Supporting Information

A Stable Bidentate Protein Binder Achieved via DNA Self-Assembly Driven Ligand Migration

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Content:

1. General experimental

All chemicals, unless specifically mentioned, were obtained from Sigma-Aldrich or Acros Organics. Solvents were purchased from Fischer Scientific. EDC·HCl, *N*-hydroxymaleimide and 4-(*p*-iodophenyl) butyric acid were purchased from Sigma Aldrich. The parent ODN sequences 1, 3 as well as precursor ODNs (p2, p2m, and p4) were synthesized using standard automated solid phase synthesis at the Keck Foundation Biotechnology Laboratory at Yale University. All phosphoramidites needed to prepare these ODNs, including fluorescein modifications (Fluorescein-dT Phosphoramidite) and synthetic handles/spacer groups (Thiol-Modifier C6 S-S, 3'-PT-Amino-Modifier C6 CPG and 3'-(6-FAM) CPG) were purchased from Glen Research. DCs 2 and 2m were synthesized as shown in Figure SI 1, DC 4 was synthesized as shown in Figure SI 2. All ODNs were purified with sephadex resin Microspin G-25 columns (GE Healthcare) and chromatographed with a Varian Prostar reverse-phase HPLC equipped with a MetaTherm column heater and an Agilent 100Å 5μm PLRP-S reverse phase column. Concentrations of stock solutions of the ODNs were quantified based on their respective electronic absorption at 260 nm (and their molar extinction coefficients were obtained by nearest neighbor calculations). Purified ODNs were characterized by Matrix-Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) mass spectrometry using a Bruker Daltonics Autoflex III. For nuclease studies, DNase I and 10X reaction buffer was purchased from New England Biolabs. HSA was obtained from Sigma-Aldrich.

2. Preparation of DCs 2 and 2m

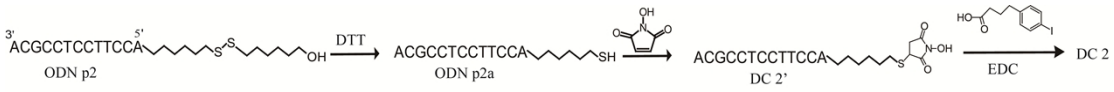
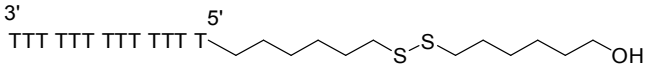


Figure SI 1. Synthetic scheme for DC 2.

As illustrated in Figure SI 1, ODN p2 was first treated with 50μL 100 mM DTT (in 60 mM $NaHCO_3$ aqueous solution, pH 8.5) at room temperature for 30 min to cleave the S-S bond and generate the free thiol functional group. After cleavage, the reaction was purified by G-25 spin column. This purification step was repeated three times. After which, a 50 μL solution containing 5 mg of *N*-hydroxymaleimide in 0.5 M MOPS buffer (3-morpholinopropanesulfonic acid) was added to the solution containing ODN p2a. After agitating for 30 min, the reaction was desalted by G-25 filtration and purified by reverse-phase HPLC. To link 4-(*p*-iodophenyl) butyric acid with NHS-tethered DC 2', 2.7 mg of 4-(*p*-iodophenyl) butyric acid was mixed with 2 mg of EDC·HCl in 100 μL DMF. After 5 min, 50 μL of this reaction mixture was added to 50 μL ODN DC 2' in 100 mM MES buffer (2-(*N*-morpholino)ethanesulfonic acid), pH 6.0. The reaction was agitated for 30 min and desalted by G-25 column three times to remove unreacted small molecules. The resultant DC 2 was characterized by MALDI-TOF.

The same synthetic route as mentioned above was applied to prepare DC 2m, using p2m (below) as the precursor sequence.



3. Preparation of DCs 4 and 5

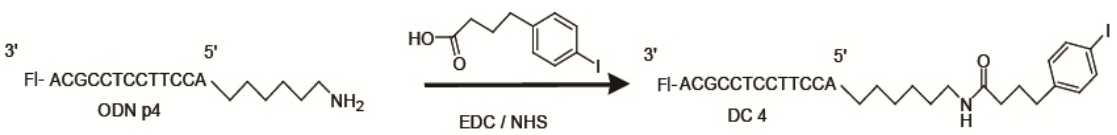
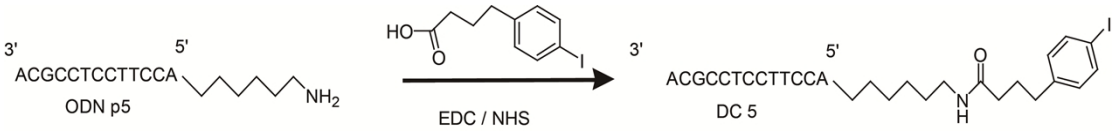


Figure SI 2. Synthetic scheme for DC 4.

As illustrated in Figure SI 2, 2.7 mg of 4-(*p*-iodophenyl) butyric acid was mixed with 2 mg of EDC·HCl and 1.2 mg NHS in 100 µL DMF. After 2 hours, 50 µL of this reaction mixture was added to 50 µL ODN p4 in 60 mM $NaHCO_3$ buffer, pH 8.5. The reaction was agitated overnight and desalted by G-25 column, followed by HPLC purification.

The same synthetic route as mentioned above was applied to prepare DC 5, using p5 (below) as the precursor sequence.



4. Ligand migration reaction via the formation of DC duplex 1:2

1.2 equivalent of DC 2 was added to 1 mL aqueous solution (50 mM MOPS and 10 mM $Mg(OAc)_2$, pH 7.5) containing 1 µM DC 1 at 25 °C. The reaction was agitated for 30 min and then heated to 80 °C for 5 min. Note: the same conditions were used when attempting to react DC 2m and DC 1.

5. DC 1':2' disassembly and hydrolysis by DNase I

A) **DC 1':2' Disassembly.** Duplex DC 1':2' (1 µM in 200 µL PBS buffer, 137 mM NaCl, 2.7 mM KCl, pH 7.4) was incubated with 10 equivalents of ODN 3 at 90 °C for 2 min and then allowed to cool down to room temperature.

B) **Nuclease hydrolysis.** For the DNase I induced hydrolysis reaction, 10 µM of purified DC 1' was incubated with 4 units of DNase I in 100 µL 1X reaction buffer (10 mM Tris-HCl, 2.5 mM $MgCl_2$,0.5 mM $CaCl_2$, pH 7.6) for 4 hours.

See Figure SI 12 for PAGE evidence for DNase I induced hydrolysis.

6. RP-HPLC

RP-HPLC purification of ODNs was carried out using a Varian Prostar HPLC system, equipped with an Agilent 100 Å 5 μm PLRP-S reverse phase column. The column was maintained at 65 °C for all runs. The flow rate was set at 1 mL/min. A gradient composed of two solvents (Solvent A is 0.1 M TEAA in 5% acetonitrile and solvent B is 100% acetonitrile) was used. UV absorption was monitored at 260 nm and 490 nm respectively.

Time (min)	Flow (mL/min)	%A	%B
0.00	1.00	100	0
2.50	1.00	100	0
7.50	1.00	88	12
12.50	1.00	80	20
32.50	1.00	50	50
40.00	1.00	0	100

Table SI 1. HPLC eluent gradient used for both ODN purification and product distribution analysis for the ligand transfer reaction.

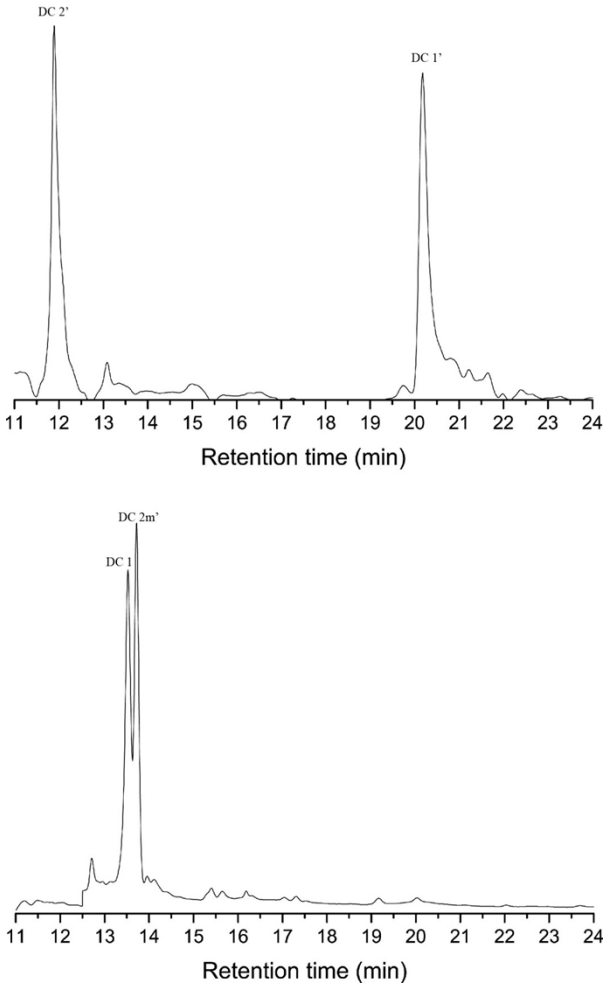


Figure SI 3. HPLC analysis of the products from the reaction of DC 1 with full complementary DC 2 (top) or non complementary DC 2m (bottom). Note: after ligand migration and/or reaction termination, DC 2 and DC 2m are converted to their precusors: DCs 2' and 2m' respectively. However, when DC 1 reacts with DC 2, a new peak appears (20 min) corresponding to DC 1'. While the reaction of DC 1 with DC 2m still shows intact reactant DC 1. Note: for these traces, the HPLC detector wavelength was set to 260 nm.

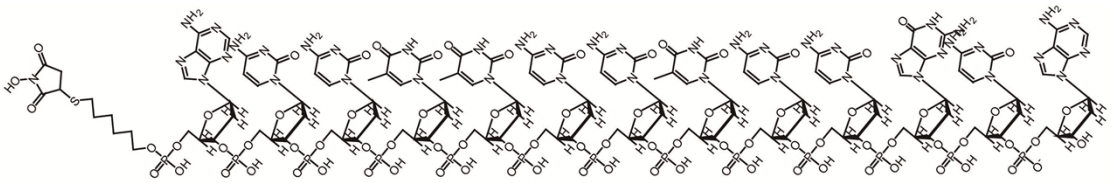
7. MALDI-TOF characterization of DCs

MALDI-TOF mass spectroscopy was performed on a Bruker Daltonics Autoflex III in a linear negative mode. The ODN samples were prepared using a 9:1:1 matrix of 2',4',6'-trihydroxyacetophenone monohydrate (THAP) (20mg/mL in 1:1 acetonitrile/water),

ammonium citrate (50mg/mL in water, 1% TFA), and ODNs in H_2O. Experimental parameters are shown in Table SI 2. The MALDI-TOF spectra and the theoretical mass to charge (m/z) are listed in the following Figures (Note: theoretical m/z = $[M-H]^-$).

Ion Source Voltage (kV)		Lens Voltage	Detector	Pulsed Ion Extraction
IS 1	IS 2	(kV)	(kV)	(ns)
20.00	18.80	6.70	20.00	250

Table SI 2. MALDI-TOF experimental parameters.



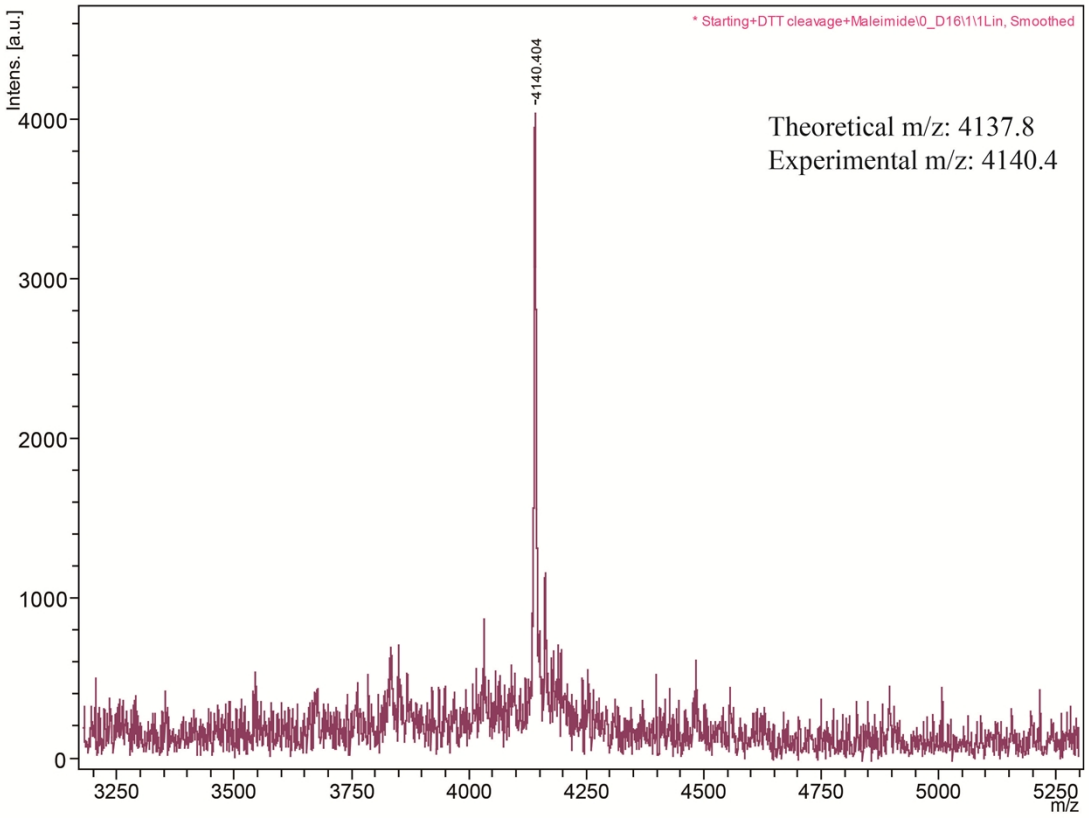


Figure SI 4. Chemical structure and MALDI-TOF spectrum of DC 2'.

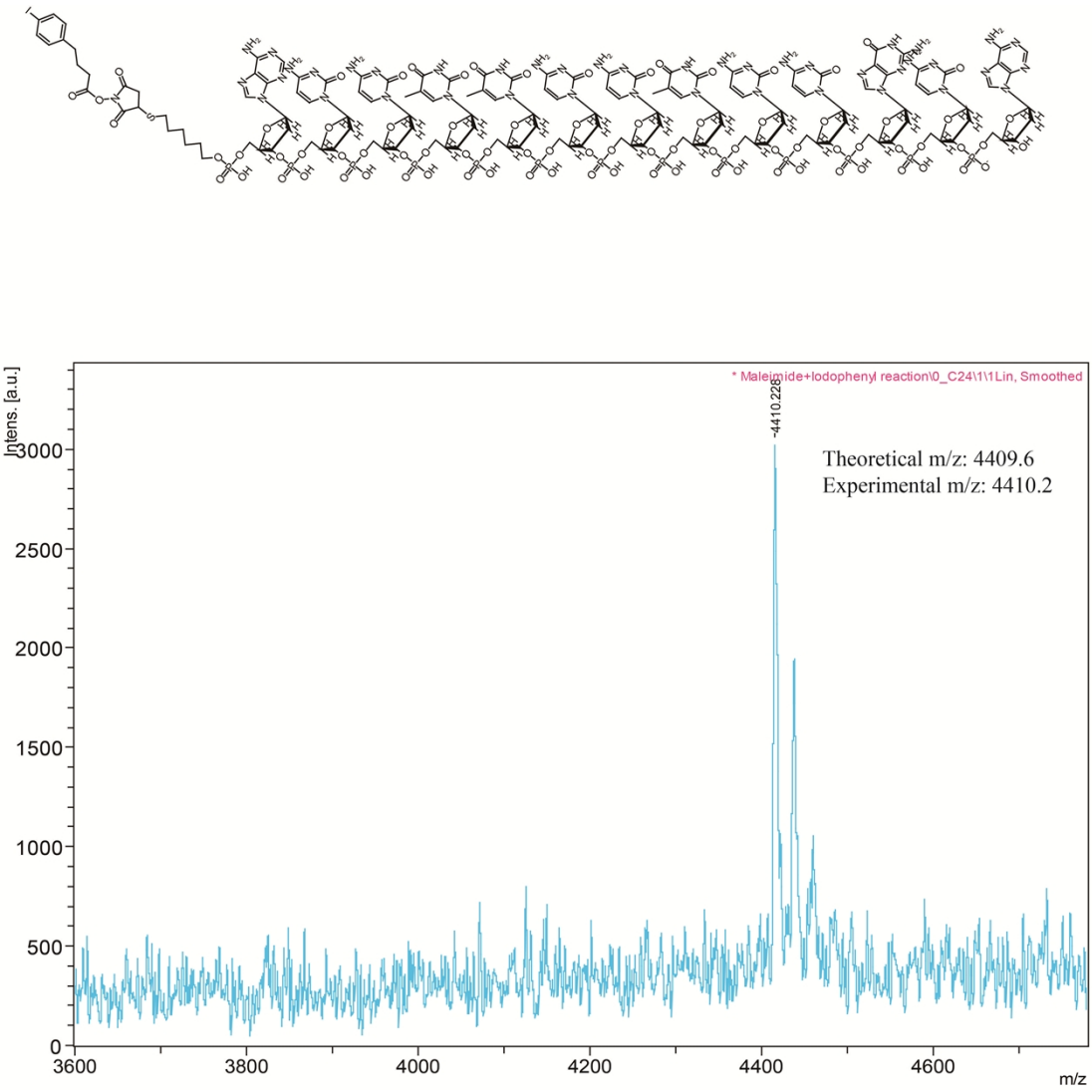
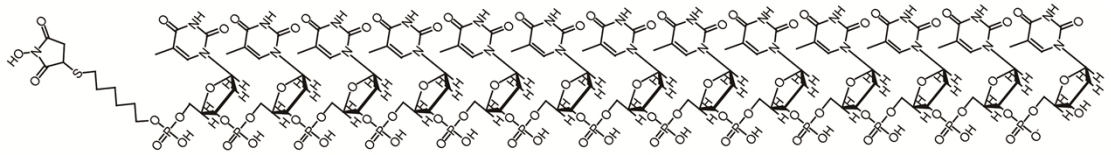


Figure SI 5. Chemical structure and MALDI-TOF spectrum of DC 2.



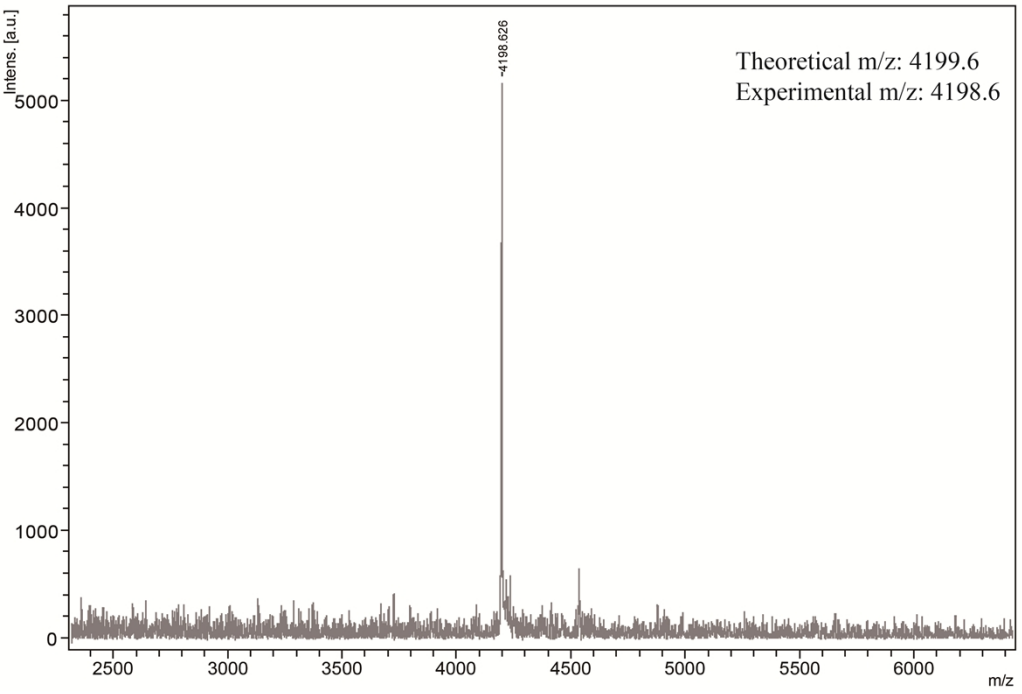


Figure SI 6. Chemical structure and MALDI-TOF spectrum of DC 2m'.

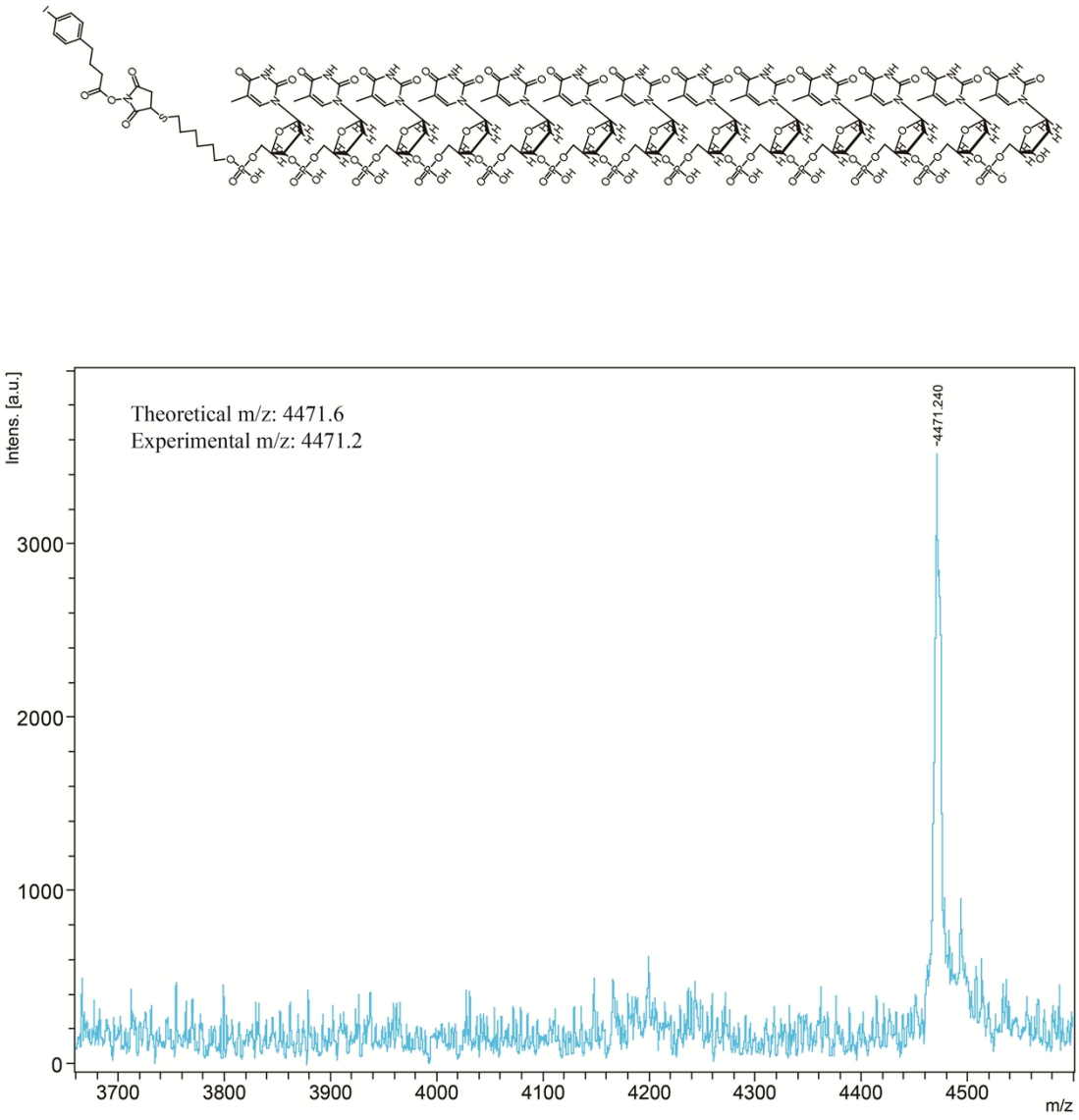


Figure SI 7. Chemical structure and MALDI-TOF spectrum of DC 2m.

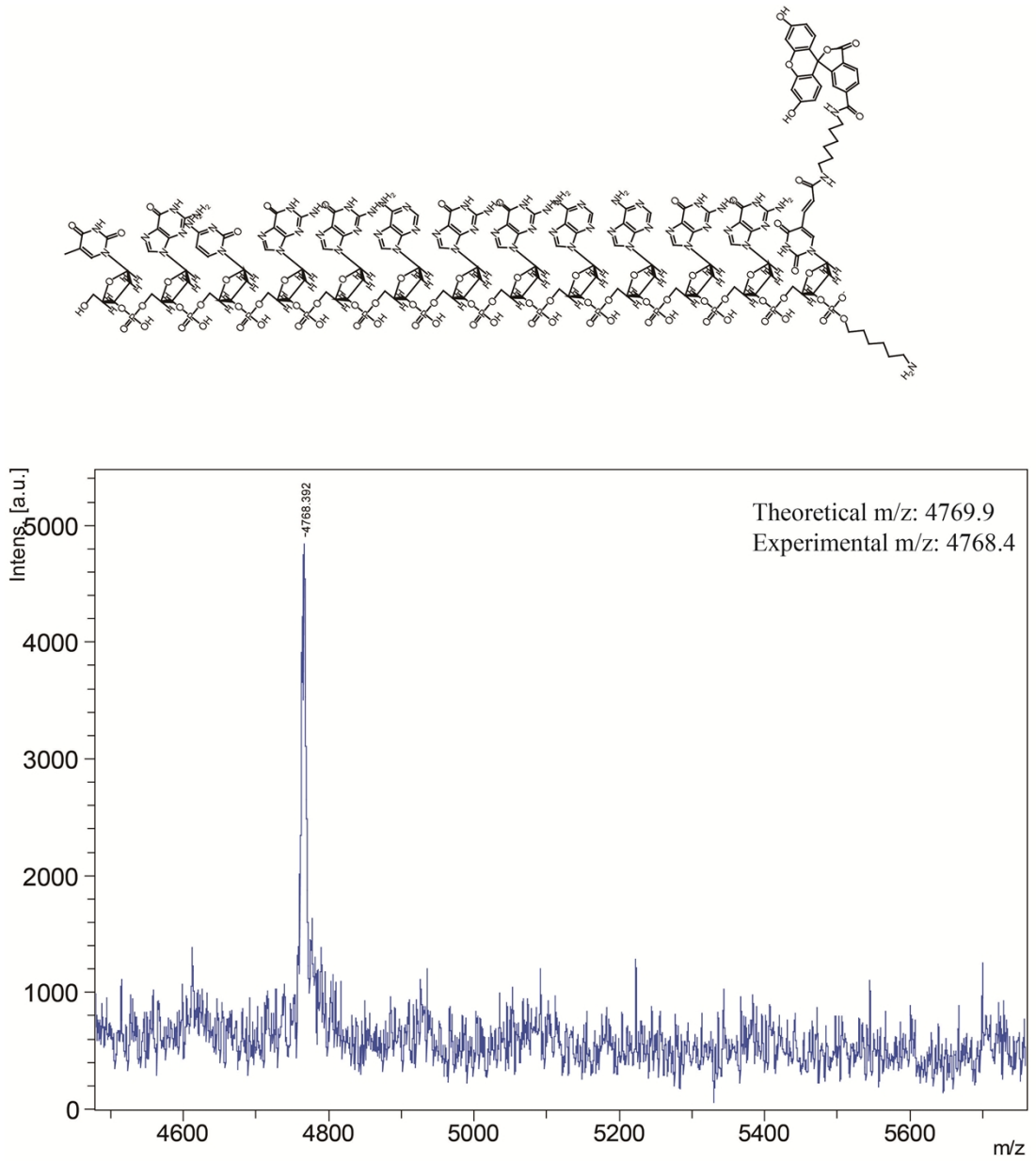


Figure SI 8. Chemical structure and MALDI-TOF spectrum of DC 1.

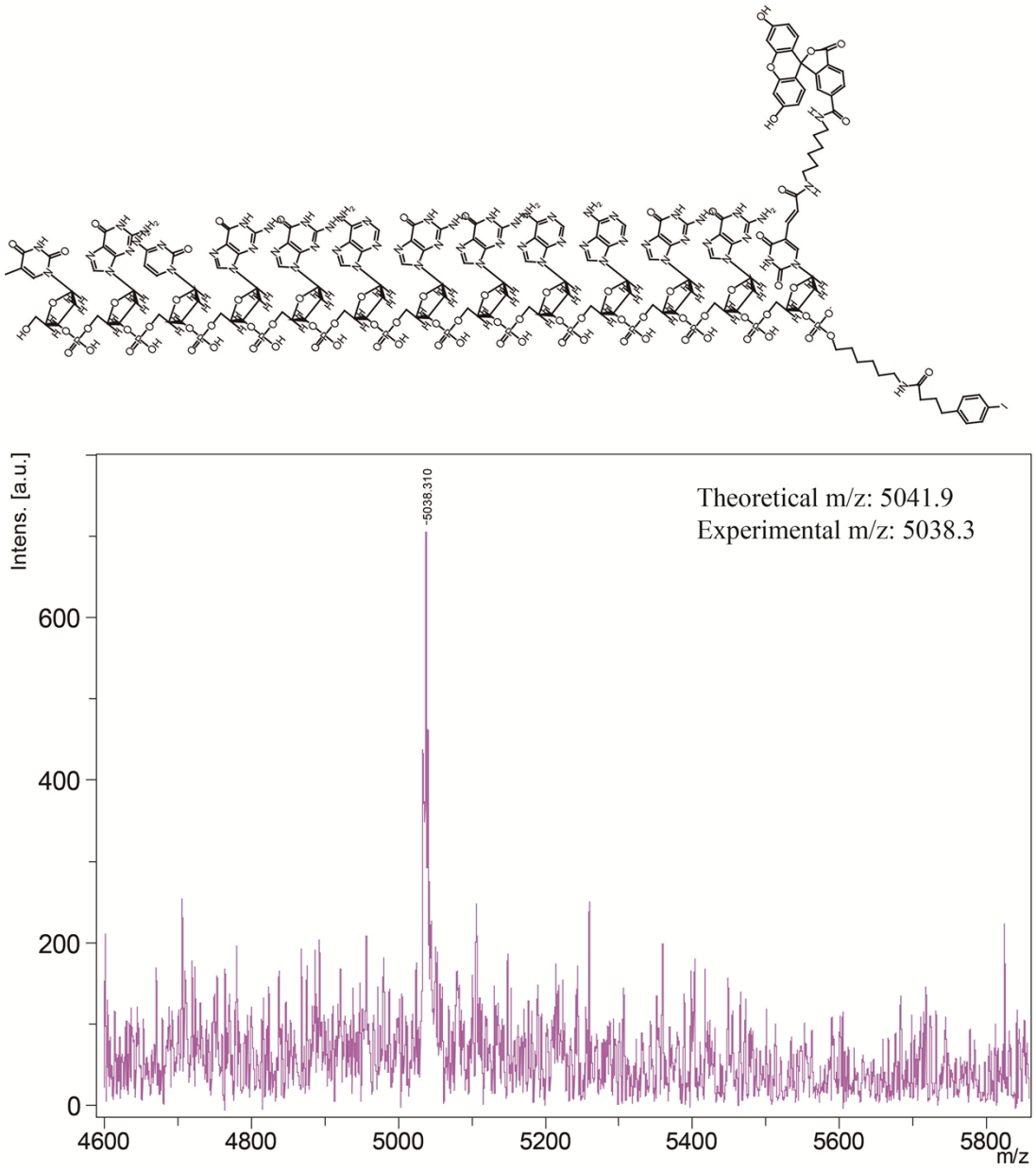


Figure SI 9. Chemical structure and MALDI-TOF spectrum of DC 1'.

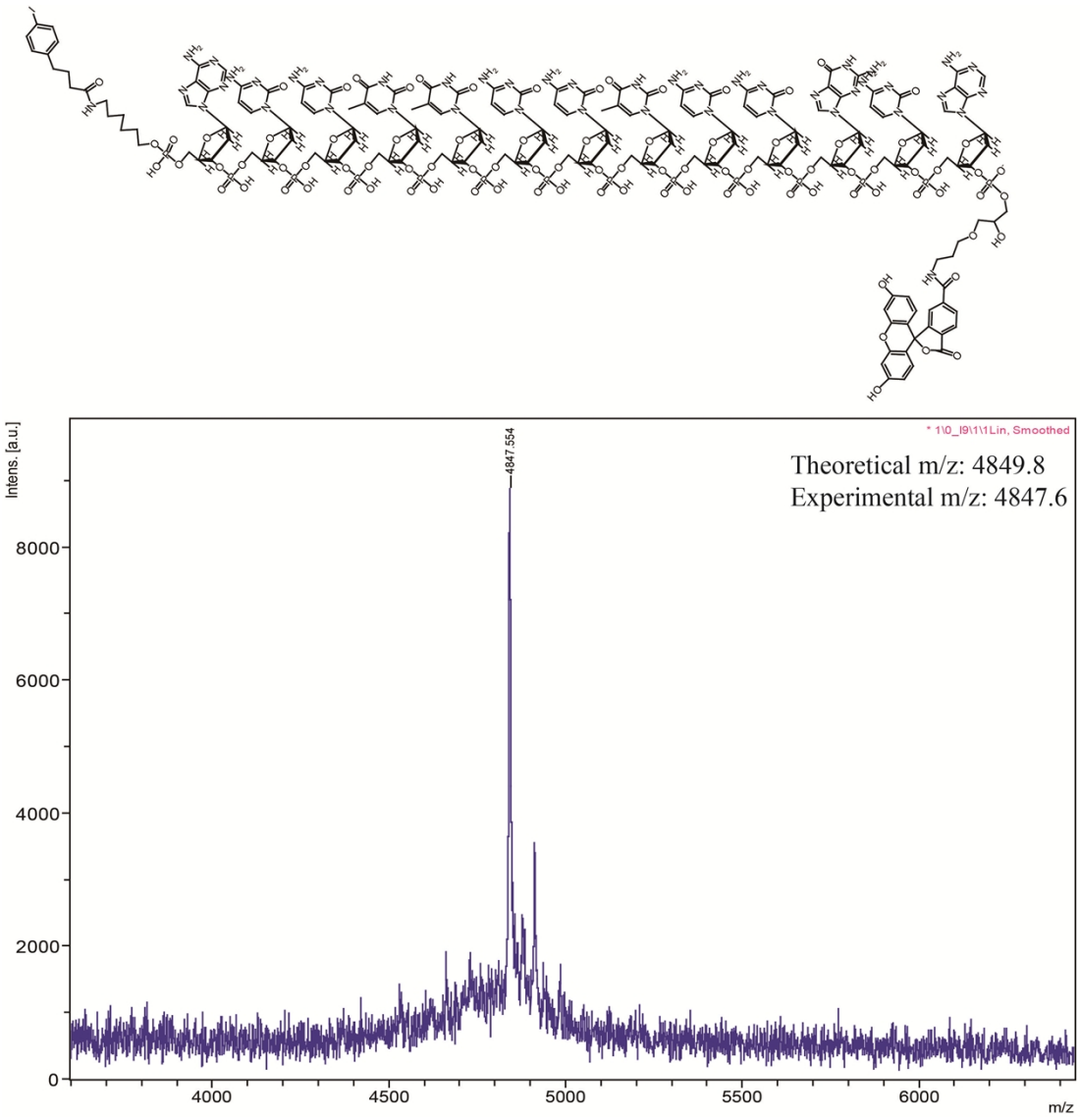


Figure SI 10. Chemical structure and MALDI-TOF spectrum of DC 4.

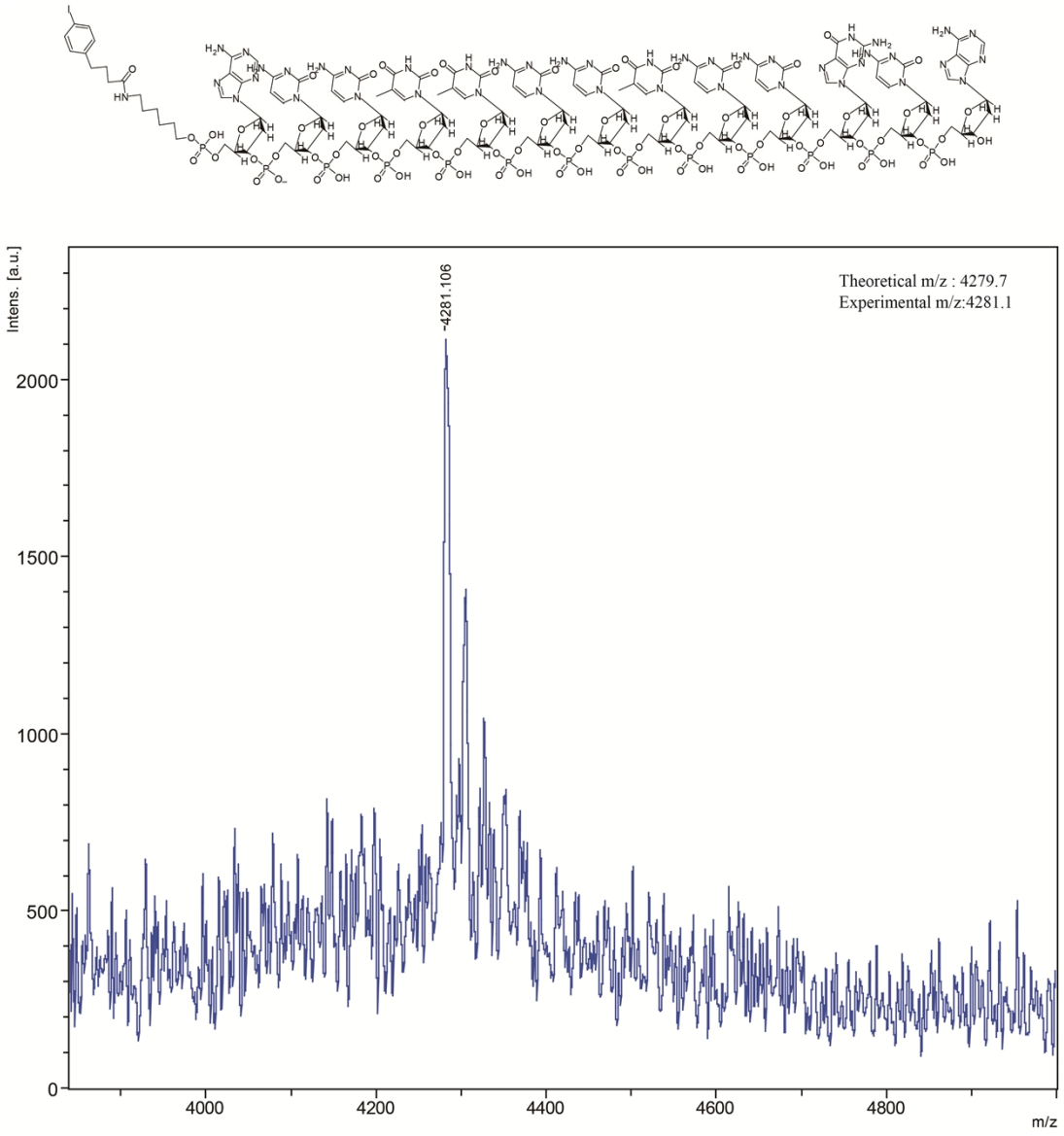


Figure SI 11. Chemical structure and MALDI-TOF spectrum of DC 5.

8. PAGE studies

A) **Mobility shift assays**

Non-denaturing polyacrylamide gel (15%) electrophoresis (PAGE) studies were performed using a BioRad mini protean tetra cell that is equipped with BioRad PowerPac HC. For the gel shown in Figure 2 (main text), in Lane 1, 0.75×10^{-4} µmole of DC 1' and 7.5×10^{-4} µmole of HSA was introduced. In Lane 2, 0.75×10^{-4} µmole of DC 1' alone was used. In Lane 3, 0.75×10^{-4} µmole of DC 1 and 7.5×10^{-4} µmole of HSA was used, and in Lane 4, 0.75×10^{-4} µmole of DC 1 was introduced. The loading buffer was 1× TBE, the loading solution of each sample also contained 13.3% glycerol. The running buffer was 1× TBE buffer at 150 V, and

the gel was run for 20 min. The gel was subsequently imaged on a Sony DSC-WX1 digital camera upon excitation with a hand held short wavelength UV-lamp.

B) **DNase I degradation assay**

To confirm the hydrolysis of DC 1' by DNase I**,** a PAGE study (Figure SI 12) was carried out (under the same PAGE conditions mentioned above).

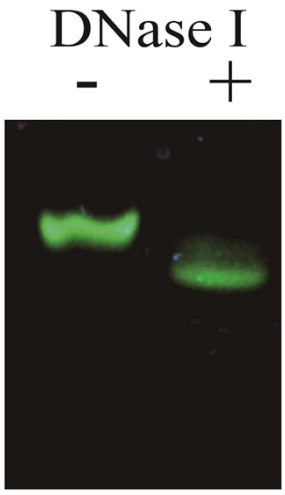


Figure SI 12. PAGE of DC 1' in the absence and presence of DNase I. Lane 1: no DNase I; Lane 2: DNase I treatment. It is clear that after DNase I addition, the nucleic acid products migrate further.

9. Fluorescence anisotropy to probe HSA binding

Fluorescence anisotropy titration was carried out in borosilicate glass culture tubes on a Molecular Probes Beacon 2000 Instrument (using 495 nm and 525 nm narrow band pass filters for excitation and emission, respectively). All fluorescence anisotropy experiments were conducted at 25 °C and were performed in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, pH 7.4). Initially fluorescence polarization (FP) values were obtained. The polarization (P) value that defines the rotational diffusion of the fluorophore in the excited state is calculated using equation 1.

Equation SI 1: $P = (I_{II} - I_I) / (I_{II} + I_I)$

Prior to the FP experiments, stock solutions of HSA were freshly prepared in 1X PBS buffer (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4). UV-absorption at 280 nm was used to determine the concentration of the HSA stock solutions (ε = 36,500 M^{-1} cm^{-1})[1]. For each experiment, the various fluorescein tagged ODNs (100 nM) were mixed with a series of different concentrations of HSA (spanning a range from 1nM to 1mM) in 150 µL PBS solution in eppendorf tubes. After gently agitating, the 150 µL solution was transferred to borosilicate glass culture tubes and allowed to incubate for 30 min at 25 °C, and then FP measurements were obtained. After each measurement, values of millipolarization obtained from the instrument were converted to millianisotropy using equation SI 2, in which A is the anisotropy value and P is the obtained polarization.

Equation SI 2: $A = 2P/(3-P)$ [2]

To determine the dissociation constant (K_d), millianisotropy was plotted against HSA (receptor protein) concentration and fitted to equation SI 3 (an equation that accounts for receptor depletion).[3] Non-linear regression analysis using the Origin 9.5 software was used for data fitting (see Figure 2 main text and Figures below).

Equation SI 3: $$A = A_f + (A_b - A_f) \times \frac{(L_T + K_d + P_t) - \sqrt{(L_T + K_d + P_t)^2 - 4 \times L_T \times P_t}}{2 \times L_T}$$

Here, A_f is the anisotropy of the free fluorescent molecule, A_b is the anisotropy of the fully bound fluorescent molecule, P_t is the total added protein concentration, L_t is the total ligand concentration added to the solution, in our case, L_t = 100 nM.

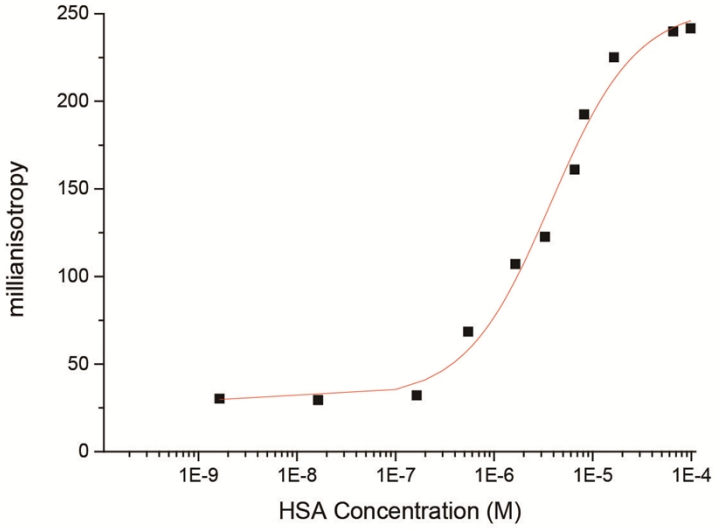


Figure SI 13. FA profile of 1 equivalent of DC 1':2' duplex in the presence of 10 equivalents ODN 3 with increasing concentrations of HSA. The line is the curve fit (K_d = 3.6 ± 0.5 × 10^{-6} M, R^2 =0.99).

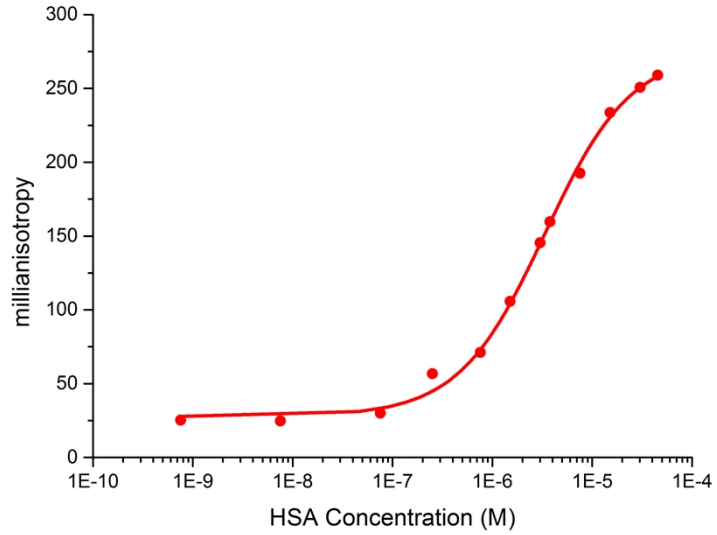


Figure SI 14. FA profile of DC 1' in the presence of DNase I. The line is the curve fit (K_d = 3.3± 0.2 × 10^{-6} M, R^2 =0.99).

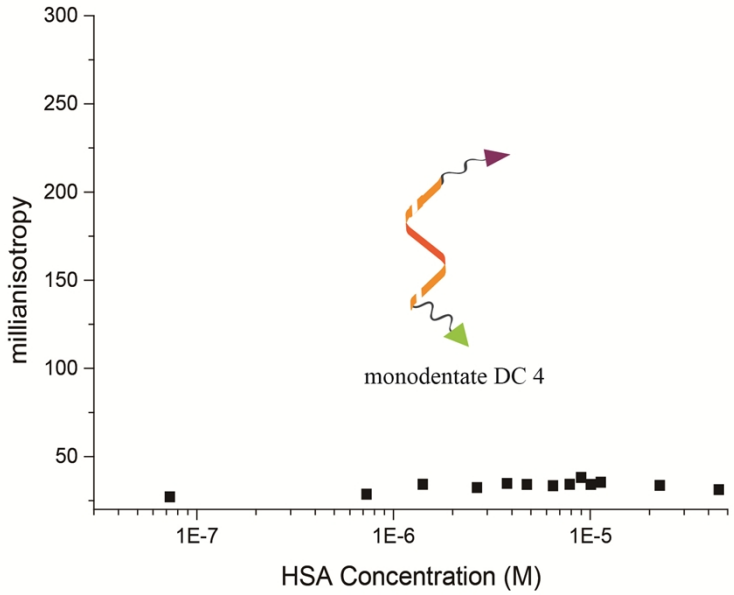


Figure SI 15. FA profile of DC 4 with increasing concentrations of HSA. This control shows that there is no significant binding to HSA when the fluorescein and **ip** head-groups are on the 3' and 5' termini of one strand, respectively.

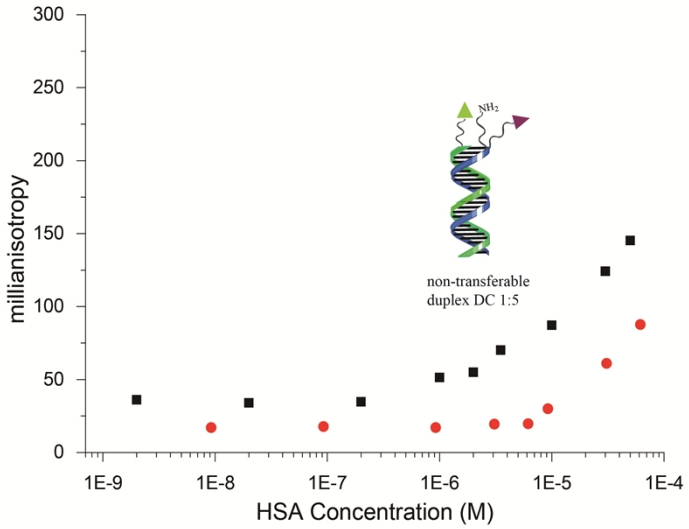


Figure SI 16. FA profile of DC 1:5 duplex (black square) with increasing concentrations of HSA. This control shows that binding to HSA is weak and there is no saturation even at high HSA concentration. This result shows that the simple association of a duplex that projects both binding

ligands (wherein the **ip** head-group is non-transferrable) is not as effective as the covalently fastened bidentate duplex 1':2'. Thus, the exact projection of the **ip** and **fl** head-groups is important for high-affinity HSA binding.

Hydrolysis of duplex 1:5, red circle (in an identical manner shown in SI-8 for duplex 1':2') further decreases the binding to HSA. Note that some binding is observed by the **fl** containing ODN fragments of DC 1 at high HSA concentrations. This likely means that the small fragments of DC 1 have a slightly better affinity for HSA versus the larger non-hydrolyzed DC 1 (Figure 2, main text).

[1]. G. Sancataldo, V. Vetri, V. Fodera, G. Di Cara, V. Militello and M. Leone, *PLoS One*, 2014, **9**, e84552.

[2].Fluorescence Polarization Technical Resource Guide (4th Edition), www.Invitrogen.com.

[3]. P. Jaru-Ampornpan, K. Shen, V. Q. Lam, M. Ali, S. Doniach, T. Z. Jia and S. O. Shan, *Nat Struct Mol Biol*, 2010, **17**, 696.