Electronic Supplementary Material (ESI) for Chemical Communications

Branched DNA nanostructures efficiently stabilised and monitored by novel pyrene-perylene 2′-α-L-amino-LNA FRET pairs

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Supplementary Information

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General. Reagents obtained from commercial suppliers were used as received. Phosphoramidite reagents for incorporation of monomers **M**^{1}, **M**^{2} and **M**^{3} were synthesized as described.[1] Reagents and solvents for click chemistry were obtained from commercial suppliers (Fluka, Lumiprobe, Sigma-Aldrich), and were used as received; TBTA,[2] 4,4'-diazido-1,1'-biphenyl **3**,[3] and perylene diimide azide **4**[4] were prepared following published procedures. Synthesis of azide **5** was performed in 4 steps starting from rosolic acid **9** (Scheme S1; overall yield of **5**: 24%). Stock solutions for click chemistry were prepared as described in recent publications.[5] Click reactions were performed in 1 mL reactor tubes under argon and vigorous stirring in Emrys Creator (Personal Chemistry). 1-Pyrenebutyric acid and perylene were used as standards for emission quantum yield measurements after recrystallization. Photochemical studies were performed using spectroquality methanol and cyclohexane. Other solvents and reagents applied in this study were used as received. Silica gel (0.040-0.063 mm) used for column chromatography and analytical silica gel TLC plates 60 F254 precoated aluminium plates were purchased from Merck.

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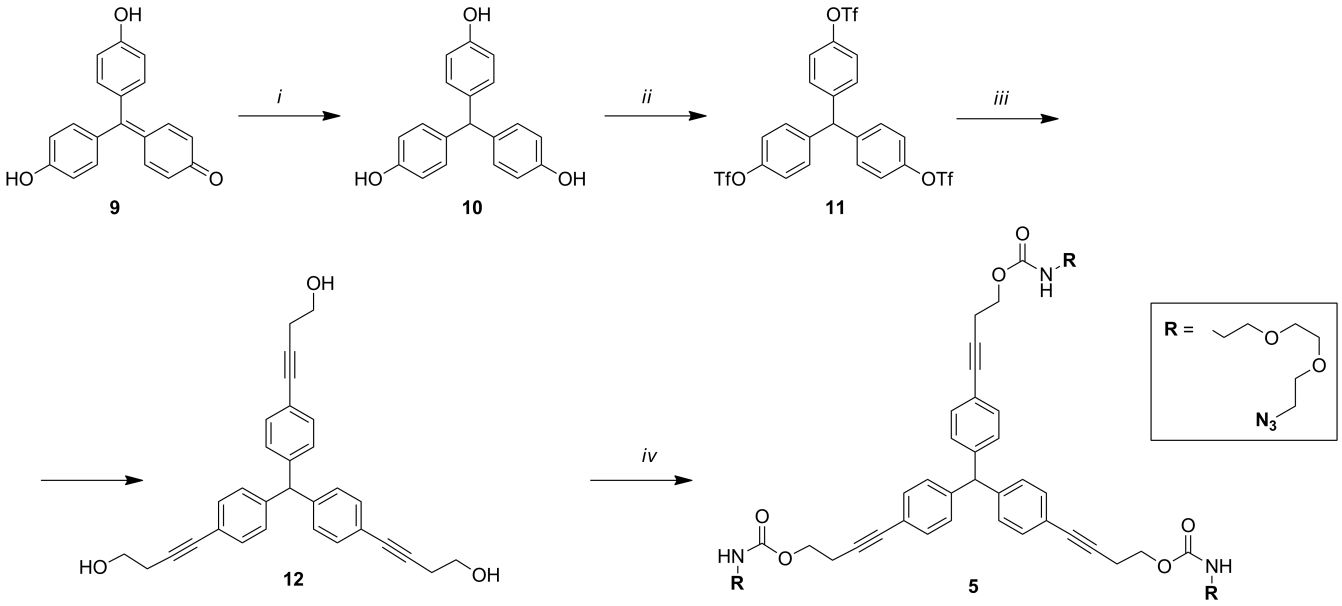
[1] Monomer **M**^{1}: I. V. Astakhova, T. S. Kumar, J. Wengel, *Collect. Czech. Chem. Commun.,* 2011, **76**, 1347; monomer **M**^{2}: T. S. Kumar, A. S. Madsen, J. Wengel, P. J. Hrdlicka, *Nucleosides Nucleotides Nucleic Acids*, 2007, **26**, 1403; monomer **M**^{3}: T. S. Kumar, J. Wengel, P. J. Hrdlicka, *ChemBioChem*, 2007, **8**, 1122.

[2] Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl] amine (TBTA): T. R. Chan, R. Hilgraf, K. B. Sharpless, V. V. Fokin ,*Org. Lett.*, 2004, **6**, 2853.

[3] M. Minato, P. M. Lahti, H. v. Willigen, *J. Am. Chem. Soc.*, 1993, **115**, 4532.

[4] A. V. Ustinov, V. V. Dubnyakova, V. A. Korshun, *Tetrahedron*, 2008, **64**, 1467.

[5] Some reviews on DNA modification with click chemistry: a) A. V. Ustinov, I. A. Stepanova, V. V. Dubnyakova, T. S. Zatsepin, E. V. Nozhevnikova, V. A. Korshun, *Russ. J. Bioorg. Chem.*, 2010, **36**, 401. b) A.H. El-Sagheer, T. Brown, *Chem. Soc. Rev.*, 2010, **39**, 1388. c) F. Amblard, J. H. Cho, R. F. Schinazi, *Chem. Rev.*, 2009, **109**, 4207.



Scheme S1. Reagents and conditions (and yields): *i*, $NaBH_4$, EtOH, AcOH (**10**, 76%); *ii*, Tf_2O, Py, 0°C→rt, 12 h (**11**, 80%); *iii*, 3-butyne-1-ol, Et_3N, $Pd(PPh_3)_4$, CuI, DMF, 12 h (**12**, 57%). *iv*, *N*,*N*-carbonyldiimidazole, CH_2Cl_2, 5 h; 2-(2-(2-azidoethoxy)ethylamine, 12 h (**5**, 70%).

Tris(4-hydroxyphenyl)methane (10). To a solution of rosolic acid **9** (5.12 g, 15 mmol) in EtOH (90 mL), $NaBH_4$ (11.32 g, 300 mmol) and acetic acid (30 mL) were added alternately in small portions until the color disappears. The mixture was evaporated; the residue was dissolved in EtOAc (200 mL) and washed with water (2×50 mL), 5% citric acid (2×50 mL), sat. NaCl (50 mL), then dried over Na_2SO_4 and evaporated. The residue was co-evaporated with toluene (100 mL) and chromatographed in 10→12% acetone in toluene on silica gel. Yield 3.35 g (76%), pinkish needles, mp 245–247°C (lit.[6] mp 240°C). R_f 0.50 (40% acetone in toluene v/v).

Tris(4-trifluoromethylsulfonyloxyphenyl)methane (11). Compound **10** (2.44 g, 8.35 mmol) was evaporated with pyridine (3×10 mL) and dissolved in dry pyridine (15 mL). The solution was cooled on NaCl/ice bath to 0°C and Tf_2O (6.7 mL, 40 mmol) was added dropwise under Ar. The reaction was allowed to warm to rt overnight and then poured into EtOAc (100 mL) and water (100 mL). The organic layer was separated, washed with water (3×100 mL), 5% $NaHCO_3$ (2×100 mL), sat. NaCl (50 mL), dried over Na_2SO_4 and evaporated. The residue was chromatographed on silica gel in 40% hexane in toluene. Yield 4.20 g (80%), colorless solidified oil. R_f 0.53 (50% hexane in toluene v/v). ^1H NMR (500 MHz, $CDCl_3$): δ 7.27 (d, 6H, *J* 8.7 Hz, H-3,5), 7.16 (d, 6H, *J* 8.5 Hz, H-2,6), 5.67

[6] S. Aitipamula, A. Nangia, R. Thaimattam, M. Jaskólski, *Acta Cryst.*, 2003, **C59**, o481.

(br. s, 1H, Ar_3CH). ^{13}C NMR (125 MHz, $CDCl_3$): δ 148.63 (3C), 142.65 (3C), 140 (m, 3C, $^1J_{CF}$ 320 Hz, CF_3), 131.15 (6C), 122.71 (6C), 54.91 (Ar_3CH).

Tris[4-(4-hydroxybutyn-1-yl)phenyl]methane (12). Triflate **11** (2.3 g, 3.34 mmol) was dissolved in DMF (10 mL) and the flask was degassed three times by evacuation followed by filling with Ar. 3-Butyn-1-ol (1.6 mL, 22 mmol), $Pd(PPh_3)_4$ (1.15 g, 1.0 mmol), CuI (380 mg, 2 mmol) and Et_3N (2.8 mL, 20 mmol) were added under Ar and the reaction was stirred for 12 h. The reaction was diluted with EtOAc (200 mL), washed with water (4×200 mL), 3% aq. $(NH_4)_2EDTA$ (2×100 mL), sat. NaCl (50 mL), dried over Na_2SO_4 and evaporated. The residue was chromatographed on silica gel in 10%→15% acetone in toluene to give **12** (849 mg, 57%) as colorless crystalline solid, mp 215–218ºC. R_f 0.53 (toluene–acetone 1:1 v/v). ^1H NMR (500 MHz, DMSO-d_6): δ 7.33 (d, 6H, *J* 8.2 Hz, H-3,5), 7.16 (d, 6H, *J* 8.2 Hz, H-2,6), 5.64 (br. s, 1H, Ar_3CH), 4.86 (t, 3H, *J* 5.6 Hz, O*H*), 3.56 (q, 6H, *J* 5.8 Hz, CH_2OH), 2.53 (t, 3H, *J* 6.7 Hz, CH_2CH_2OH). ^{13}C NMR (125 MHz, DMSO-d_6): δ 142.88 (3C), 131.39 (6C), 129.13 (6C), 121.39 (3C), 88.50 (3C), 80.76 (3C), 59.76 (3C), 54.81 (Ar_3CH), 23.25 (3C).

Tris{[4-(4-{2-[2-(2-azidoethoxy)ethoxy]ethylaminocarbonyloxy}butyn-1-yl)]phenyl}-methane (5). Triol **12** (246 mg, 0.55 mmol) was dissolved in CH_2Cl_2 (10 mL) under argon and *N*,*N*-carbonyldiimidazole (384 mg, 2.37 mmol) was added in one portion; the mixture was stirred for 4 h. Then 2-[2-(2-azidoethoxy)ethoxy]ethylamine[7] (600 mg, 3.4 mmol) was added and the mixture was kept for 12 h at rt. The mixture was evaporated and the residue was chromatographed on silica gel eluting with 40% acetone in toluene (v/v). Yield 406 mg (70%), yellowish viscous oil. R_f 0.64 (toluene–acetone 1:1 v/v). ^1H NMR (500 MHz, DMSO-d_6): δ 7.19 (t, 3H, *J* 5.4 Hz, N*H*), 7.10 (d, 6H, *J* 8.2 Hz, H-3,5), 7.05 (d, 6H, *J* 8.0 Hz, H-2,6), 5.66 (br.s, 1H, Ar_3CH), 4.09 (t, 6H, *J* 6.4 Hz), 3.63–3.46 (m, 20H), 3.44–3.34 (m, 10H), 3.13 (app. q, 6H, *J* 5.8 Hz, CH_2NH), 2.70 (t, 6H, *J* 6.4 Hz). ^{13}C NMR (125 MHz, DMSO-d_6): δ 156.00 (3C, CO), 143.06 (3C), 131.43 (6C), 129.15 (6C), 121.07 (3C), 87.18 (3C), 81.10 (3C), 72.34 (3C), 69.71 (3C), 69.77 (3C), 69.60 (3C), 69.53 (3C), 69.21 (3C), 69.07 (3C), 54.82 (Ar_3CH), 49.97 (3C), 19.77 (3C).

[7] A. W. Schwabacher, J. W. Lane, M. W. Schiesher, K. M. Leigh, C. W. Johnson, *J. Org. Chem.*, 1998, **63**, 1727.

Synthesis and purification of modified oligonucleotides. LNA phosphoramidites (A^L and $^{5\text{-Me}}C^L$) and 5′-terminal alkyne phosphoramidite **2** were obtained from commercial suppliers (Exiqon and Lumiprobe, respectively), and were incorporated into synthetic oligonucleotides following manufactures' protocols. Oligonucleotide synthesis was carried out on a PerSpective Biosystems Expedite 8909 instrument in 200 nmol scale using standard manufacturer's protocols, except for hand-coupling and increased coupling time for monomers $\mathbf{M^1}$–$\mathbf{M^3}$ (30 min using 0.6 M pyridine hydrochloride as activator), and LNA[8] (10 min using 1*H*-tetrazole in MeCN as activator). Coupling yields based on the absorbance of the dimethoxytrityl cation released after each coupling were approximately 87–92% for modified monomers $\mathbf{M^1}$–$\mathbf{M^3}$, and 99% for LNA, 5′-terminal alkyne phosphoramidite (monomer $\mathbf{M^4}$) and unmodified DNA phosphoramidites. Cleavage from solid support and removal of nucleobase protecting groups were performed using standard conditions (32% aqueous ammonia for 12 h at 55 ºC). Unmodified DNA strands were obtained from commercial suppliers and used without further purification, while all the modified oligonucleotides were purified by DMT-OFF RP-HPLC using the Waters Prep LC 4000 equipped with Xterra MS C18-column (10 μm, 300 mm × 7.8 mm). Elution was performed starting with an isocratic hold of A-buffer for 2 min followed by a linear gradient to 70 % B-buffer over 40 min at a flow rate of 1.0 mL/min (A-buffer: 0.05 M triethyl ammonium acetate, pH 7.4; B-buffer: 25% buffer A, 75% CH_3CN). RP-purification was followed by precipitation (acetone, -18 ºC, 12 h) and washing with acetone (2 × 0.5 mL). The identity and purity of the products were verified by MALDI-TOF mass spectrometry (Ultraflex II, Bruker) and IE HPLC, respectively. The IE HPLC was performed using LaChrom L-7100 pump (VWR International) equipped with Dionex DNAPac Pa-100 column (4 mm × 250 mm). Elution was performed as follows: 1) isocratic hold of 10% C- and 2% B-buffers in A-buffer for 2 min; 2) linear gradient over 22 min in isocratic hold

[8] a) A. A: Koshkin, S. K. Singh, P. Nielsen, V. K. Rajwanshi, R. Kumar, M. Meldgaard, C. E. Olsen, J. Wengel, *Tetrahedron*, 1998, **54**, 3607. b) S. Obika, D. Nanbu, Y. Hari, J. Andoh, K. Morio, T. Doi, T. Imanishi, *Tetrahedron Lett.*, 1998, **39**, 5401.

of 10% C-buffer: B-buffer - from 2 % to 60 %, A-buffer from 88% to 30%); 3) isocratic hold of 60% B-buffer, 30% A-buffer and 10% C-buffer for 8 min (A-buffer: MQ water; B-buffer: 1 M $NaClO_4$; C-buffer: 25mM Tris-Cl; pH 8.0; flow rate 1.0 mL/min). MALDI-TOF mass-spectrometry analysis was performed using a MALDI-LIFT system on the Ultraflex II TOF/TOF instrument from Bruker and using HPA-matrix (10 mg 3-hydroxypicolinic acid, 50 mM ammonium citrate in 70% aqueous acetonitrile).

Table S1. MALDI MS of modified oligonucleotides.

#	Sequence, 5′→3′	Found *m/z* $[M-H]^-$	Calc. *m/z* $[M-H]^-$
ON1	$\mathbf{M^4}$ CGT GA$\mathbf{M^1}$ ATA TAA$\mathbf{^L}$ A	4541	4544
ON2	$\mathbf{M^4}$ TTT ATA $\mathbf{M^2}$AT CA$^{\mathbf{5\text{-}Me}}\mathbf{C^L}$ G	4460	4462
ON3	$\mathbf{M^4}$ TTT ATA $\mathbf{M^3}$AT CA$^{\mathbf{5\text{-}Me}}\mathbf{C^L}$ G	4476	4476

General method for microwave-assisted CuAAC reactions. Starting oligonucleotide **ON1**–**ON3** (30 nmol) was dissolved in fresh MQ water (300 μL) in a microwave-tube. DMSO (500 μL), 2M triethylammonium acetate buffer (pH 7.4; 50 μL), corresponding azide (**3**–**5**; 50 μL of 10 mM solution in DMSO), ascorbic acid (10 μL of 50 mM freshly prepared stock solution) and Cu(II)-TBTA equimolar complex (50 μL of 10 mM stock solution) were subsequently added. The resulting mixture was tightly closed, mixed on vortex and subjected to microwave conditions (microwave reactor, 60 °C, 15 minutes). The reaction was afterwards cooled to room temperature and filtrated through Illustra NAP-10 column (GE Healthcare) following manufacture's protocol. The resulting solution was evaporated followed by precipitation of the product conjugates from cold acetone (-18 ºC, 12 h) and subsequent washing with acetone two times. The resulting conjugates **C1**−**C5** were analyzed by MALDI TOF mass spectrometry, 13% denaturing PAGE (7M urea)[4] and IE HPLC (Table S2, Figures S1-S2). Yields: 72% (**C1**), 79% (**C2**), 63% (**C3**), 65% (**C4**), 68% (**C5**).

Table S2. MALDI-MS of branched conjugates **C1**−**C5** prepared by click chemistry.

Starting oligonucleotide, 5′→3′	Azide#	Product Conjugate	Found *m/z* $[M-H]^-$	Calc. *m/z* $[M-H]^-$
M^{4} CGT GA**M**^{1} ATA TAA^{L} A (**ON1**)	**3**	**C1**	9399	9394
M^{4} CGT GA**M**^{1} ATA TAA^{L} A (**ON1**)	**4**	**C2**	9715	9705
M^{4} CGT GA**M**^{1} ATA TAA^{L} A (**ON1**)	**5**	**C3***	4890	14681 $[M-H]^-$/3: 4894
M^{4} TTT ATA **M**^{2}AT CA$^{5\text{-Me}}$C^{L} G (**ON2**)		**C4***	4802	14435 $[M-H]^-$/3: 4812
M^{4} TTT ATA **M**^{3}AT CA$^{5\text{-Me}}$C^{L} G (**ON3**)		**C5***	4817	14477 $[M-H]^-$/3: 4826

* For 3-way branched conjugates **C3**–**C5** a high-power laser beam was required in order to obtain a peak in MALDI-TOF MS spectra which resulted in observation of the corresponding to $[M-H]^-$/3 values.[9]

[9] For example, K. Chughatai, R. M. A. Heeren, *Chem. Rev*., 2010, **110**, 3237.

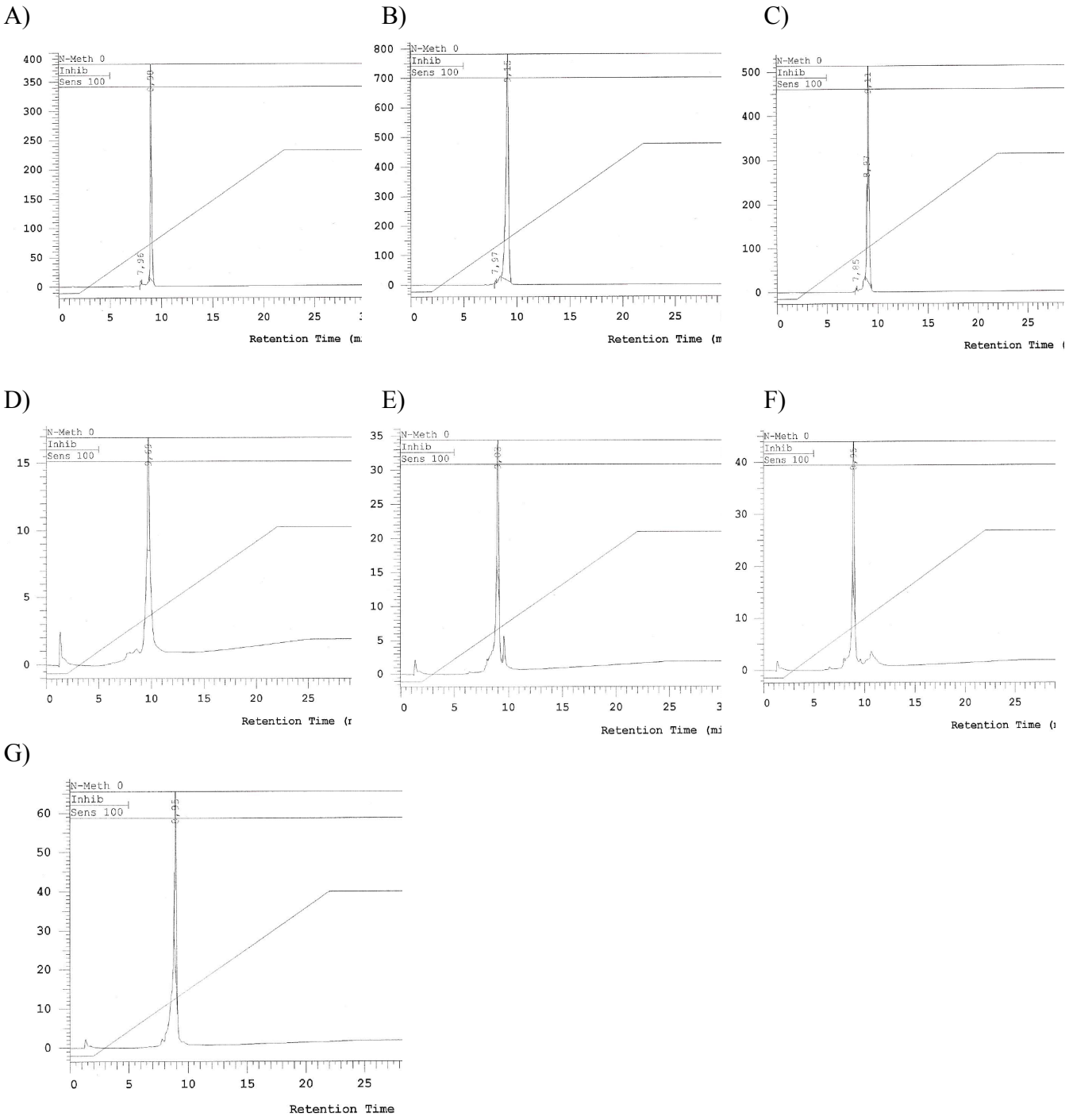
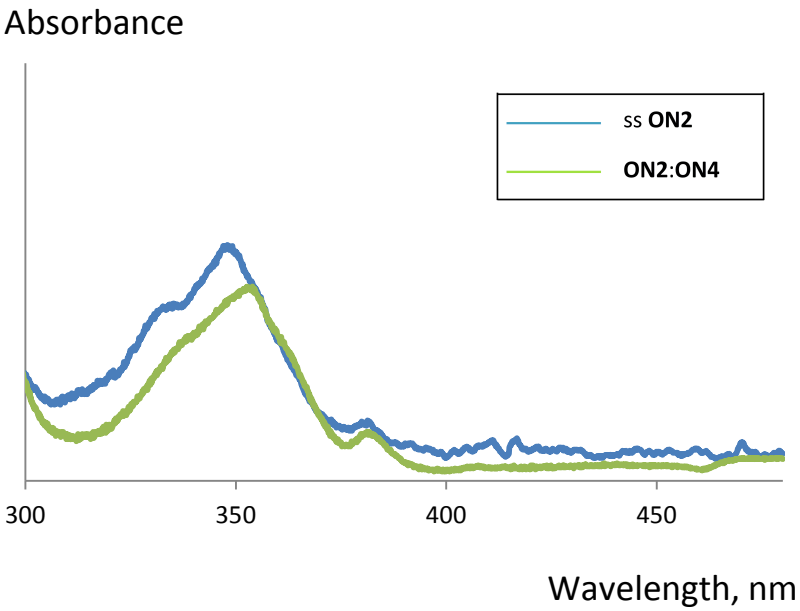


Figure S1. Representative IE HPLC traces of oligonucleotides and double- and tri- branched conjugates: **ON1** (A), **ON2** (B), **ON3** (C); **C2** (D), **C3** (E), **C4** (F), **C5** (G).

Preparation of nanostructures; UV-visible absorbance and thermal denaturation studies. Annealing of nanostructures was performed in the following media: medium salt phosphate buffer (100 mM sodium chloride, 10 mM sodium phosphate, 0.1 mM EDTA, pH 7.0), high salt phosphate buffer (700 mM sodium chloride, 10 mM sodium phosphate, 0.1 mM EDTA, pH 7.0), low salt phosphate buffer (10 mM sodium chloride, 10 mM sodium phosphate, 0.1 mM EDTA, pH 7.0), aqueous magnesium chloride solution of 500 mM, 50 mM, 5 mM concentrations. Additionally, 50 mM aqueous magnesium chloride solution containing 110 mM sodium chloride was used. Concentrations of oligonucleotides and branched conjugates were calculated using the nearest-neighbour method and the following extinction coefficients (OD_{260}/μmol): G, 10.5; A, 13.9; T/U, 7.9; C, 6.6; **M**^{1}, 33.2; **M**^{2} and **M**^{3}; 20.2; **6**, 16.0; **7**, 45.0; **8**, 25.5. Complementary conjugates were annealed at the desired concentration and in equimolar ratio (reference duplexes and complexes by **C3**−**C5**), or in a molar ratio 3:1 for complexes of bis-conjugates **C1**−**C2** with trivalent conjugates **C4**−**C5**, respectively. The complementary strands were thoroughly mixed in a buffer of choice, denatured by heating to 85 °C for 10 min and subsequently cooled over 2 h or overnight to the starting temperature of spectroscopic experiment or prior to loading on a gel. Alternatively, the complexes annealed as described above were kept at + 4 °C for additional 6 h before spectroscopic experiment or loading on gel.

UV-visible absorption spectra and thermal denaturation experiments were performed on a Perkin Elmer Lambda 35 UV/VIS Spectrometer equipped with PTP 6 (Peltier Temperature Programmer). Thermal denaturation temperatures (T_m values, ºC) were determined as the maxima of the first derivative of the thermal denaturation curve (A_{260} vs. temperature). Reported T_m values are an average of two measurements within ± 0.5 ºC.



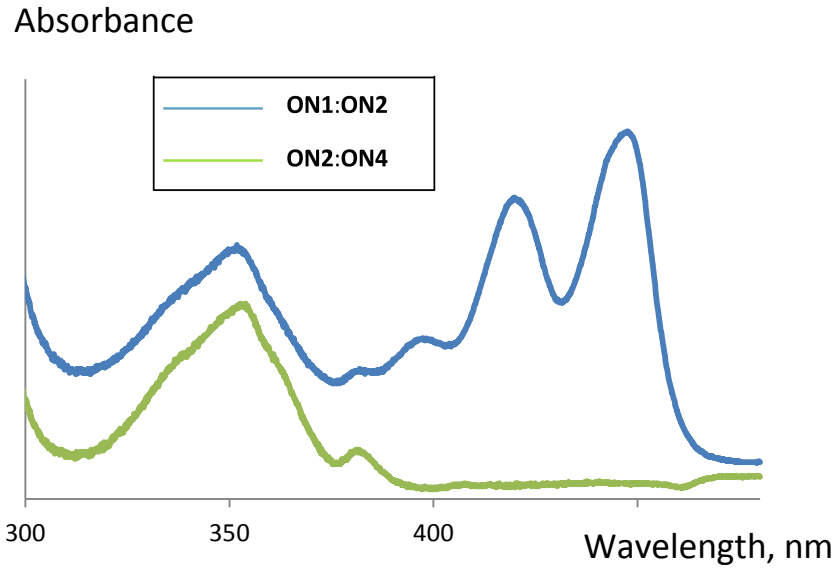
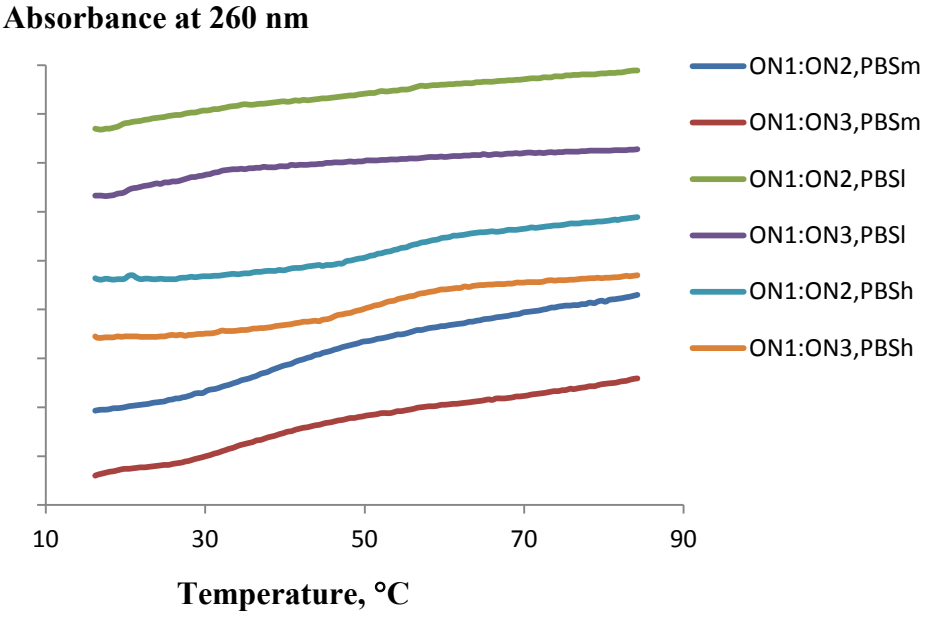


Figure S2. Representative visible absorbance spectra of modified oligonucleotides and duplexes (not normalized). Spectra were obtained in medium salt buffer at 19 ºC using 0.25 μM concentrations of single-stranded oligonucleotides and complementary strands.



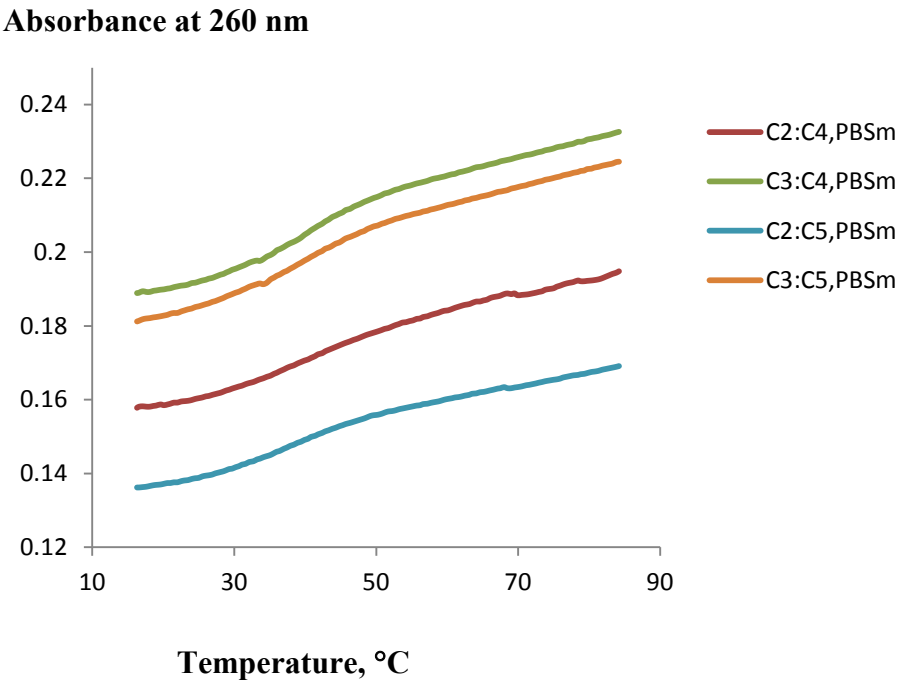


Figure S3. Thermal denaturation curves of duplexes containing monomers $\mathbf{M^1}$–$\mathbf{M^3}$ recorded in medium, low and high salt phosphate buffers using 0.25μM concentration of oligonucleotides (PBSm, PBSl and PBSh, respectively).

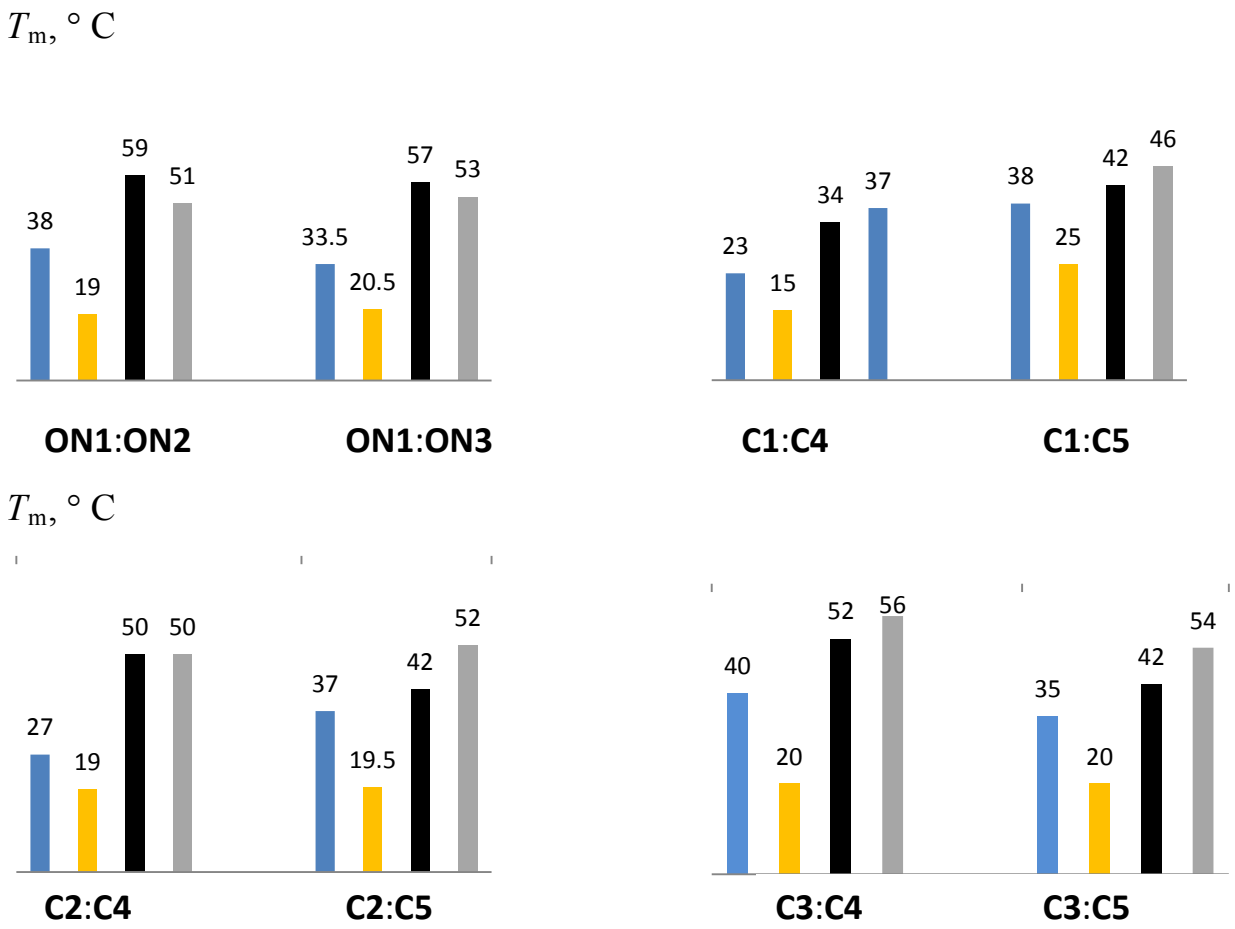


Figure S4. Thermal denaturation values of reference duplexes and nanostructures containing monomers $\mathbf{M^1}$–$\mathbf{M^3}$ recorded in medium, low and high salt phosphate buffers (blue, yellow and black bars, respectively), pH 7.0, and in 50 mM aqueous solution of magnesium chloride, pH 7.0 (grey bars).

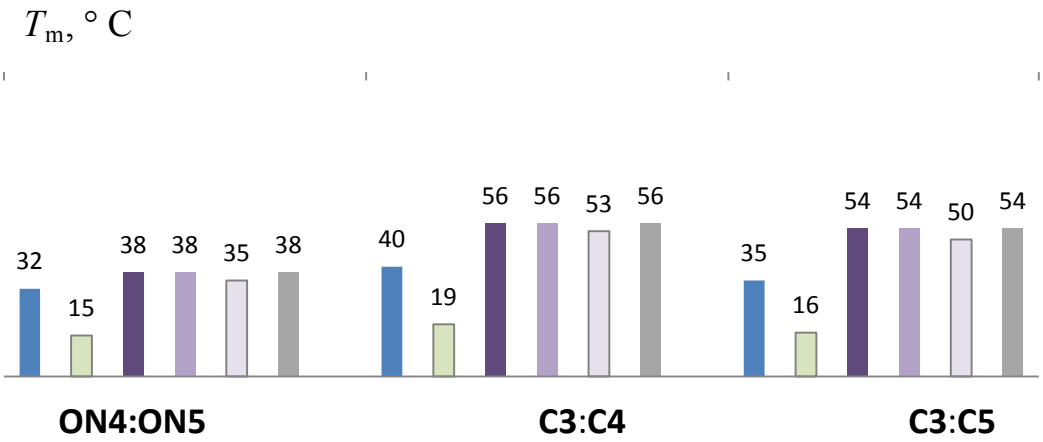
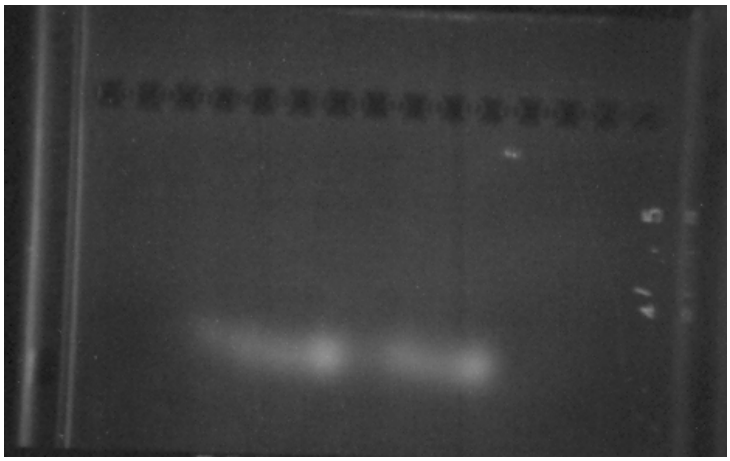


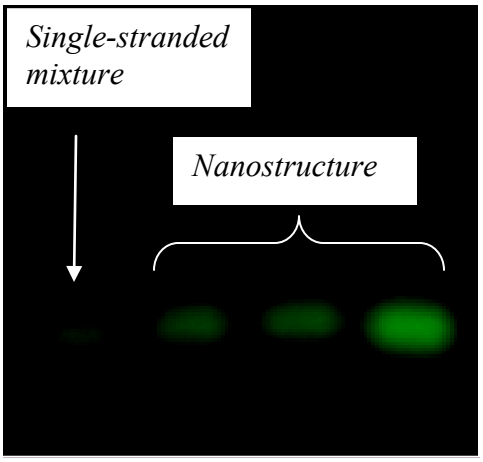
Figure S5. Representative thermal denaturation values of reference unmodified duplex **ON4**:**ON5** and nanostructures containing monomers $\mathbf{M^1}$–$\mathbf{M^3}$ annealed and recorded in various media (bars from left to right): medium salt phosphate buffer, water; 500 mM, 50 mM and 5 mM magnesium chloride, 50 mM magnesium chloride solution containing 110 mM sodium chloride, pH 7.0. **ON4** = 5′-d(CGT GAT ATA TAA A), **ON5** = 5′-TTT ATA TAT CAC G.

A)



1 2 3 4 5 6 7

B)



1 2 3 4

Figure S6. Representative agarose (A, 1% agarose, 50 mM $MgCl_2$, 1x TBE, slow run) and native PAGE (B, 5%, run at + 4°C) analysis of branched fluorescent nanostructures.

Gel A, visualization by staining with EtBr. Lanes from left to right as follows: marker double-stranded DNA 39 bp (*1,2*), **C1**:**C4** (*3*), **C2**:**C4** (*4*), reference **C5**:**ON4** (*5*), **C1**:**C5** (*6*), **C2**:**C5** (*7*). **ON4** = 5′-d(CGT GAT ATA TAA A)

Gel B, excitation and emission wavelengths of 365 nm and 500 nm, respectively, were applied for bands' visualization. Lanes from left to right as follows: single-stranded mixture **C2**+**C5** (*1*; 0.5 nmol, quenched signal) and upon annealing: 0.1 nmol, 0.25 nmol, and 0.5 nmol (lanes *2*→*4*, respectively)

Fluorescence steady-state emission and excitation studies. Determination of quantum yield, FRET efficiency and limit of detection values. Fluorescence spectra were obtained using a PerkinElmer LS 55 luminescence spectrometer equipped with a Peltier temperature controller using an excitation wavelength of 330 nm, excitation slit of 4.0 nm, emission slit of 2.5 nm, scan speed of 120 nm/min and 0.25 μM (0.75 μM for **C1**−**C2**) concentrations of the single-stranded probe or the corresponding complementary complex in the buffer of choice described above. Excitation spectra were obtained recording emission of acceptor FRET at 500 nm. The fluorescence quantum yields (Φ_f) were measured by the relative method[10] using standards of highly diluted solutions of 1-pyrenebutyric acid (Φ_f = 0.07) in methanol,[11] perylene (Φ_f = 0.93)[12] and 9,10-diphenylantracene[13] (Φ_f = 0.95) in cyclohexane. The samples used in quantum yield measurements were not degassed; concentrations were 0.25 μM. FRET efficiency values *E* for pyrene-perylene FRET pairs were calculated using equiation:

$E = \varepsilon_A/\varepsilon_D \times (I_{AD}/I_A - 1)$,[14]

Where ε_A and ε_D are the molar extinction coefficients of the acceptor and donor at the excitation wavelength, and I_{AD} and I_A are the fluorescence intensities at the acceptor emission wavelength in the presence and absence of the donor, respectively.

Limit of detection (LOD) values were determined by series of dilution experiments following previously described protocol.[15] Relevant fluorescent oligonucleotides at concentrations 500, 250, 100,

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[10] J. R. Lakowicz, *Principles of Fluorescence Spectroscopy*, 3th. Ed., Springer, Singapore, 2006.

[11] P. J. Hrdlicka, B. R. Babu, M. D. Sørensen, N. Harrit, J. Wengel, *J. Am. Chem. Soc*., 2005, **127**, 13293.

[12] N. Nijegorodov, R. Mabbs, W. S. Downey, *Spectrochim. Acta, Part* A, 2001, **57**, 2673.

[13] J. V. Morris, M. A. Mahaney, J. R. Huber, *J. Phys. Chem.*, 1976, **80**, 969.

[14] D. Lindegaard, A. S. Madsen, I. V. Astakhova, A. D. Malakhov, B. R. Babu, V. A. Korshun, J. Wengel, *Bioorg. Med. Chem.*, 2008, **16**, 94.

[15] Department of Health and Human Services, Food and Drug Administration, Center for Biologics Evaluation and Research (CBER), *Guidance for industry in the manufacture and clinical evaluation of in vitro tests to detect nucleic acid sequences of human immunodeficiency viruses type 1 and 2* (Office of Communication, Training and Manufactures Assistance (HFM-40), Rockville, MD), 1999, pp. 4.

50, 20, 10 and 1 nM were mixed in a medium salt phosphate buffer in molar ratios described above. Upon annealing, a fluorescence signal was measured at λ^{fl} = 450 nm. LOD value of each complex was then defined as a lowest complex concentration such that the fluorescence signal to noice ratio (S/N) relative to the blank solution of a medium salt phosphate buffer was minimum three. Resulting LOD values (complex): 20 nM (**ON1**:**ON2**), < 10 nM (**C1**:**C4**), < 10 nM (**C2**:**C4**), < 10 nM (**C3**:**C4**), 10 nM (**ON1**:**ON3**), 10 nM (**C1**:**C5**), 10 nM (**C2**:**C5**), < 10 nM (**C3**:**C5**).

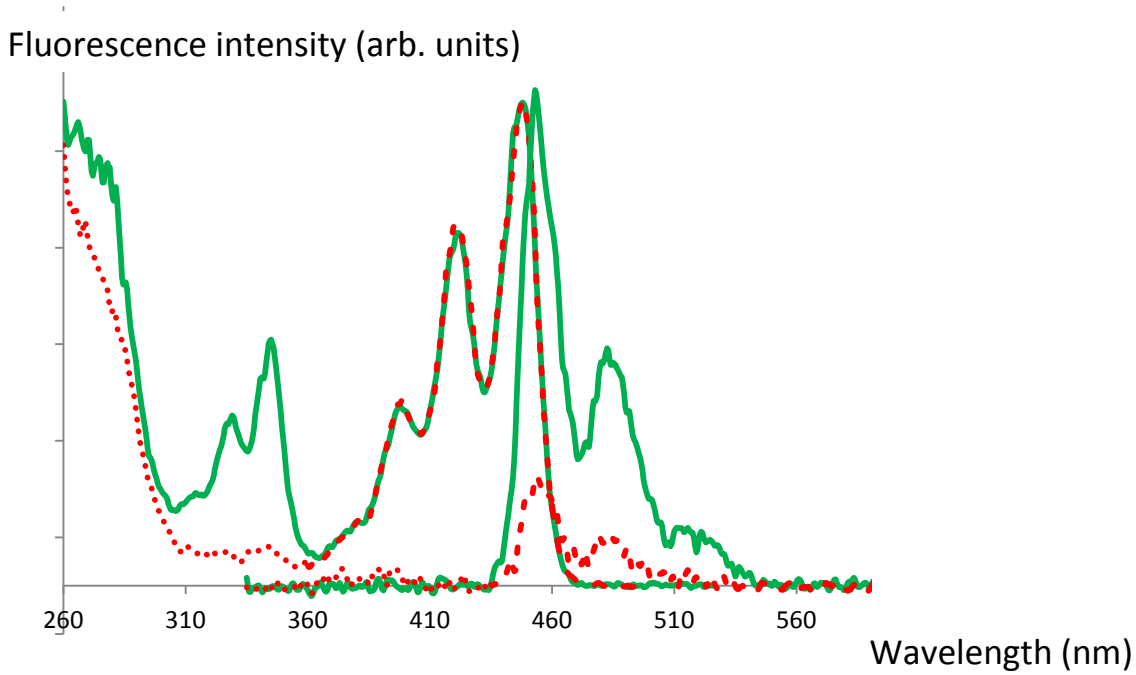

Figure S7. Representative excitation and steady-state fluorescence emission spectra of 0.25 μM single-stranded mixture **C2**+**C5** (dotted red lines) and upon assembly (green lines).

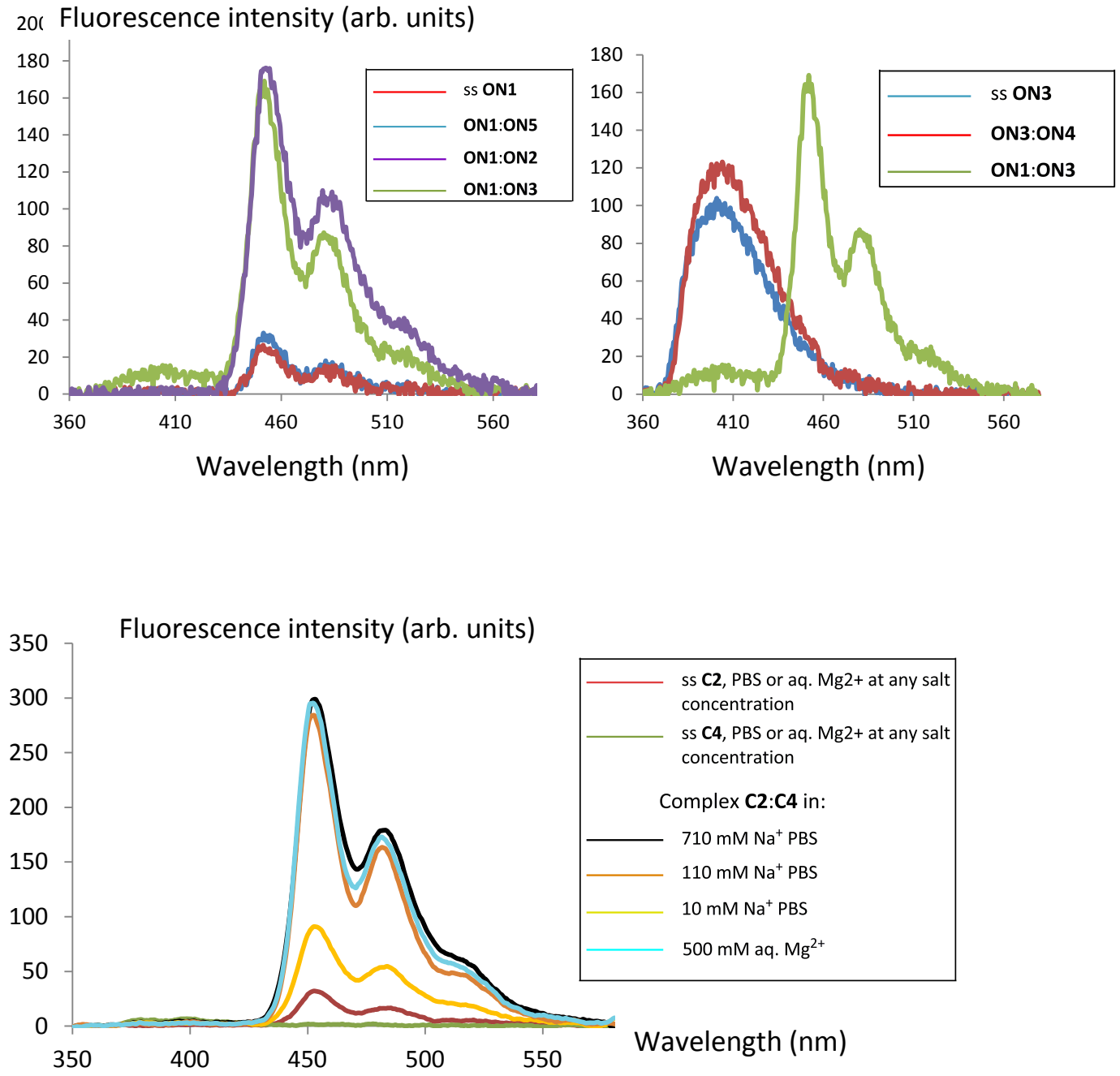

Figure S8. Representative steady-state fluorescence emission spectra of nanostructures obtained in various annealing buffers at 19 ºC using excitation wavelength of 330 nm. **ON4** = 5′-d(CGT GAT ATA TAA A), **ON5** = 5′-TTT ATA TAT CAC G.

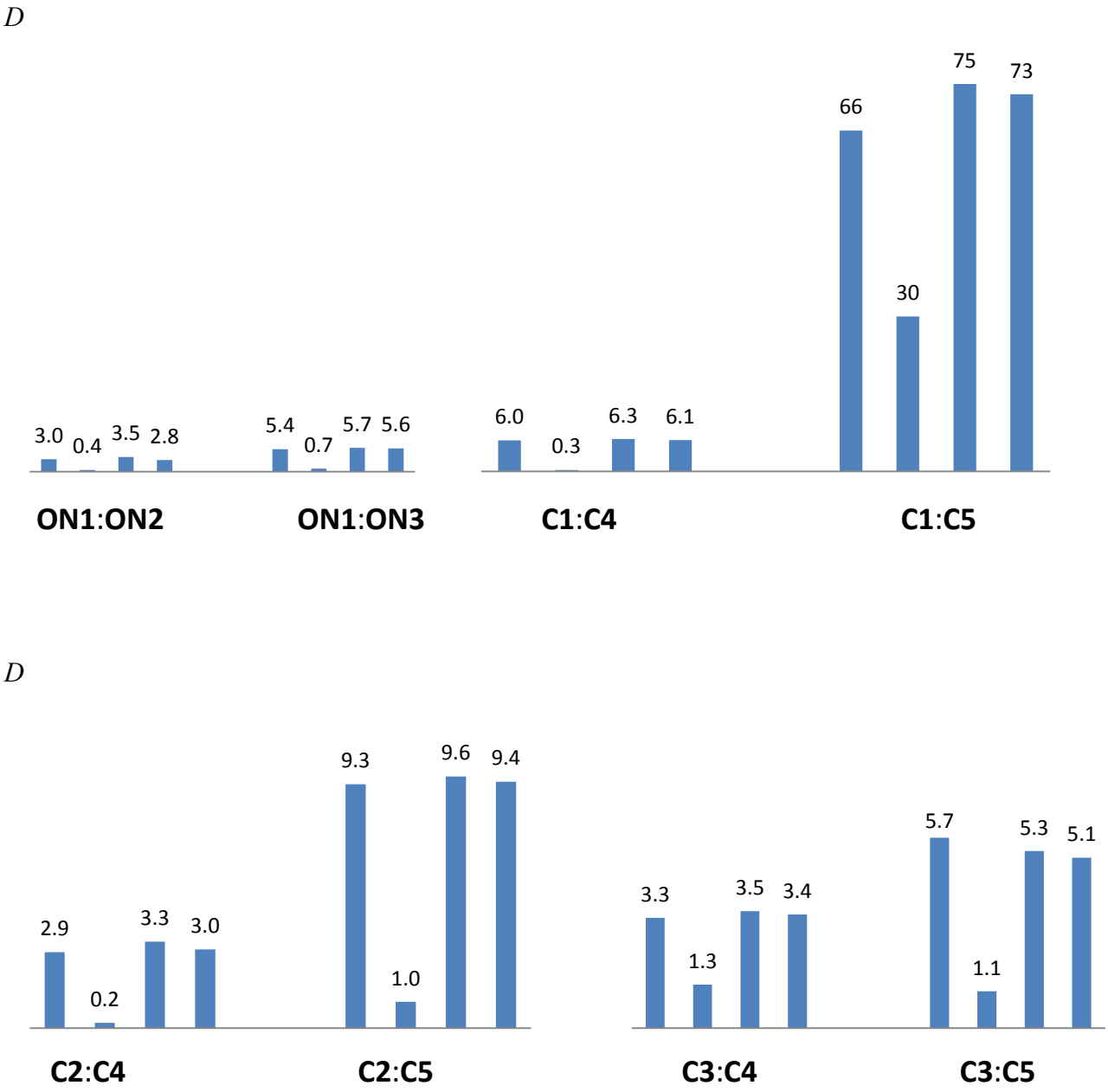

Figure S9. Discrimination factor values *D* determined as ratio of fluorescence intensity I_{ds}/I_{ss} at λ^{fl}_{max} = 450 nm for double-stranded complexes to corresponding single strand mixtures. Fluorescence measurements were performed in medium salt phosphate buffer using excitation wavelength of 330 nm and 0.25 μM concentration of complementary strands.

Table S3. Properties of the reference complexes **ON1**:**C4** and **ON1**:**C5** in a medium salt buffer.

Complementary complex	T_m, °C	λ^{abs}_{max}, (nm)	λ^{fl}_{max} (nm)	*D*	Φ_f	FB	*E*
ON1:**C4**	24	443,415	450	5.2	0.42	39.0	1.00
ON1:**C5**	40	447,419	450	66.0	0.39	37.2	1.00

Molecular modeling. Duplex **ON1**:**ON3** was built in standard B-type helical geometry and further analyzed by MacroModel V9.1.[16] When doing this the structure of **ON1**:**ON3** was minimized using the Polak–Ribiere conjugate gradient method, the all-atom AMBER force field[17], and GB/SA solvation model[18] as implemented in MacroModel V9.1. Non-bonded interactions were treated with extended cut-offs (van der Waals 8.0 Å and electrostatics 20.0 Å). The minimized structures were then (using the same constraints as described above) submitted to 3 ns of stochastic dynamics (simulation temperature 300 K, time step 2.2 fs, SHAKE all bonds to hydrogen), during which 300 structures were sampled and subsequently minimized.

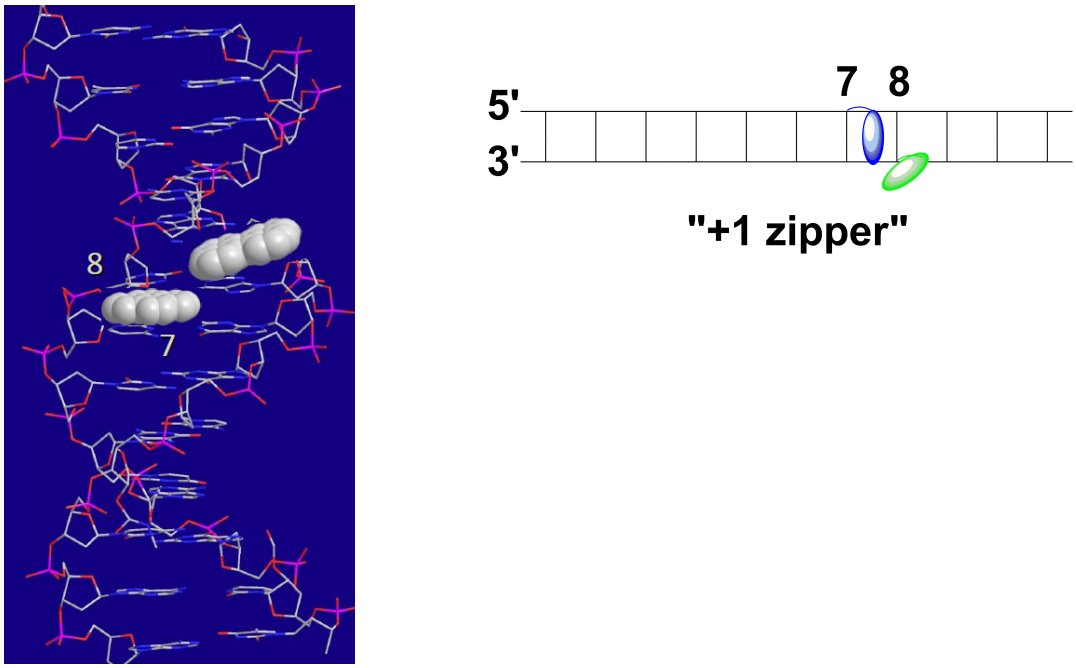

Figure S10. Representative molecular model of the duplex **ON1**:**ON3** containing $\mathbf{M^3/M^1}$ FRET pair (*left*) and illustration of the duplex structure as suggested by molecular modeling (*right*).

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[16] *MacroModel*, version 9.1, Schrödinger, 2005, LLC, New York, NY.

[17] S. J. Weiner, P. A. Kollman, D. A. Case, U. C. Singh, C. Ghio, G. Alagona, S. Profeta, P. J. Weiner, *J. Am. Chem. Soc.*, 1984, **106**, 765.

[18] S. J. Weiner, P. A. Kollman, D. T. Nguen, D. A. Case, *J. Comput. Chem.*, 1986, **7**, 230.

Calculation of Förster radius and spectral overlap integral values

Förster distances R_0 were determined using Förster Energy Transfer module of PhotochemCAD ver. 2.0 software[19] (Fig. S11) and using the following equations:[20]

$$R_0 = (8.79 \times 10^{-5}\, J\, \phi_D\, n^{-4}\, \kappa^2)^{-1/6} \text{ (in Å)} \qquad \textbf{(1)}$$

$$J = \int \varepsilon_A(\lambda)\, f_D(\lambda)\, \lambda^4\, d\lambda \,/\, \int f_D(\lambda)\, \lambda^4 \text{ (in cm}^6\text{ mmol}^{-1}\text{)} \qquad \textbf{(2)}$$

Where J is the normalized spectral overlap of the donor emission spectrum and the acceptor absorption spectrum, ϕ_D is the quantum yield for donor emission in absence of the acceptor, n is the refractive index of the solution, κ^2 is an orientation factor depending on the relative orientation of the emission dipole of the donor and the excitation dipole of the acceptor, $\varepsilon_A(\lambda)$ is the extinction coefficient of the acceptor (M^{-1} cm^{-1}) and $f_D(\lambda)$ is the corrected emission spectrum of the donor per unit wavelength interval. Steady-state fluorescence emission and absorbance spectra of the corresponding complementary oligonucleotides containing donor and acceptor FRET were uploaded into the PhotochemCAD database (Fig. S11), as well as FRET efficiency and distance D–A values estimated by fluorescence measurements and molecular modeling described below, respectively. Resulting values using κ^2 value of 2/3 (lit.[20]): R_0 = 21.5 Å, $J = 3.2 \times 10^{-14}$ cm^6 $mmol^{-1}$, pair $\mathbf{M^2/M^1}$; R_0 = 20.5 Å, $J = 3.2 \times 10^{-14}$ cm^6 $mmol^{-1}$, pair $\mathbf{M^3/M^1}$. Reference values for pyrene–perylene FRET pair in cyclohexane: R_0 = 31.3 Å, $J = 2.3 \times 10^{-14}$ cm^6 $mmol^{-1}$ (2.4×10^{-14} cm^6 $mmol^{-1}$, lit.[20]).

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[19] J. M. Dixon, M. Taniguchi, J. S. Lindsey, *Photochem. Photobiol.,* 2005, **81**, 212.

[20] M. Masuko, S. Ochuchi, K. Sode, H. Ohtani, A. Shimadzu, *Nucleic Acids Res*., 2000, **28**, e34.

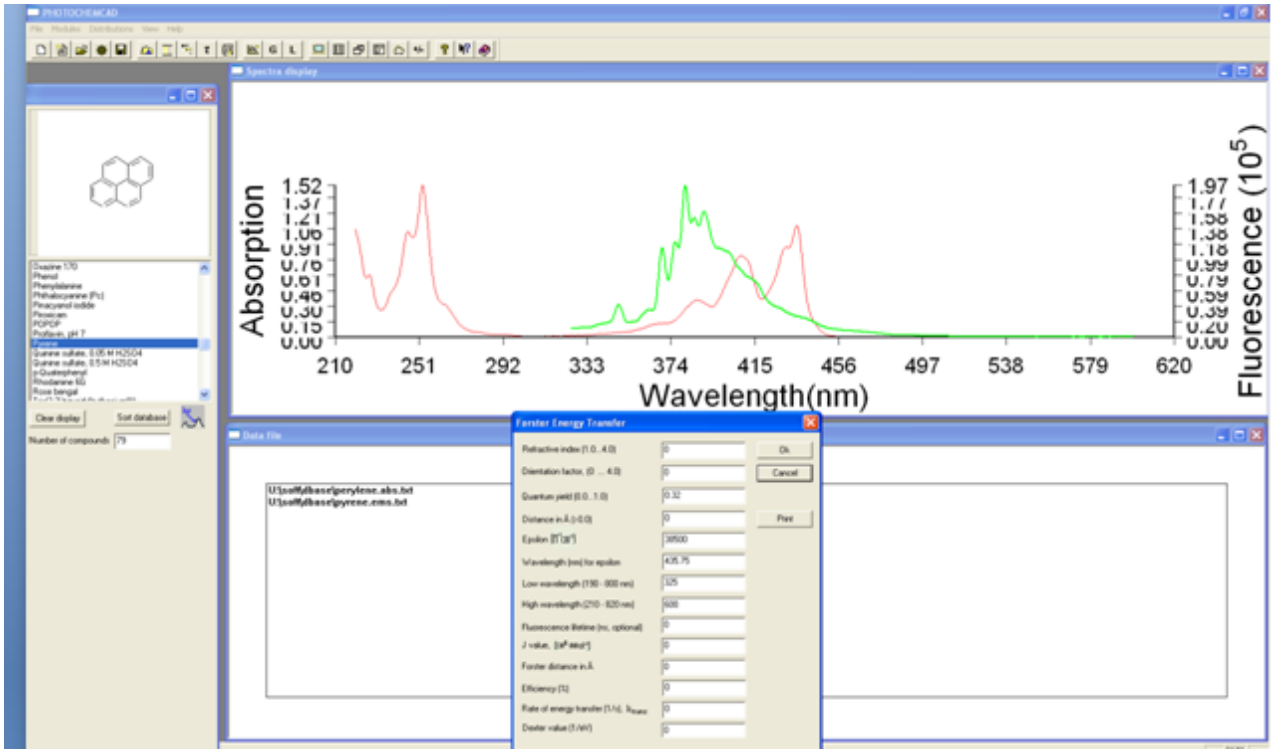

Figure S11. Analysis of pyrene–perylene FRET pair using PhotochemCAD ver. 2.0., Förster Energy Transfer module.