TOF mass spectrum of AdA-modified GFP.

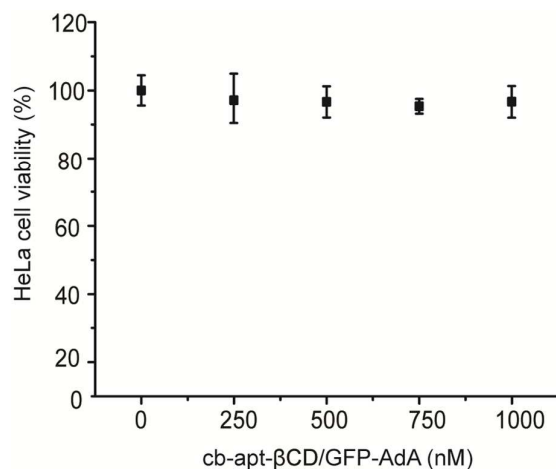


Figure S4. Cell viability of HeLa cells treated with various concentrations of cb-apt-βCD/GFP-AdA conjugates in full culture medium. The molar ratio of cb-apt-βCD to GFP-AdA was fixed to 1. Cell viability was determined using MTS assay 36 h post-delivery. The error bars represent the standard deviations of three parallel measurements.

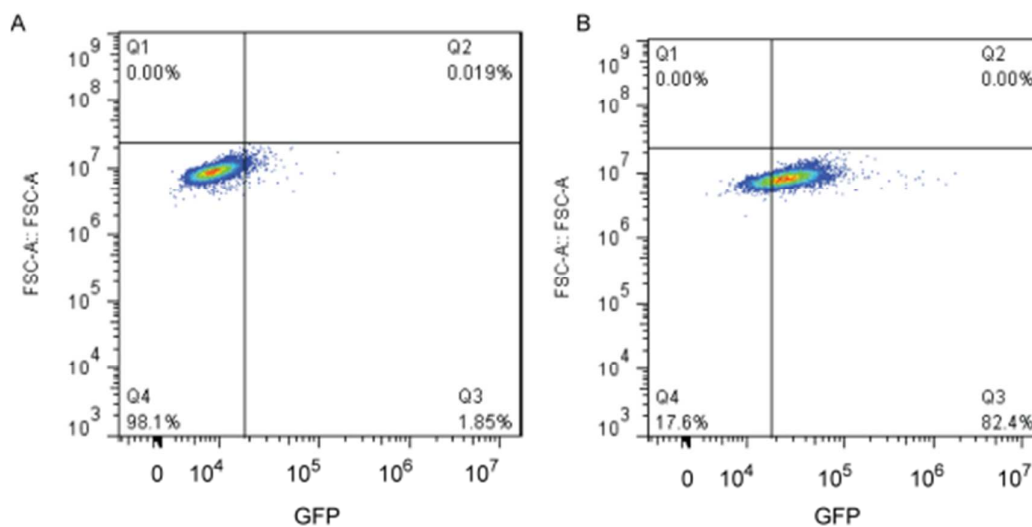


Figure S5. Flow cytometry plots of GFP-positive HeLa cells. HeLa cells treated with PBS (A), or 600 nM cb-apt-βCD/GFP-AdA conjugates (B), for 16 hours in full culture medium were harvested for flow cytometry analysis.

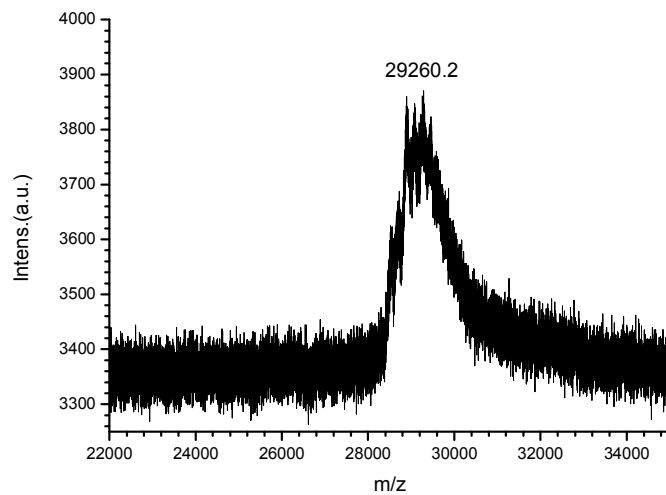


Figure S6. MALDI-TOF mass spectrum of AdA-modified saporin.

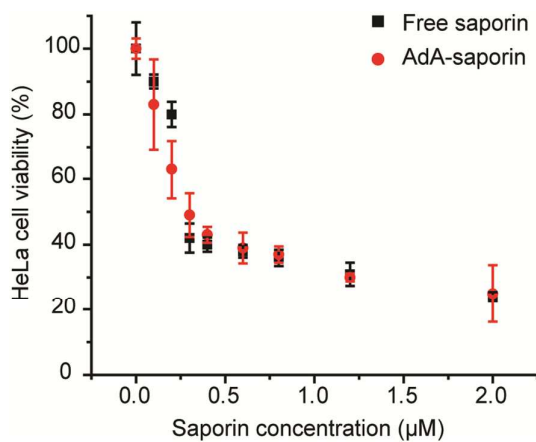


Figure S7. Viability of HeLa cells treated with saporin and AdA-modified saporin at the indicated concentrations for 36 hours, followed by the determination of cell viability by MTS assay. The error bars represent the standard deviations of three parallel measurements.

References

- (1) Wall, K. P.; Dillon, R.; Knowles, M. K. *Biochem. Mol. Biol. Educ.* **2015**, *43*, 52.