Multivalent ultrasensitive interfacing of supramolecular 1D nanoplatforms

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SUPPORTING INFORMATION

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Experimental methods.

Materials. Solvents and reagents were obtained from commercial sources and used without further purification. All oligonucleotides were obtained HPLC-pure from IDT Integrated DNA Technologies and dissolved in the appropriate amount of Invitrogen™ UltraPure™ DNase/RNase-Free Distilled Water to reach a concentration of 100 μM. The solutions were shaken for 2h at room temperature and subsequently stored at -20°C. The monomers 4-Disc, 5-Disc, 5-Disc lacking the Cy3-dye and the Inert-Disc were obtained as previously reported.[2] All the experiments were performed using UltraPure™ DNase/RNase-Free Distilled Water and RNase-Free $MgCl_2$ (1M) obtained from ThermoFisher Scientific. Corning® 384-well Low Volume Black Round Bottom Polystyrene Not Treated Microplate was obtained from Fisher Scientific. DNA LoBind tubes were obtained from Eppendorf. The 100 kDa MWCO 0.5 mL Amicon centrifugal filters were obtained from Merck Millipore. $Mg(CH_3COO)_2$, $Na(CH_3COO)_2$, $AgNO_3$ and $NaBH_4$ were obtained from Sigma Aldrich.

Protocol for the assembly of the DNA-duplex based ligand and receptor nanoscaffold. The assembly of the quencher-labeled ligands was performed by using an equimolar amount of quencher-dye and the corresponding amounts of DNA backbone, following the combinations in Table S1. The titration curves of the assembly of both nanoplatforms (m-Disc and mL_n ligands) were performed by the titration of the pre-assembled quencher-labeled ligands to a constant concentration of m-Disc (10 nM), using UltraPure™ DNase/RNase-Free distilled water containing 5 mM of RNase-Free $MgCl_2$ as the buffer. The mixture was left to incubate in the dark at room temperature for 2 hours and measured in a plate reader at a temperature of 21°C. The receptor density studies were performed by incubation of 10 nM of m-Disc with the appropriate amount of Inert-Disc (0-1μM) needed to reach the desired percentage of functionalized m-Disc in the final assembly. The solution was left to incubate in the dark at room temperature for 2 hours with the subsequent addition of the DNA-based ligands. This mixture was incubated at room temperature for an additional hour and measured in a plate reader (see below).

Transmission electron microscopy.
Visualization by TEM was performed with a Tecnai G2 Sphera by FEI operating at an acceleration voltage of 80 kV. 3 μL of the Disc-DNA solutions was drop-casted on a 400 square mesh copper grid with a carbon support film and dried for one minute. DNA-Disc monomers with DNA sequence that form silver nanoclusters in the presence of $AgNO_3$ and $NaBH_4$ were used to provide for sufficient contrast intensities of the columnar assemblies.

Silver-stained receptor nanoscaffold design. The receptor nanoscaffold visualization by TEM was carried out by using a DNA-Disc monomer with DNA sequence (TTTACCCGAACCTGGGCTACCACCCTTAATCCCC) that is specifically known to form silver nanoclusters in the presence of $AgNO_3$ and $NaBH_4$.[1] The DNA sequence which bears an azido hexanoic amide in the 5´-end, was coupled to the Disc using Strain Promoted Click Chemistry. The resulting DNA-Disc conjugate (silver nanocluster-Disc) was purified as previously reported.[2]

Silver-stained receptor nanoscaffold assembly protocol. (Figure S1a) A 10 μL solution of silver nanocluster-Disc at a concentration of 10 μM was prepared using 10 mM Tris-acetate as the buffer. The solution was left to equilibrate for 30 minutes. Subsequently, $AgNO_3$ (62 μM) was added and incubated for 15 minutes before addition of $NaBH_4$ (62 μM). The resulting samples were diluted by half with the buffer to a total concentration of 5 μM of silver nanocluster Disc.

Silver-stained receptor nanoscaffold-ligand complex assembly. (Figure S1b) The silver nanocluster-Disc and 5-Disc lacking the Cy3-dye were mixed in a 1:1 ratio, at a concentration of 10 μM each, using 10 mM Tris-acetate buffer with 5 mM of magnesium acetate as buffer. The solution was left to equilibrate for 1 hour in order to ensure intermixing of the two discotic monomers. $5L_6$ ligand was added to the mixture to reach an equimolar receptor-ligand complex concentration of 10 μM. The mixture was incubated for 1 hour to ensure receptor-ligand assembly. In the final step, before imaging the silver nanocluster-discs were stained by the addition of, first $AgNO_3$ (62 μM) with an incubation time of 15 minutes, and the subsequent addition of $NaBH_4$ (62 μM). The resulting sample was diluted by half with buffer to a total concentration of 5 μM for each disc.

Silver-stained receptor nanoscaffold-AuNP-labeled ligand complex assembly. (Figure S1c) The silver nanocluster-Disc and 5-Disc lacking the Cy3-dye were mixed in a 1:1 ratio, at a concentration of 10 μM each, using 10 mM Tris acetate buffer with 5 mM of magnesium acetate as the buffer. The solution was left to equilibrate for 1 hour in order to ensure intermixing of the two discotic monomers. 1 μL (30 nM) of AuNP-labeled ligand (AuNP-$5L_6$) (See AuNP Ligand labeling protocol below) was added to the mixture and incubated for 1 hour. In the final step the silver nanocluster-discs were stained by the addition of first $AgNO_3$ (62 μM) with an incubation time of 15 minutes, and the subsequent addition of $NaBH_4$ (62 μM). The resulting sample was diluted by half with buffer to a total concentration of 5 μM for each disc.

AuNP Ligand labeling protocol. Cytodiagnostics 10 nm NHS-Activated Gold Nanoparticle Conjugation Kit was obtained from Sigma-Aldrich and used according to their conjugation protocol. In short, the backbone solution (B_6, with a linker sequence bearing an amine moiety NH_2-TTAATT in the 5´-end) (250 μM in DNase/RNase-free water (Invitrogen))was diluted with the kit's resuspension buffer to 20 μM. This solution was transferred, together with the

kits reaction buffer, to the lyophilized NHS-activated gold nanoparticles and subsequently incubated for 2 hours at room temperature while shaking at 600 rpm. The kits quencher solution was added and 6 µL of the two handle strands (100 µM in DNase/RNase-free water (Invitrogen)) were added to form the ligand. The excess backbone and handle strands were removed using 100 kDa MWCO 0.5 mL Amicon centrifugal filters (Merck Millipore). Briefly, a filter was pre-wetted with 450 µL storage buffer (10 mM Tris, 5 mM $Mg(CH_3COO)_2$, 100 mM $Na(CH_3COO)_2$, pH 8.0). The gold nanoparticle mixture was diluted to 450 µL with storage buffer, added to the filter and centrifuged at 4°C for 5 min at 5,000 g. This step was repeated for a total of three washing steps. The concentrate was recovered by inverting the filter and spinning for 2 min at 1,000 g. The gold nanoparticle concentration was determined by measuring the absorption at 520 nm, assuming an extinction coefficient of 1.01×108 $M^{-1}cm^{-1}$. The purified ligand gold nanoparticles were stored in DNA LoBind tubes (Eppendorf) at 4 °C until use.

Fluorescence spectroscopy. Fluorescence measurements were measured using a Spark® Tecan 10M. The titration experiments were performed with single fluorescence intensity measurements exciting the samples at a wavelength of 540 nm and the fluorescence was recorded at a wavelength of 590 nm, with excitation and emission bandwidth of 20 nm, 50% mirror and 100 of gain at a temperature of 21 ^{0}C. The full spectra scans were performed using 100 of gain and 50% mirror. The excitation wavelength used for the excitation of the receptor nanoscaffold was 345 nm, recording from 400 to 650 nm. The excitation wavelength for the excitation of the Cy3-dye was 495 nm, recording from 540 to 700 nm. All samples were prepared in a Corning® 384-well Low Volume Black Round Bottom Polystyrene Not Treated Microplate, using a total sample volume of 10 µL.

Temperature-dependent UV-Vis and fluorescence measurements. Temperature-dependent UV-Vis measurements were carried out in a Varian Cary 50 UV-Vis Spectrophotometer from Agilent Technologies. The measurements were performed with a scan rate of 600 nm/min and data interval of 1 nm. The temperature-dependent fluorescence measurements were carried out with a Cay Eclipse Fluorescence Spectrophotometer from Agilent Technologies, using an excitation wavelength of 345 nm, with emission wavelength recording between 400 and 650 nm. The excitation and emission slits were 5 nm, with a scan rate of 1200 nm/min and data interval 2 nm. The temperatures for both experiments was controlled with a Peltier temperature controller featuring steps of 10ºC for each experiment, letting the temperature of the cell equilibrate for 10 minutes before each measurement.

Ligand-receptor complex ratio determination, binding affinities, K_a, ΔG^o. The quencher-labeled ligands (mL_n) were titrated against the Cy3-labelled m-Disc, incubated as described above and subsequently the fluorescence of the samples was measured. The quenching of the fluorescence intensity was reported as complex ratio, calculated from equation **S1**, and then plotted over ligand concentration.

The *fluorescence intensity of the Cy3 dye in the unbound state* in equation **S1** is the average fluorescence intensity over triplicates of a solution of m-Disc at a concentration of 10 nM. The data points were fitted using Origin 2015 of OriginLab Corporation with a non-linear curve fitting, category: Growth Sigmoidal fit using a function of Dose Response and iteration algorithm of Levenberg Marquardt. The association constants were obtained using equation **S2**. The IC_{50} values were obtained from the non-linear curve fit, where the IC_{50} values correspond to the concentration of ligand where the complex ratio is equal to 0,5. The IC_{50} was converted to K_n^{multi} using equation **S2** in order to calculate the Gibbs free energy of each interaction (ΔG^o). The ΔG^o was then derived from the association constant values (K_n^{multi}), using equation **S3** with T = 21ºC.

$$\text{complex ratio} = 1 - \left(\frac{\text{Remaining Fluoresecence intensity Cy3 dye} - \text{bound state}}{\text{Maximum Fluorescence intensity Cy3 dye} - \ 100\% \text{ unbound state}}\right) \quad \text{(Eq. S1)}$$

$$K_n^{multi} = \frac{1}{IC_{50}} \quad \text{(Eq. S2)}$$

$$\Delta G = -RT \cdot \ln(K_n^{multi}) \quad \text{(Eq. S3)}$$

Agarose gel protocol. The agarose gel was prepared by dissolving 1.5 grams of MetaPhor™ Agarose from Lonza in 50 mL of 1x TBE with 5 mM of $MgCl_2$. The mixture was heated for 2 minutes in the microwave to obtain a homogeneous solution. 5 µl of SYBR Safe was added to the solution and the mixture was poured in the agarose gel tray. The possible formed bubbles were removed with a pipette tip and the well comb placed in the tray. The solution was left to cool down for 20-40 minutes to form the gel. 1x TBE with 5 mM of $MgCl_2$ was used as the running buffer. The gel was pre-run for 15 minutes at 65 V. The samples were prepared using Gel Loading Dye, Purple (6X), no SDS. Subsequently, the gel was run for 90 minutes at 65 V. The image was taken using an ImageQuant 350 imager with UV excitation and SYBR Safe emission filter.

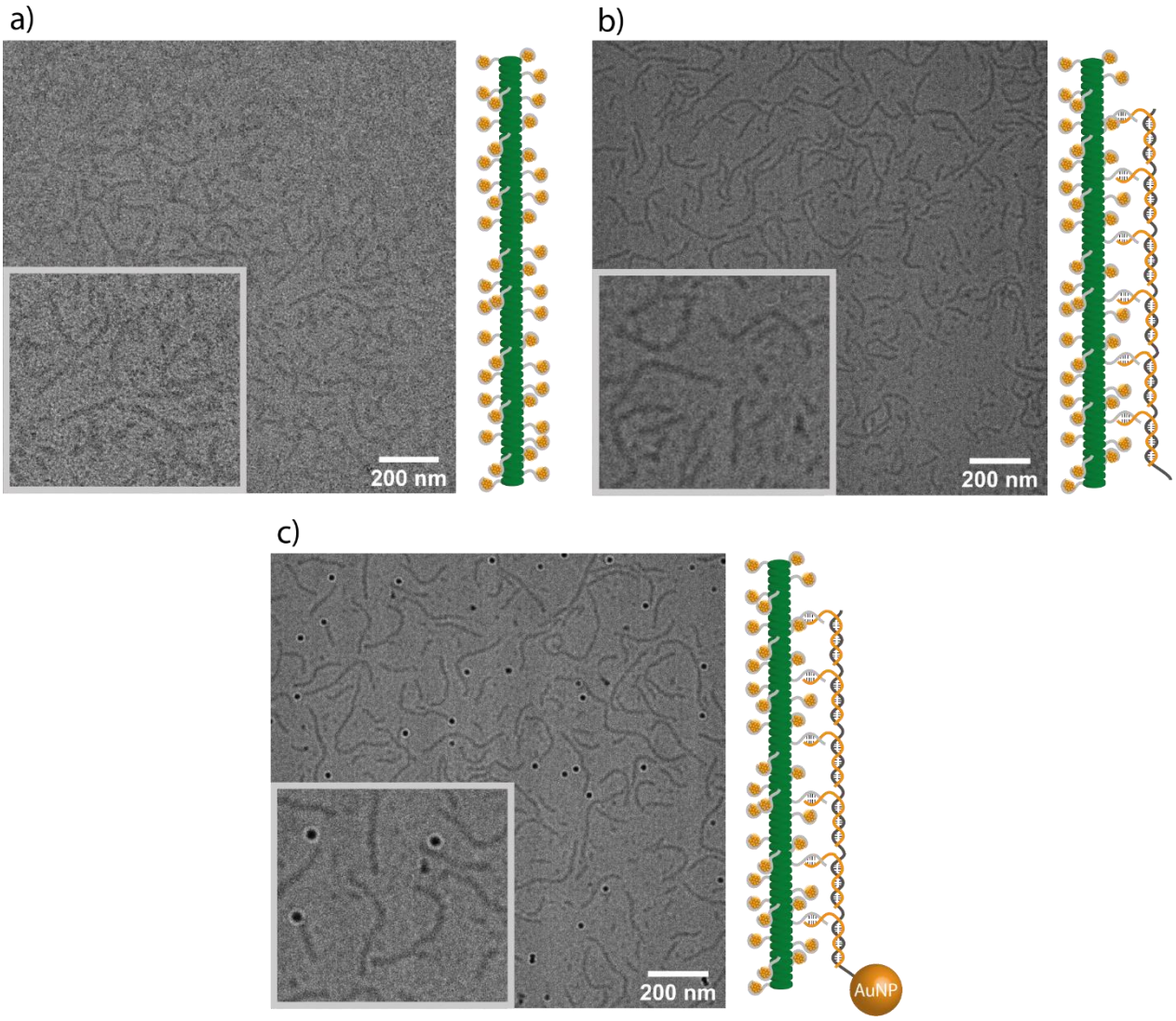

Figure S1. TEM images of the columnar assemblies formed by the receptor nanoscaffold in absence and presence of the DNA-duplex based ligand. a) TEM image, and zoom in thereof of the supramolecular assemblies formed by DNA-appended discotic monomers alone, featuring DNA sequences that form silver nanoclusters (silver nanocluster-Disc, 5 μM). b) TEM image, and zoom in thereof of the supramolecular assemblies formed by an equimolar mixture of 5 μM silver nanocluster-Disc, 5-Disc lacking and the multivalent DNA-based ligand ($5L_6$), followed by silver-staining of the silver nanocluster-Disc, c) TEM image, and zoom in thereof of the pre-assembled supramolecular assemblies formed by an equimolar mixture of 5 μM silver nanocluster-Disc and 5-Disc and 30 nM of the AuNP-labeled multivalent DNA-ligand (AuNP-$5L_6$), followed by silver-staining.

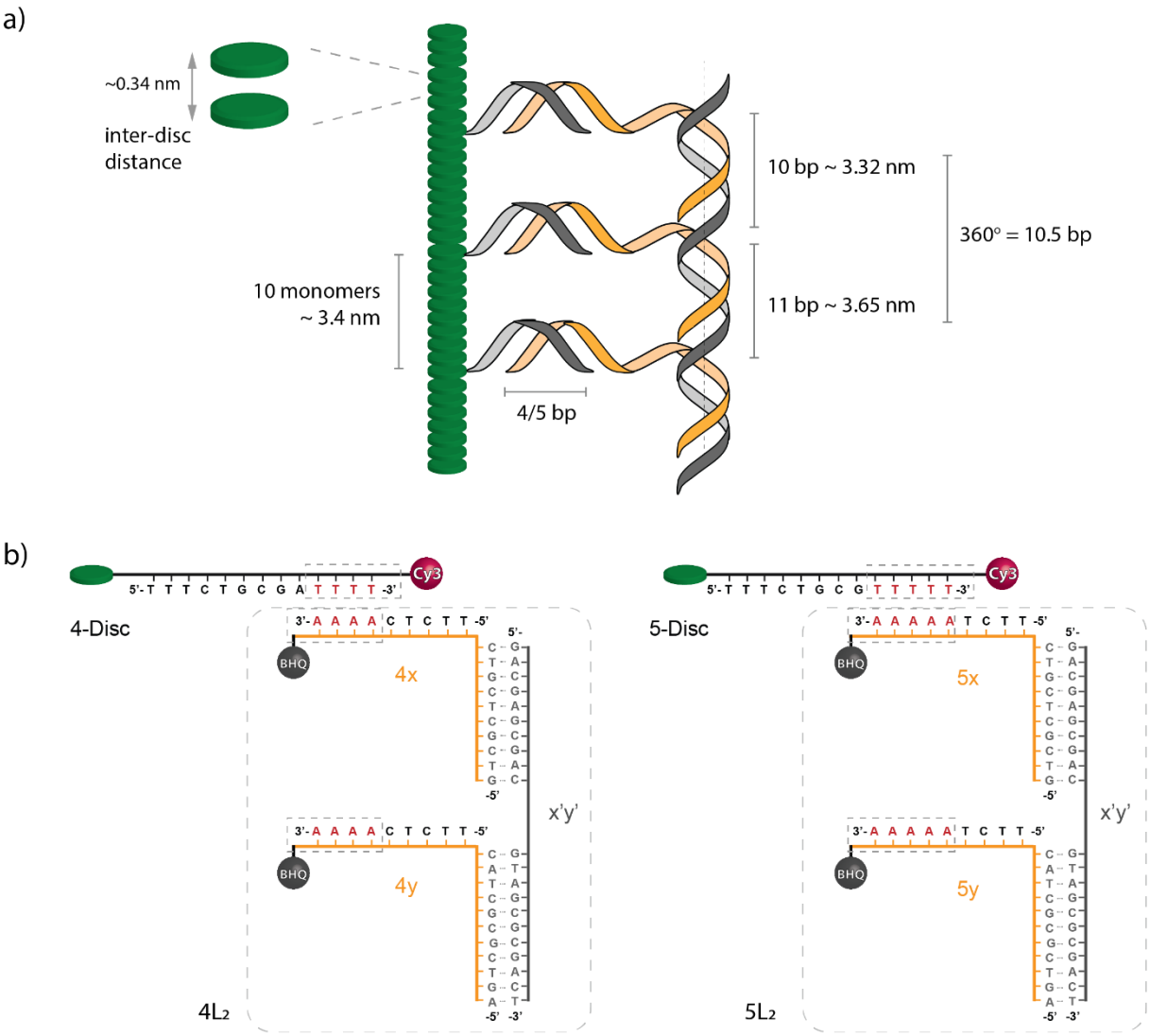

Figure S2. a) Design and geometric distribution of the DNA-based ligands. In green, the discotic monomers. Previous studies of the central bipyridine core have estimated that there are 28 molecules (monomers) for a 360°-turn. (120°-turn is 9.35 molecules).[3] b) Exact DNA sequences of the DNA-functionalized discotic monomers (4-Disc and 5-Disc)[2] and the DNA-based ligands of Series I (4x and 4y) and of Series II (5x and 5y).

Table S1. Ligand composition table, backbone (B_2-B_6) and branches (mx and my) sequences for the ligands (L_2-L_6).

Ligand	Backbone		Branch m**x**	Branch m**y**
Name	Name and Sequence	Equivalents	Equivalents	Equivalents
L_2	B_2 (**x'y'**)	1	1	1
L_3	B_3 (**y'x'y'**)	1	1	2
L_4	B_4 (**x'y'x'y'**)	1	2	2
L_5	B_5 (**y'x'y'x'y'**)	1	2	3
L_6	B_6 (**y'x'y'x'y'x'**)	1	3	3

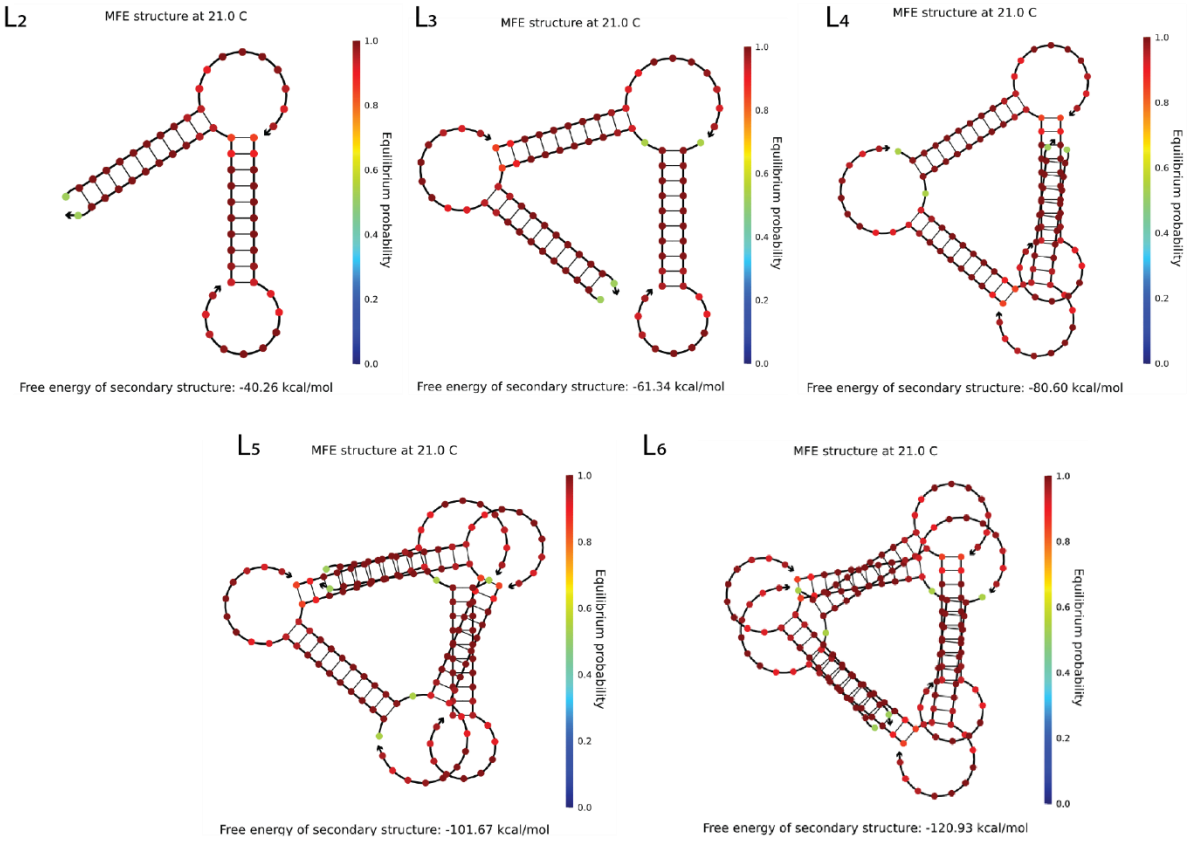

Figure S3. Simulation analysis with the NuPack software of the formation of the DNA-duplex based ligands. The backbone strands (B_2-B_6) are assembled with the branches 5x and 5y for the formation of the DNA-based ligands (L_2-L_6) at the experimental conditions (21°C and 10 nM of total quencher-labeled branch) (also see Table S1). In each case, a clearly defined 10 or 11 base-paired double-helical segments can be identified together with a non-paired single-stranded DNA overhang.

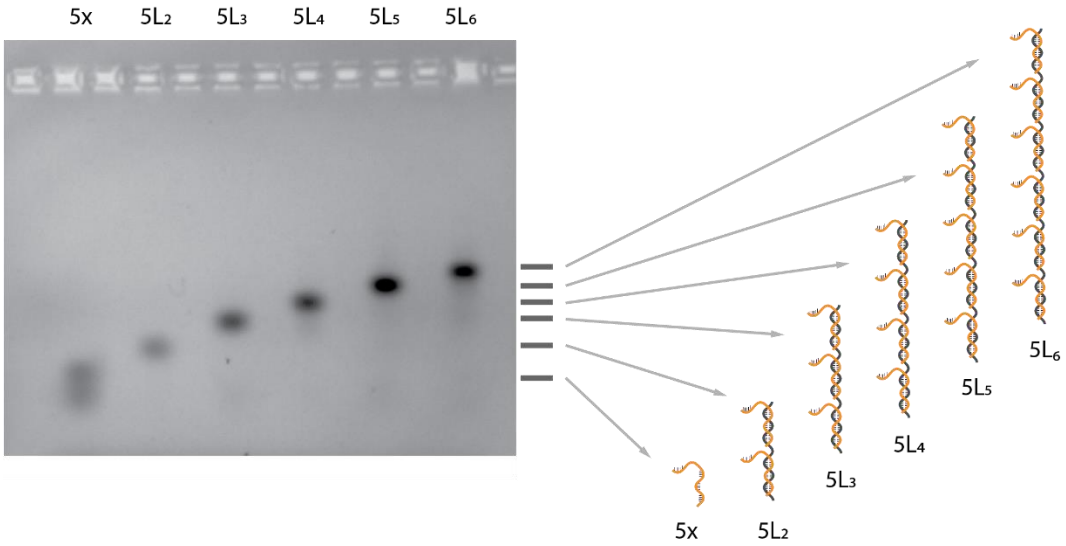

Figure S4. Ligand assembly confirmation by agarose gel of the library of ligands from series II (L_2-L_6). The ligands are formed by the assembly between the different backbones B_2-B_6 (at a constant concentration of 1 μM) and the complementary branches 5x and 5y, following the ratios established in Table S1. The agarose gel (3%) was run using TBE with 5 mM $MgCl_2$ as the running buffer. The gel was run at 65V for 90 minutes. The presence of the different backbones shows the shift of the branch spot in the gel, due to an increase in molecular weight with the consequent decrease in the electrophoretic mobility.

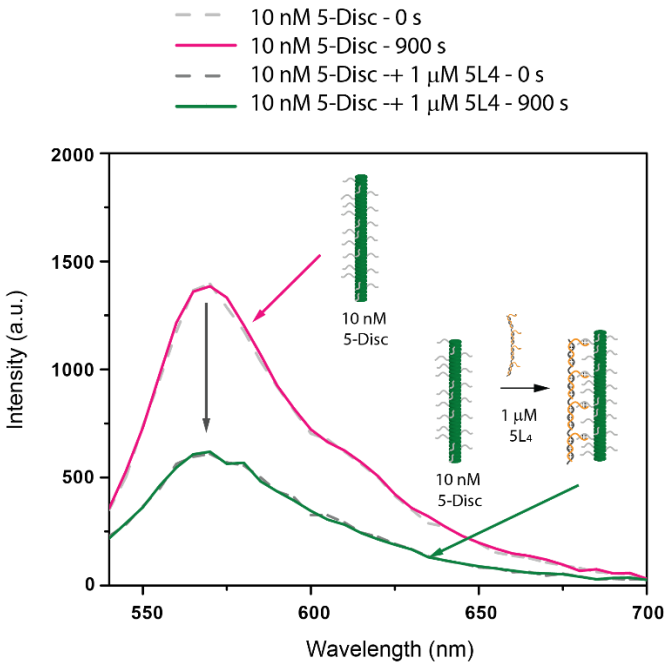

Figure S5. Cy3-labeled receptor nanoscaffold fluorescence with and without quenching ligand direct after mixing and after 10 minutes. The supramolecular nanoscaffold (5-Disc) concentration is 10 nM while the ligand ($5L_4$) has a total quencher concentration of 1 μM. The solid pink line is the fluorescence of the 5-Disc appended Cy3 dye before addition of the ligand. Upon addition, the Cy3-dye fluorescence is quenched resulting in a lower fluorescence intensity (green solid line). The fluorescence after 15 minutes of both samples gave the same intensity profile.

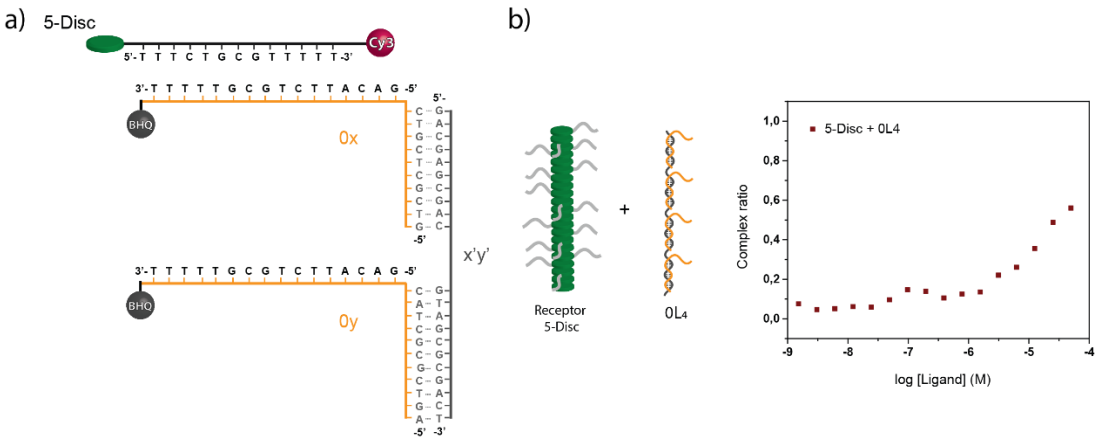

Figure S6. a) DNA sequences of the DNA-functionalized discotic monomer (5-Disc) and a control DNA-based ligand with branches 0x and 0y and with 0 bases of complementarity (m=0). b) The control titration experiment was performed using backbone B_4 and performed at a receptor nanoscaffold concentration of 10 nM and at a temperature of 21ºC. Addition of the ligand does not lead to quenching of the Cy3-appended dye on the receptor (as expressed in complex ration on the y-axis). Only at high concentrations above 10 μM, some background dye quenching occurs because of the high concentration of quencher.

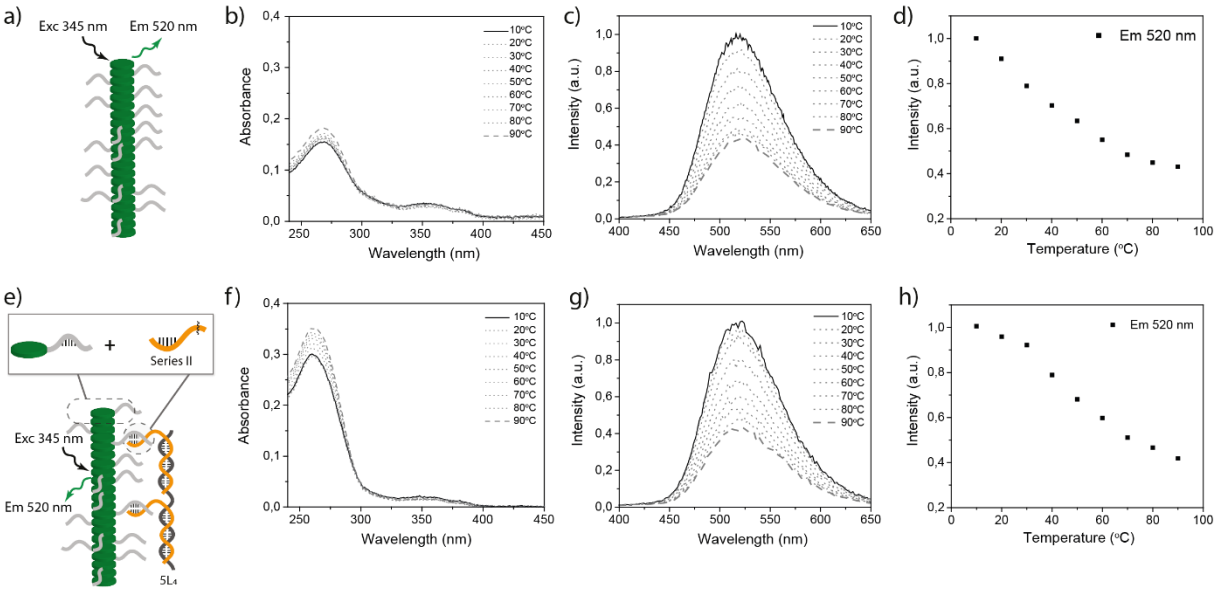

Figure S7. Temperature-dependent UV-Vis and fluorescence optical properties of the receptor nanoscaffold in the absence and presence of DNA-duplex based ligand. The supramolecular nanoscaffold has a characteristic absorption maximum at 345 nm and a fluorescence maximum at 520 nm in the aggregated state. This characteristic spectroscopic footprint was studied in a-d) the absence and e-h) presence of the tetravalent ligand ($5L_4$). b) UV-vis of a 2.5 μM solution of 5-Disc (without Cy3-dye), shows the characteristic peaks of the oligonucleotide at 260 nm and the disc at 260 and 345 nm, over the range of temperatures (10ºC-90ºC) studied. c) The fluorescence spectrum of the same sample shows a decrease of fluorescence upon increase in temperature, due to a shift of the equilibrium from the aggregated to the monomeric state at higher temperatures. d) Emission intensity at the fluorescence maximum (520 nm) versus the temperature. f-h) The same set of experiments in the presence of an equimolar amount of ligand ($5L_4$) showed: f) a similar UV-Vis temperature response (the higher intensity at 260 nm results from the absorption of the added DNA ligand); g-h) a similar fluorescence intensity temperature response, with indications for a stabilizaiton effect by the DNA ligand at temperatures up to 40ºC.

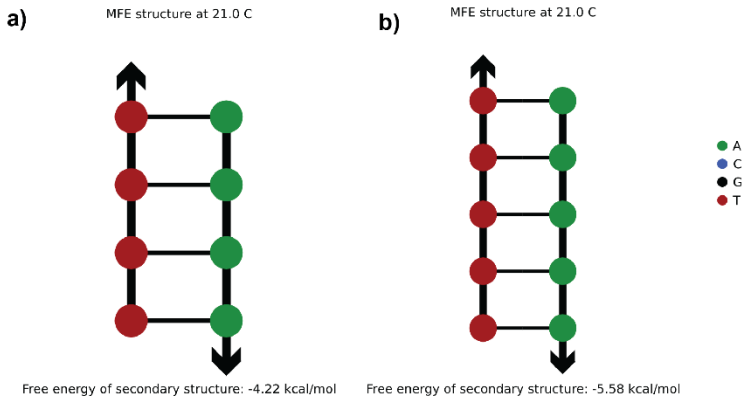

Figure S8. Nupack simulation analysis of the ΔG^o resulting from the monovalent interaction with a) four and b) five bases of complementarity. The simulation was performed at a temperature of 21ºC and a concentration of 1 mM for both DNA strands. The resulting ΔG^o values for Series I ($4L_1$) and Series II ($5L_1$) are -4.2 and -5.6 Kcal/mol respectively. These values can be converted to association constants using $\Delta G = - RT \cdot \ln (K_a)$, see Table 1.

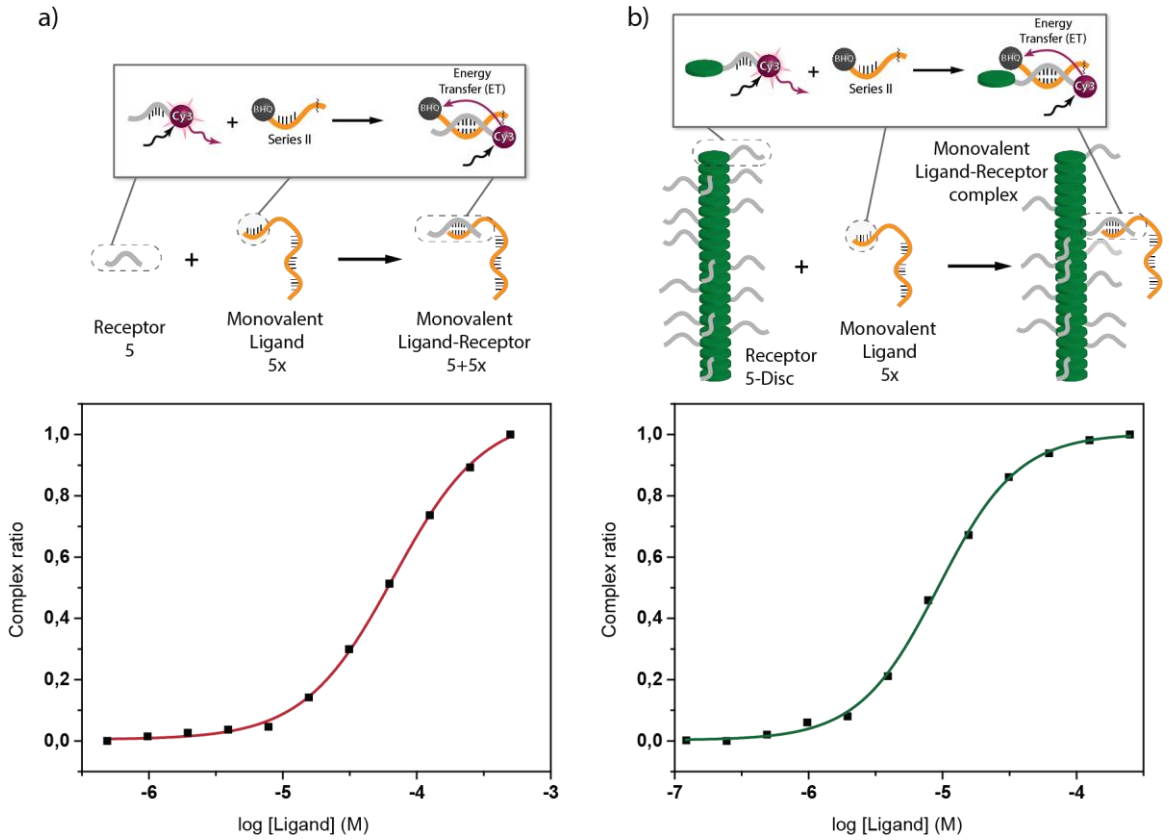

Figure S9. Complex ratio formation of the monovalent interaction within Series II monitored by the quenching of Cy3-labelled a) Receptor (5) and b) Receptor 5-Disc with the monovalent branch of the DNA-ligand featuring 5 complementary base pairs (5x). The experiment was carried out at a temperature of 21°C and a receptor concentration of 10 nM. For resulting K_a values, see Table S2.

Table S2. K_a values of the monovalent Receptor (5) and Receptor Disc (5-Disc) interaction with the DNA-based Ligand (5x). *Calculated value using the program Nupack, using at a temperature of 21°C and a concentration of both components, ligand and receptor, of 1 mM.

Monovalent interaction Series II	K_a (M^{-1})	ΔG^o (Kcal/mol)
$5L_1$ + 5 (calculated*)	$1.40 \cdot 10^4$	-5.6
$5L_1$ + 5 (experimental)	$1.50 \cdot 10^4$	-5.7
$5L_1$ + 5-Disc (experimental)	$1.07 \cdot 10^5$	-6.8

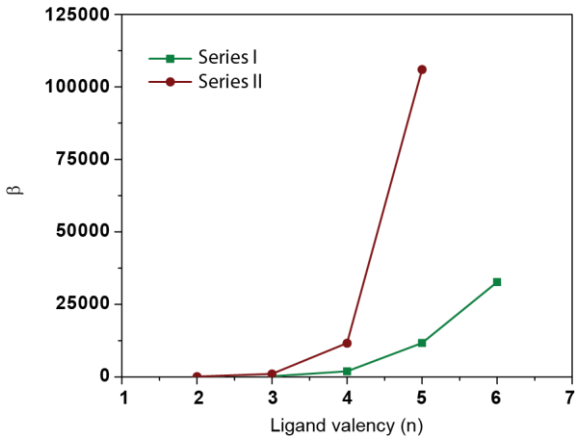

Figure S10. β enhancement parameter of the ligand of Series I and II (values in Table 1) plotted against the ligand valency (n) for both Series I and II.

Table S3. Free energies (ΔG°) in Kcal/mol at different receptor densities, calculated from the binding isotherms shown in Figure 3; n.d. not determined.

Free energy (ΔG°) in Kcal/mol					
Receptor density (Θ_R)	$5L_2$	$5L_3$	$5L_4$	$5L_5$	$5L_6$
100	-8.4	-9.7	-11.1	-12.4	n.d.
50	-7.7	-8.9	-9.9	-11.4	-11.5
10	-7.5	-8.8	-9.4	-10.5	-10.3
2	-7.2	-8.1	-8.3	-9.7	-9.8
1	-6.8	-8.0	-8.2	-9.6	-9.6

Table S4. β enhancement factor of Series II at different receptor densities, calculated using equation 1. K_n^{multi} is calculated from the IC50 values from the titration curves while K_{mono} is obtained from the experimental values for the monovalent interactions obtained in Figure S9.

	β enhancement factor				
Receptor density (Θ_R)	β ($5L_2$)	β ($5L_3$)	β ($5L_4$)	β ($5L_5$)	β ($5L_6$)
100	$1.1 \cdot 10^2$	$1.0 \cdot 10^3$	$1.2 \cdot 10^4$	$1.1 \cdot 10^5$	n.d.
50	$3.3 \cdot 10^1$	$2.8 \cdot 10^2$	$1.6 \cdot 10^3$	$2.1 \cdot 10^4$	$2.2 \cdot 10^4$
10	$2.5 \cdot 10^1$	$2.5 \cdot 10^2$	$6.3 \cdot 10^2$	$4.4 \cdot 10^3$	$3.1 \cdot 10^3$
2	$1.6 \cdot 10^1$	$7.5 \cdot 10^1$	$9.8 \cdot 10^1$	$1.0 \cdot 10^3$	$1.3 \cdot 10^3$
1	$7.0 \cdot 10^0$	$6.0 \cdot 10^1$	$8.6 \cdot 10^1$	$9.6 \cdot 10^2$	$9.1 \cdot 10^2$

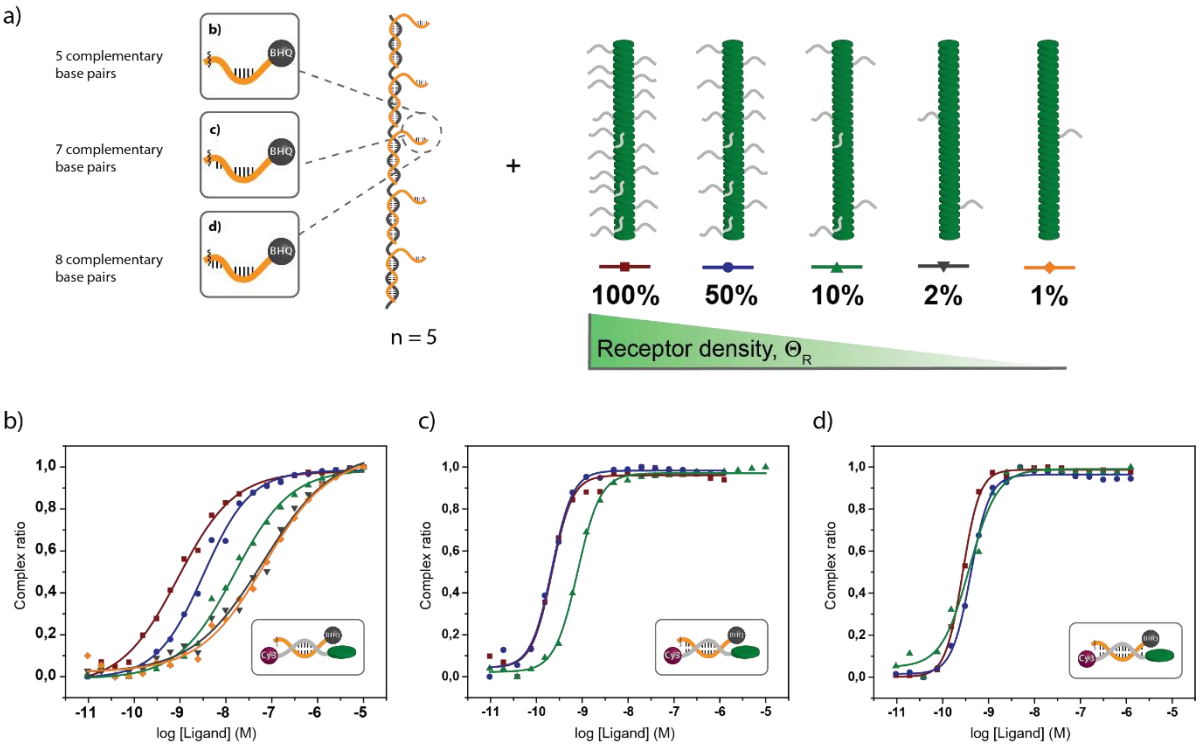

Figure S11. a) Schematic representation of receptor nanoscaffold with different complementary base-pairs and with densities of DNA overhangs. b-d) Titration curves of pentavalent ligands $5L_5$, $7L_5$, and $8L_5$ (with five, seven, and eight complementary base pairs) to the receptor nanoscaffolds with differing receptor density (for color coding see Figure S11a). The concentration of DNA-Disc was kept constant at 10 nM with increasing amounts of Inert-Disc.

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