LNA for optimization of fluorescent oligonucleotide probes: improved spectral properties and target binding

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SUPPORTING INFORMATION

EXPERIMENTAL PROCEDURES

General. 3′-*O*-(*N*,*N*-Diisopropylamino-2-cyanoethoxyphosphinyl)-5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-[3(4)-(pyren-1-ylethynyl]phenylmethylaminocarbonyl)uracil-1-*β*-D-arabinofuranosides **1** (*1*) and **2** (*1*), 5-(pyrene-1-ylethynyl)-2-deoxyuridine (*2*) were prepared as described; LNA phosphoramidites were obtained from commercial supplier (Exiqon).

Synthesis and purification of modified oligonucleotides. Oligonucleotide (ON) synthesis was carried out on a Perseptive Biosystems Expedite 8909 instrument in 200 nmol scale using manufacturer's standard protocols, except for increased coupling time for modified phosphoramidies **1** and **2** (*1*) and LNA phosphoramidites (*3–7*) (10 min using 0.6 M pyridine hydrochloride or 1*H*-tetrazole in MeCN as activator, respectively). Coupling yields based on the absorbance of the dimethoxytrityl cation released after each coupling were approximately 99% for modified phosphoramidites **1** and **2**, LNA phosphoramidites, 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/RNA strands were obtained from commercial suppliers and used without further purification, while all the modified ONs were purified by DMT-ON RP-HPLC using 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 5 min followed by a linear gradient to 55 % B-buffer over 75 min at a flow rate of 1.0 mL/min (A-buffer: 95% 0.1 M NH_4HCO_3, 5% CH_3CN; B-buffer: 25% 0.1 M NH_4HCO_3, 75% CH_3CN). RP-purification was followed by detritylation (80% aq. AcOH, 20 min), precipitation (abs. EtOH, -18 ºC, 12 h) and washing with abs. EtOH three times. The identity and purity of ONs were verified by MALDI-TOF mass spectrometry (recorded on an Applied Biosystems Voyager-DE STR spectrometer) and IC-HPLC, respectively. Measured masses $[M-H]^-$ of the ONs (calculated masses *m/z* for $[M-H]^-$): **ON2**: 4938 (4941); **ON3**: 4913 (4913); **ON4**: 4910 (4913); **ON5**: 4912 (4913); **ON6**: 5006 (5005); **ON7**: 5006 (5008); **ON8**: 5006 (5006); **ON9**: 5006 (5006); **ON10**: 5006 (5006); **ON11**: 5006 (5006); **ON13**: 4776 (4774).

UV-visible absorption and thermal denaturation studies. 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) element in a medium salt buffer (100 mM sodium chloride, 10 mM sodium phosphate, 0.1 mM EDTA, pH 7.0). Concentrations of ONs were calculated using the following extinction coefficients (OD_{260}/μmol): G, 10.5; A, 13.9; T/U, 7.9; C, 6.6; **X**, 17.3; **Y**, 20.0. ONs (1.0 μM each strand) were thoroughly mixed, denaturated by heating and subsequently cooled to the starting temperature of experiment. Thermal denaturation temperatures (T_m values, ºC) were determined as the maxima of the first derivative of the thermal denaturation curves (A_{260} vs. temperature). Reported T_m values are an average of two measurements within ± 1.0 ºC.

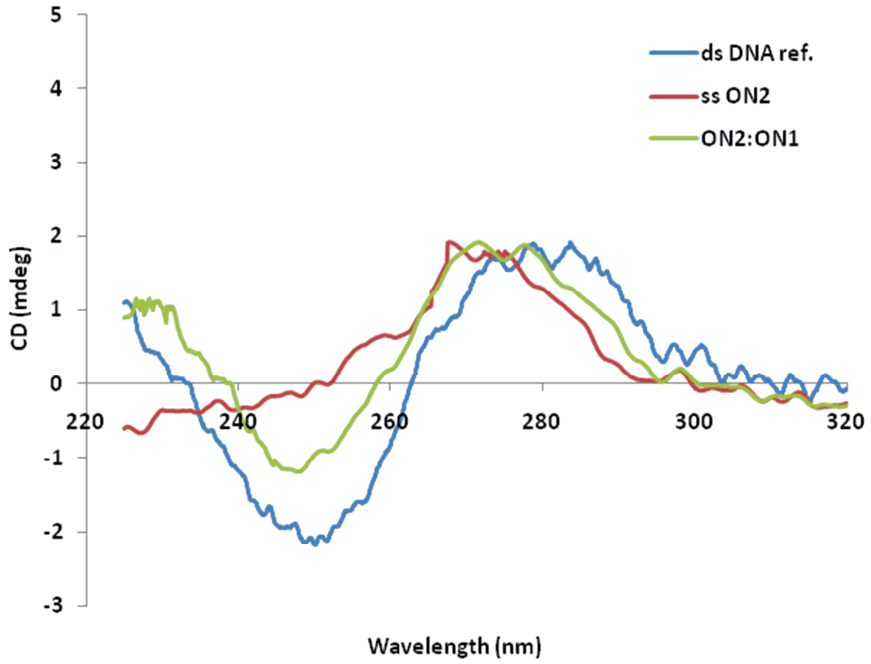
Fluorescence steady-state emission studies, and quantum yield and fluorescence lifetime determinations. Fluorescence spectra were obtained in a medium salt buffer (see above) using a PerkinElmer LS 55 luminescence spectrometer equipped with a Peltier temperature controller using an excitation wavelength of 360 nm, excitation slit of 4.0 nm, emission slit of 2.5 nm, scan speed of 120 nm/min and 0.2 μM concentrations of the single-stranded probe or the corresponding duplex. The fluorescence quantum yields (Φ_f) were measured by the relative method (*8*) using 5-(pyrene-1-ylethynyl)-2-deoxyuridine (Φ_f 0.45) (*2*) in abs. EtOH as a standard. The optical density of the solutions in a 1-cm quartz cuvette at the excitation wavelength did not exceed 0.1. The values of Φ_f were corrected with the refractive index of the solvent; the measured refractive indexes of abs. ethanol and phosphate buffer were 1.361 and 1.334 at 20 ºC, respectively. Fluorescence lifetimes were determined by the method of time-correlated single-photon counting using a PRA model 3000 nanosecond lifetime fluorometer. The parameters of the fluorescence decays were analyzed using T900 software (Edinburgh Instruments). The samples used in quantum yield and lifetime fluorescence measurements were not degassed; concentrations were 3.0×10^{-6} M.

CD measurements. CD spectra were recorded on a JASCO J-815 CD Spectrometer equipped with CDF 4265/15 temperature controller. Samples for CD measurements were prepared as described for the

thermal denaturation studies section except that a concentration of 1.5 μM of the two complementary ONs was used. Quartz optical cells with a path-length of 0.5 cm were used.

Molecular modelling. ON4 was built in standard B-type helical geometry and further analyzed by MacroModel V9.1 (*9*). When doing this the structure of **ON4** was minimized using the Polak–Ribiere conjugate gradient method, the all-atom AMBER force field (*10–11*), and the GB/SA solvation model (*12*) 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.

A)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ON2:ON1
CD (mdeg)
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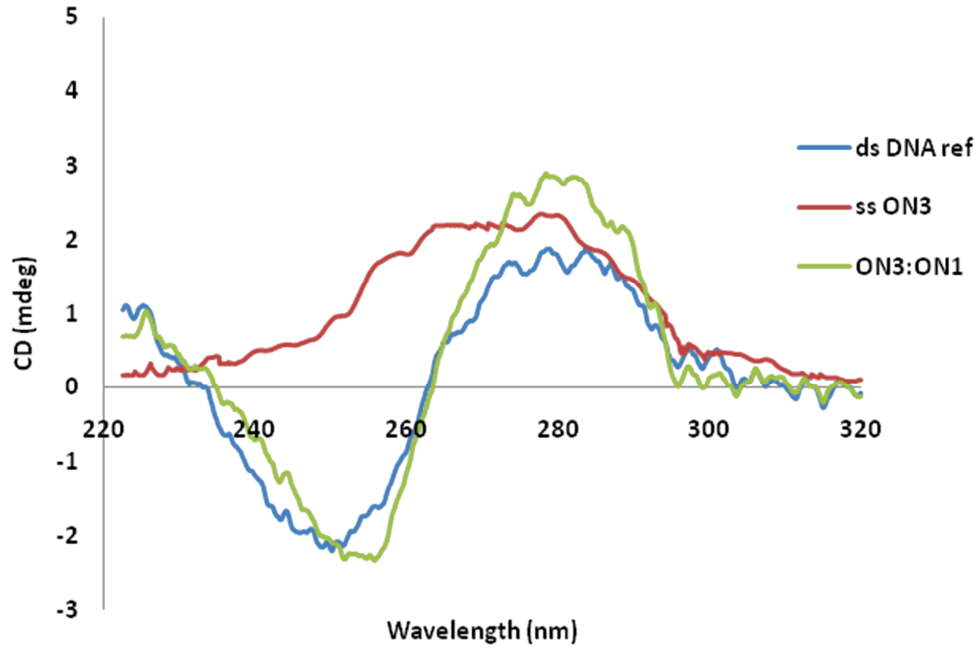
320

B)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C)

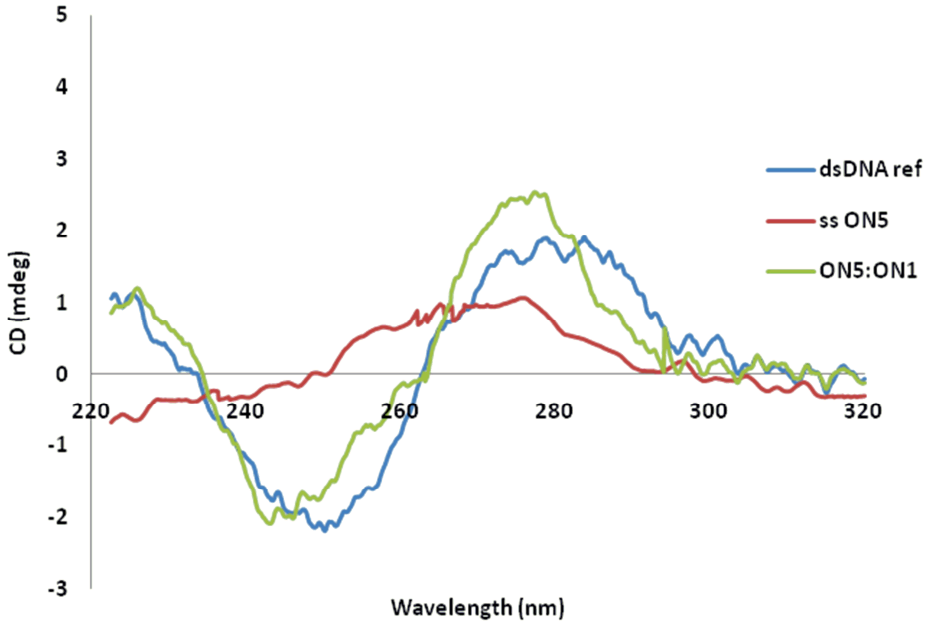

Figure S1. CD spectra of modified ONs and of duplexes. The spectra were recorded in medium salt buffer at 19 ºC using 1.5 µM concentration of strands. dsDNA ref = unmodified DNA duplex reference **ON12**:**ON1**.

References

(1) Astakhova, I. V., Malakhov, A. D., Stepanova, I. A., Ustinov, A. V., Bondarev, S. L., Paramonov, A. S., and Korshun, V. A. (2007) 1-Phenylethynylpyrene (1-PEPy) as refined excimer forming alternative to pyrene: case of DNA major groove excimer. *Bioconjugate Chem. 18*, 1972–1980.

(2) Korshun, V. A., Manasova, E. V., Balakin, K. V., Prokhorenko, I. A., Buchatskii, A. G., and Berlin, Yu. A. (1996) 5-(1-Pyrenylethynyl)-2'-Deoxyuridine, a Novel Fluorescent Nucleoside Analogue. *Russ. J. Bioorg. Chim. 22*, 807–809.

(3) Koshkin, A. A., Singh, S. K., Nielsen, P., Rajwanshi, V. K., Kumar, R., Meldgaard, M., Olsen, C. E., and Wengel, J. (1998) LNA (Locked Nucleic Acids): Synthesis of the Adenine, Cytosine, Guanine,

5-Methylcytosine, Thymine, and Uracil Bicyclonucleoside Monomers, Oligomerisation, and Unprecedented Nucleic Acid Recognition. *Tetrahedron 54*, 3607–3630.

(4) Obika, S., Nanbu, D., Hari, Y., Andoh, J., Morio, K., Doi, T., and Imanishi, T. (1998) Stability and structural features of the duplexes containing nucleoside analogues with a fixed *N*-type conformation, 2′-*O*,4′-*C*-methyleneribonucleosides. *Tetrahedron Lett. 39*, 5401–5404.

(5) Koshkin, A. A., Nielsen, P., Meldgaard, M., Rajwanshi, V. K., Singh, S. K., and Wengel, J. (1998) LNA (Locked Nucleic Acid): An RNA Mimic Forming Exceedingly Stable LNA:LNA Duplexes. *J. Am. Chem. Soc. 120*, 13252–13253.

(6) Bondensgaard, K., Petersen, M., Singh, S. K., Rajwanshi, V. K., Wengel, J., and Jacobsen, J. P. (2000) Structural Studies of LNA:RNA Duplexes by NMR: Conformations and Implications for RNase H Activity. *Chem. Eur. J. 6*, 2687–2695.

(7) Astakhova, I. V., Korshun, V. A., and Wengel, J. (2008) Highly Fluorescent Conjugated Pyrenes in Nucleic Acid Probes: (Phenylethynyl)pyrenecarbonyl-Functionalized Locked Nucleic Acids. *Chem. Eur. J. 14*, 11010–11026.

(8) Lakowicz, J. R. (2006) Principles of Fluorescence Spectroscopy, 3th Ed., Springer, Singapore.

(9) *MacroModel*, version 9.1 (2005). Schrödinger, LLC, New York, NY.

(10) Weiner, S. J., Kollman, P. A., Case, D. A., Singh, U. C., Ghio, C., Alagona, G., Profeta, S., and Weiner, P. J. (1984) A new force field for molecular mechanical simulation of nucleic acids and proteins. *J. Am. Chem. Soc. 106*, 765–784.

(11) Weiner, S. J., Kollman, P. A., Nguyen, D. T., and Case, D. A. (1986) An All Atom Force Field for Simulations of Proteins and Nucleic Acids. *J. Comput. Chem.* 7, 230–252.

(12) Still, W. C., Tempczyk, A., Hawley, R. C., and Hendrickson, T. (1990) Semianalytical treatment

of solvation for molecular mechanics and dynamics. *J. Am. Chem. Soc. 112*, 6127–6129.