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Supporting Information

69451 Weinheim, Germany

Addressing Single Molecules on DNA Nanostructures

Justin D. Cohen, John P. Sadowski, and Peter B. Dervan

Abbreviations. *N*,*N*-dimethylformamide (DMF), *N*,*N*-diisopropylethylamine (DIEA), *rac*-dithiothreitol (DTT), *N*-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluoro-phosphate (HBTU), trifluoroacetic acid (TFA), Trishydroxymethylaminomethane (TRIS), ethylenediaminetetraacetic acid (EDTA).

Materials. Boc-β-Ala-PAM resin was purchased from Peptides International. Trifluoroacetic acid (TFA) was purchased from Halocarbon. Methylene Chloride (DCM) was obtained from Fisher Scientific and *N*,*N*-dimethylformamide (DMF) from EMD. Ethylenediaminetetraacetic dianhydride was purchased from Aldrich. EZ-Link TFP-PEO_3-Biotin was purchased from Pierce. DTT and Ultra Pure TRIS were purchased from ICN. Magnesium Acetate 4-hydrate was obtained from J.T. Baker. Ferrous Ammonium Sulfate was purchased from Fisher Scientific. Magnesium Chloride 6-Hydrate was obtained from Mallinckrodt. Water (18 MΩ) was purified using a aquaMAX-Ultra water purification system. Biological experiments were performed using Ultrapure Water (DNase/RNase free) purchased from USB. The pH of buffers was adjusted using a Thermo Orion 310 PerpHect Meter. All buffers were sterilized by filtration through a Nalgene 0.2 μm cellulose nitrate filtration. DNA oligonucleotides were purchased from Integrated DNA Technologies. [γ-^{32}P]-adenosine-5'-triphosphate ($\geq$ 7000 Ci/mmol) was obtained from MP Biomedicals. Calf thymus DNA was from Amersham and all enzymes were obtained from Roche.

Polyamide Synthesis. Polyamide monomers were prepared as described previously.[1] Synthesis was performed using established protocols and all polyamides were characterized by MALDI-TOF and analytical HPLC.

1: (MALDI-TOF-MS) $[M+H]^+$ calc. for $C_{69}H_{90}N_{25}O_{17}^+$ 1540.7, observed 1540.6

2: (MALDI-TOF-MS) $[M+H]^+$ calc. for $C_{83}H_{117}N_{28}O_{17}S^+$ 1809.9, observed 1810.0

Preparation of Labeled DX Tiles. In all cases 5' radiolabeling of 60 pmol of a single DNA strand was done using Polynucleotide Kinase. The labeled strand was then added to the three unlabelled strands in Affinity Cleavage Buffer (20 mM HEPES, 60 mM NaCl, 62.5 mM $MgCl_2$, pH 7.3) and a total volume of 20 μL. Several samples were made to titrate the labeled stand against the unlabelled strands in order to maximize formation of the four strand DX complex. The strands were heated to 95°C for 10 min and then allowed to cool slowly to room temperature over several hours. Purity was assessed by running 1 μL of the sample on a 6% polyacrylamide gel. The gel was then dried and exposed to a phosphor screen which was visualized using a Molecular Dynamics 400S Phosphorimager. In all cases the DX tile that was used was greater than 90% pure.

Affinity Cleavage on DX complexes. Affinity Cleavage experiments were done following previously established protocols.[2] The total reaction volume was 50 μL. The polyamide was allowed to equilibrate with DX (12,000 cpm / lane) for one hour in Affinity Cleavage Buffer (see above). A solution of freshly prepared ferrous ammonium sulfate was added to a final concentration of 1 μM and allowed to sit for 30min. To initiate the reaction, DTT was added to a final concentration of 5 mM. The reaction was allowed to proceed for 40min and stopped by the addition of 1.25 μL of precipitation buffer (Glycogen 2.8mg / ml, 140 μM bp calf thymus DNA). The DNA was then isolated by ethanol precipitation and run on an 8% polyacrylamide denaturing gel.

Electrophoretic Mobility Shift Assay (EMSA). 60 pmol of DNA strands DUPLEX1 and DUPLEX2 were first annealed by heating to 95°C for 10 min and allowing them to cool slowly to room temperature over several hours. Using Polynucleotide Kinase they were then 5' radiolabelled. The labeled DNA (3,000 cpm/lane) was then incubated with polyamide for 1 hr in TAEMg buffer followed by incubation with streptavidin for 30 min. The total volume for the reaction was 10 μl which was then run on a 6% polyacrylamide gel and imaged.

Affinity determination by quantitative DNase I footprinting. Reactions were carried out in a volume of 400 μL in aqueous TKMC buffer according to published protocols.[2] Standard molecular biology techniques were used to insert a 75bp DNA sequence into the *Bam*HI/*Hind*III restriction site of pUC19.[3] This plasmid was used to generate a 5' ^{32}P labeled 283bp *Eco*RI/*Pvu*II restriction fragment which was used for all footprinting experiments. Developed gels were imaged using storage phosphor autoradiography using a Molecular Dynamics 400S Phosphorimager. Equilibrium association constants were determined as previously described.[2]

Strand 1	5'-TCACTCTACCGCACGAGAATGGAGAT-3'
Strand 4	5'-CATACCGATCCGTGGCTACTGTCTTG-3'
DX-Up-2 (Tile A)	5'-CATTCTCGACGCCAATAGTTTGCACGTAACTTAGGTCACCTGCGGTAG-3'
DX-Up-3 (Tile A)	5'-CAGTAGCCTGCAAACTATTGGCGTGGTGACCTAAGTTACGACGGATCG-3'
DX-In-2	5'-CATTCTCGACGCCATAACTAAGCACGTATAGGTCATTGCCTGCGGTAG-3'
DX-In-3	5'-CAGTAGCCTGCTTAGTTATGGCGTGGCAATGACCTATACGACGGATCG-3'
DX-Down-2	5'-CATTCTCGACGCCTTTAGGTCACACGTAACTTCATTTGCCTGCGGTAG-3'
DX-Down-3	5'-CAGTAGCCTGTGACCTAAAGGCGTGGCAAATGAAGTTACGACGGATCG-3'
DX-Out-2	5'-CATTCTCGACGCTAGGTCACAGCACGTAACTTCATTTGCCTGCGGTAG-3'
DX-Out-3	5'-CAGTAGCCTGCTGTGACCTAGCGTGGCAAATGAAGTTACGACGGATCG-3'
DX-Tile B-1	5'-GTATGGCGAAGCGTGTAGAGCCAAGA-3'
DX-Tile B-2	5'-GCTCTACAGGATCTGGTAAGTTCGTGTAACGTCGGCTTGTCGCTTCGC-3'
DX-Tile B-3	5'-GCGGTTGTCGAACTTACCAGATCCACAAGCCGACGTTACAGGATTGCC-3'
DX-Tile B-4	5'-AGTGAGGCAATCCACAACCGCATCTC-3'
DUPLEX1	5'-CATTCTCGACGCTAGGTCACAGCAGGCTACTGTCTTG-3'
DUPLEX2	5'-CAGTAGCCTGCTGTGACCTAGCGTCGAGAATGGAGAT-3'

Supplemental Table 1. DNA strands used.

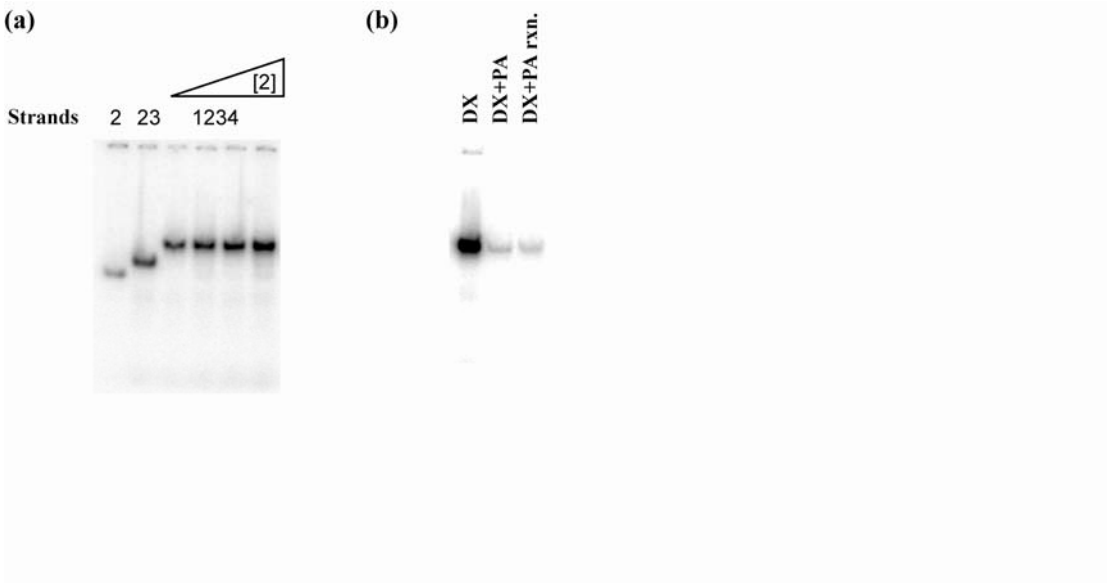

Figure S1. (a) Representative native gel showing the formation of the four stranded DX-In tile. Lane 1: strand 2. Lane 2: strands 2 and 3. Lanes 3-6: .7, .9, 1.0, and 1.2 equivalents of strand 2 with 1 equivalent of strands 1, 3, and 4 (b) Representative native gel showing stability of the fully formed complex. Lane 1: DX-In. Lane 2: DX-In and 1 μM polyamide **1** in affinity cleavage buffer. Lane 3: DX-Up and 1 μM polyamide **1** in affinity cleavage buffer after the cleavage reaction has occurred.

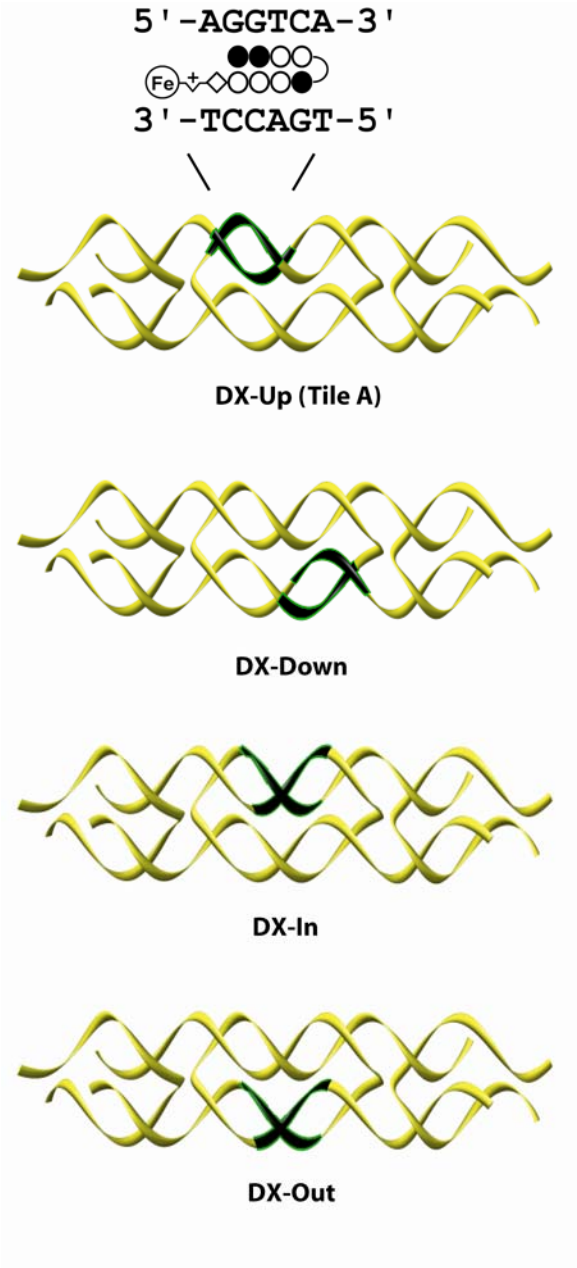

Figure S2. Models of four DX-tiles used to study a variety of binding orientations. Each DX consists of four individual DNA strands shown in yellow with the 6 bp polyamide binding site in black. The designations of each molecule refer to how the minor groove of each binding site, and thus the polyamide are situated.

(a)

Labelled Strand

DX-Up (Tile A)	**5' - CAT TCT CGA CGC CAA TAG TTT GCA CGT AAC TTA GGT CAC CTG CGG TAG - 3'**
DX-In	**5' - CAT TCT CGA CGC CAT AAC TAA GCA CGT ATA GGT CAT TGC CTG CGG TAG - 3'**
DX-Down	**5' - CAT TCT CGA CGC CTT TAG GTC ACA CGT AAC TTC ATT TGC CTG CGG TAG - 3'**
DX-Out	**5' - CAT TCT CGA CGC TAG GTC ACA GCA CGT AAC TTC ATT TGC CTG CGG TAG - 3'**

(b)

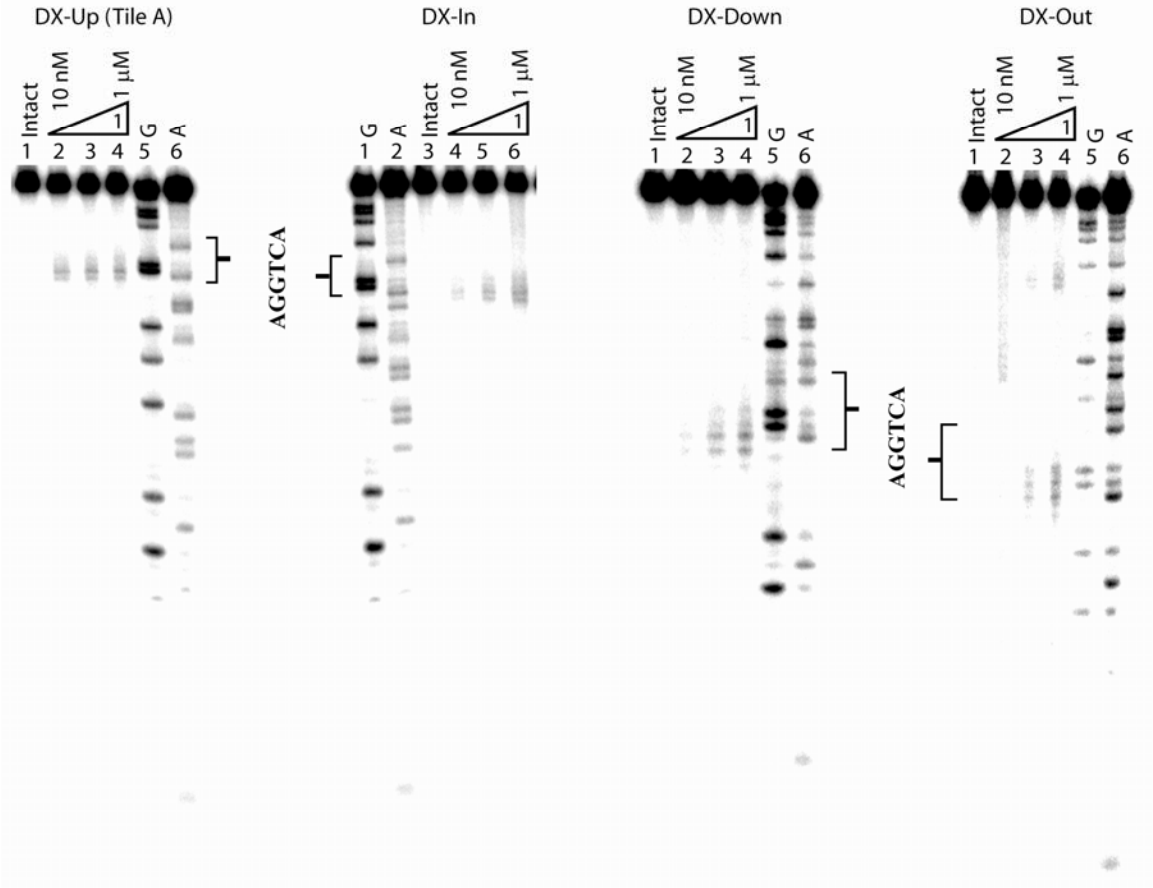

Figure S3. (a) List of DNA sequences for the labeled strand in each DX tile. The binding site for **1** is highlighted in red. (b) Affinity Cleavage on each DX tile. Strand 2 of each DX was ^{32}P-radiolabeled and affinity cleavage was done using polyamide **1**; the complex was then denatured and visualized by gel electrophoresis. The target binding site is indicated. A and G sequencing lanes are shown for each gel. Polyamide **1** was added at concentrations of 10 nM, 100 nM, and 1 µM. Intact lanes were not incubated with **1**.

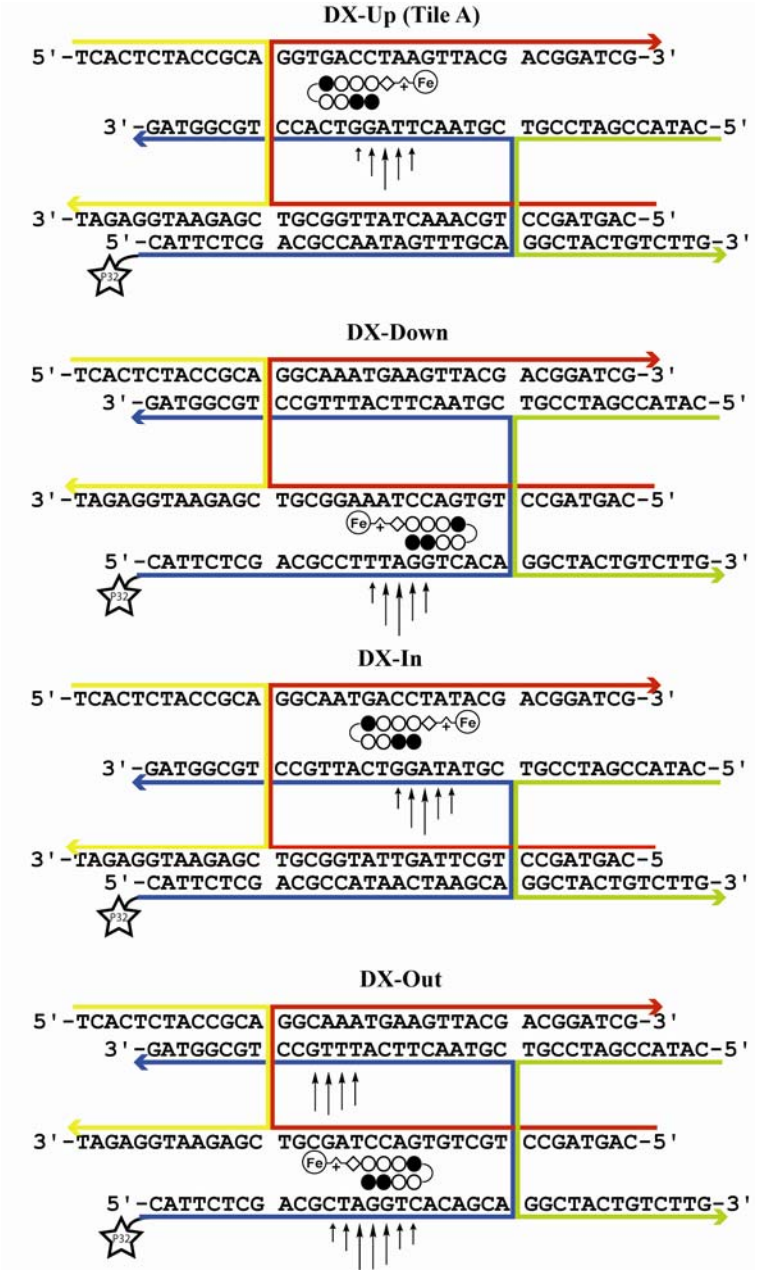

Figure S4. Representations of each DX tile with polyamide **1**. Arrows represent the extent of cleavage at the indicated base position.

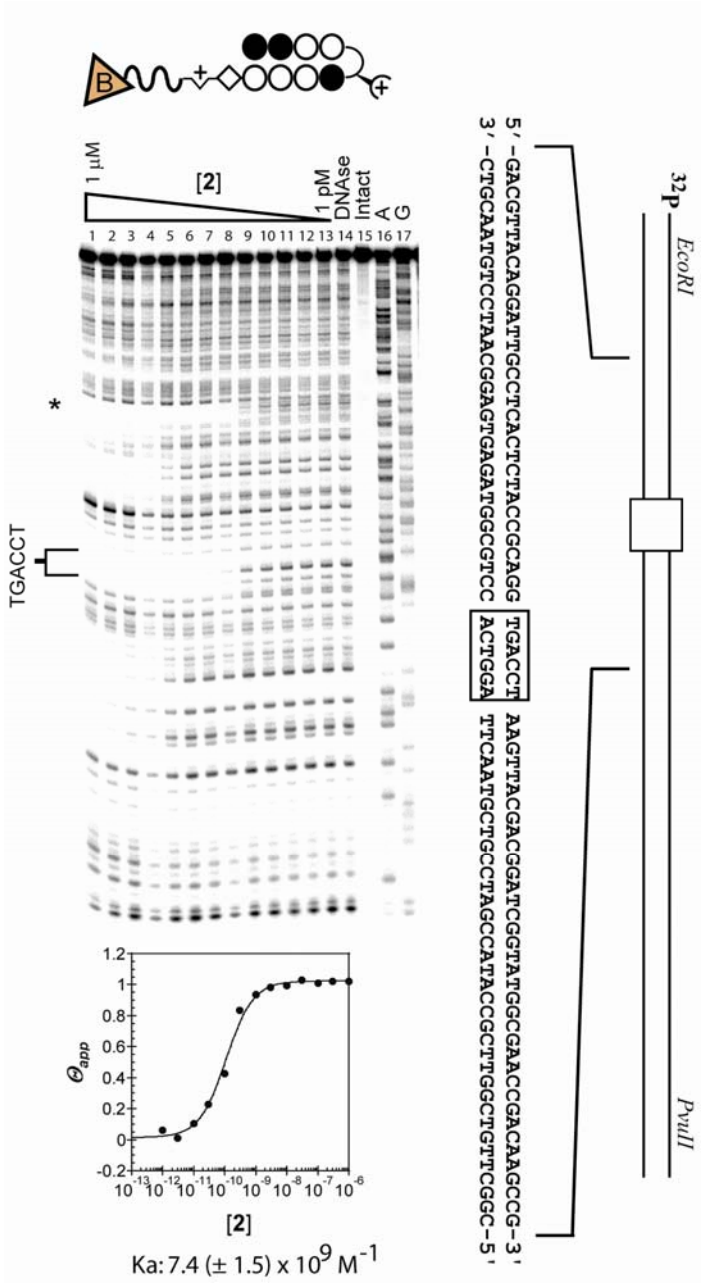

Figure S5. DNAse I Quantitative Footprinting with polyamide **2**. The 75 bp insert and schematic of the plasmid are shown with the binding site boxed. Lanes 1-13: 1 μM, 300 nM, 100 nM, 30 nM, 10 nM, 3 nM, 1 nM, 300 pM, 100 pM, 30 pM, 10 pm, 3 pm, and 1 pm of polyamide. Lane 14: DNAse standard. Lane 15: Intact DNA. Lane 16: A-sequencing lane. Lane 17: G-sequencing lane. Representative isotherm for polyamide binding and the calculated Ka value is shown. The experiment was repeated in triplicate. The DNA sequence used was designed to mimic (with the exception of crossover junctions) the sequence formed by half of tile B, tile A, and half of tile B as would be present in a DX array. An additional binding site consisting of the sequence 5'-TGGTCA-3' is contained in the plasmid and indicated by the asterisk above.

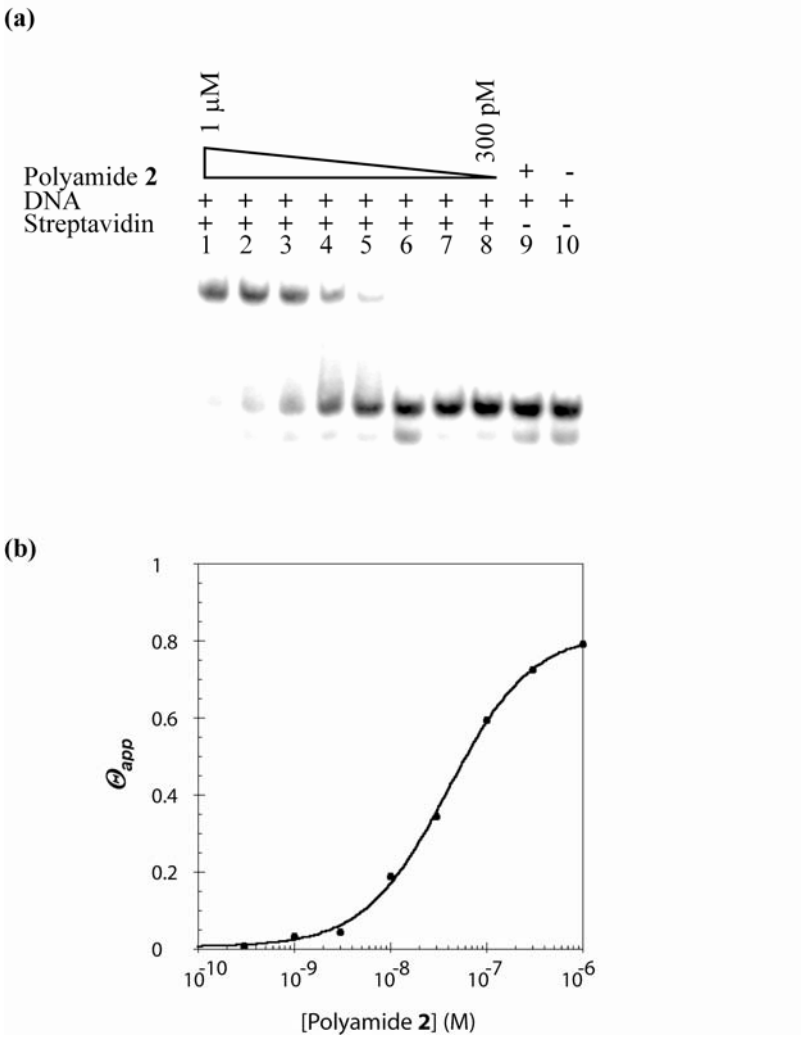

Figure S6. (a) EMSA for polyamide **2** in the presence and absence of 10 µM streptavidin. Lanes 1-8: 1 µM, 300 nM, 100 nM, 30 nM, 10 nM, 3 nM, 1 nM, 300 pM polyamide **2**. Lane 9: Control containing 1 µM polyamide **2** and DNA**.** Lane 10: Control containing only DNA. (b) Isotherm for streptavidin recruitment by polyamide **2**. The duplex DNA used was designed to mimic the sequence present on one helix of DX-Out (with the exception of the crossover junctions).

References:

[1] E. E. Baird, P. B. Dervan, *J. Am. Chem. Soc.* **1996**, *118*, 6141.

[2] J. W. Trauger, P. B. Dervan, in *Drug-Nucleic Acid Interactions, Vol. 340*, **2001**, pp. 450.

[3] J. Sambrook, E. F. Fritsh, T. Maniatis, *Molecular Cloning: Standard Protocols for DNA Manipulation. A laboratory Manual 2nd ed.*, Cold Spring Harbor Laboratory, Plainview, NY, **1989**.