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Novel insights into the use of Glowing LNA as nucleic acid detection probes—Influence of labeling density and nucleobases

Michael E. Østergaard [a], Jyotirmoy Maity [b,c], B. Ravindra Babu [b], Jesper Wengel [b], Patrick J. Hrdlicka [a,*]

[a] Department of Chemistry, University of Idaho, Moscow, Idaho 83844-2343, USA
[b] Nucleic Acid Center, Department of Physics and Chemistry, University of Southern Denmark, 5230 Odense M, Denmark
[c] Department of Chemistry, University of Delhi, Delhi 110 007, India

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ABSTRACT

Appropriately designed 2′-*N*-(pyren-1-yl)carbonyl-2′-amino-LNA (locked nucleic acid) display large increases in fluorescence intensity and remarkably high quantum yields upon hybridization with nucleic acid targets. Thermal denaturation and fluorescence spectroscopy studies on ONs modified with known thymine monomer **X** and novel 5-methylcytosine monomer **Y** provide new insights into the design principles and mechanism of these Glowing LNA nucleic acid detection probes.

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Homogeneous fluorescence assays for detection of nucleic acids are widely used for identification of single nucleotide polymorphisms,[1] imaging of RNA in cells[2] and in quantitative PCR.[3] The success of these assays is linked to their simplicity and lack of labor-intensive processing steps.[4] However, probes must produce pronounced hybridization-induced differences in fluorescence intensity as excess probe cannot be washed out. Popular approaches toward this end include molecular beacons,[5] binary probes,[6] base-discriminating probes,[7] and hybridization probes.[8] In the latter approach, fluorescently labeled oligonucleotides are designed to display (a) low fluorescence in the single stranded state through fluorescence quenching by neighboring nucleobases,[9] and (b) high fluorescence upon hybridization with nucleic acid targets by positioning fluorophores in non-quenching microenvironments.

Motivated by the interest in pyrene-modified oligonucleotide probes[7d,10] and locked nucleic acid (LNA),[11,12] we recently characterized a variety of pyrene-functionalized LNA.[8a,8b,8f,13] Oligodeoxyribonucleotides (ONs) containing at least two next-nearest neighbor incorporations of 2′-*N*-(pyren-1-yl)carbonyl-2′-amino-LNA thymine monomer **X**, emerged as particularly interesting hybridization probes which form brightly fluorescent duplexes with emission quantum yields approaching unity (Scheme 1).[8a,8b] Accordingly, the term 'Glowing LNA' has been coined to these probes.[14] Interestingly, among the studied fluorophore-variants of 2′-*N*-(pyren-1-yl)carbonyl-2′-amino-LNA monomer **X**,[8f,13,15] the parent monomer appears to combine structural and electronic features most favorably for potential diagnostic purposes.[8a,8b]

Herein, experiments providing novel insight into the design principles and mechanism of Glowing LNA[†] are described. First, the influence of labeling density on photophysical properties of Glowing LNA was studied. Next, since nearby nucleobase moieties quench pyrene fluorescence differently depending on their electronic nature,[9] we set out to study Glowing LNA modified with novel 5-methylcytosine monomer **Y** (Scheme 1).

The phosphoramidite corresponding to 2′-*N*-(pyren-1-yl) carbonyl-2′-amino-LNA thymine monomer **X** was obtained as previously described.[8a] The N4-benzoyl-5-methylcytosine phosphoramidite of monomer **Y** was obtained from known nucleoside **1Y**[16] (Scheme 1). Briefly described, **1Y** was O5′-dimethoxytritylated and N2′-acylated to provide nucleoside **2Y**. Next, a two-step thymine to 5-methylcytosine conversion was accomplished using

* Corresponding author. Tel.: +1 208 885 0108.
E-mail address: hrdlicka@uidaho.edu (P.J. Hrdlicka).

[†] The terms 'Glowing LNA' and '2′-*N*-(pyrene-1-yl)carbonyl-2′-amino-LNA' are used interchangeably and are defined as an oligonucleotide containing one or more 2′-*N*-(pyrene-1-yl)carbonylamino]-2′-deoxy-2′-*N*,4′-*C*-methyleneribofuranosyl monomers.

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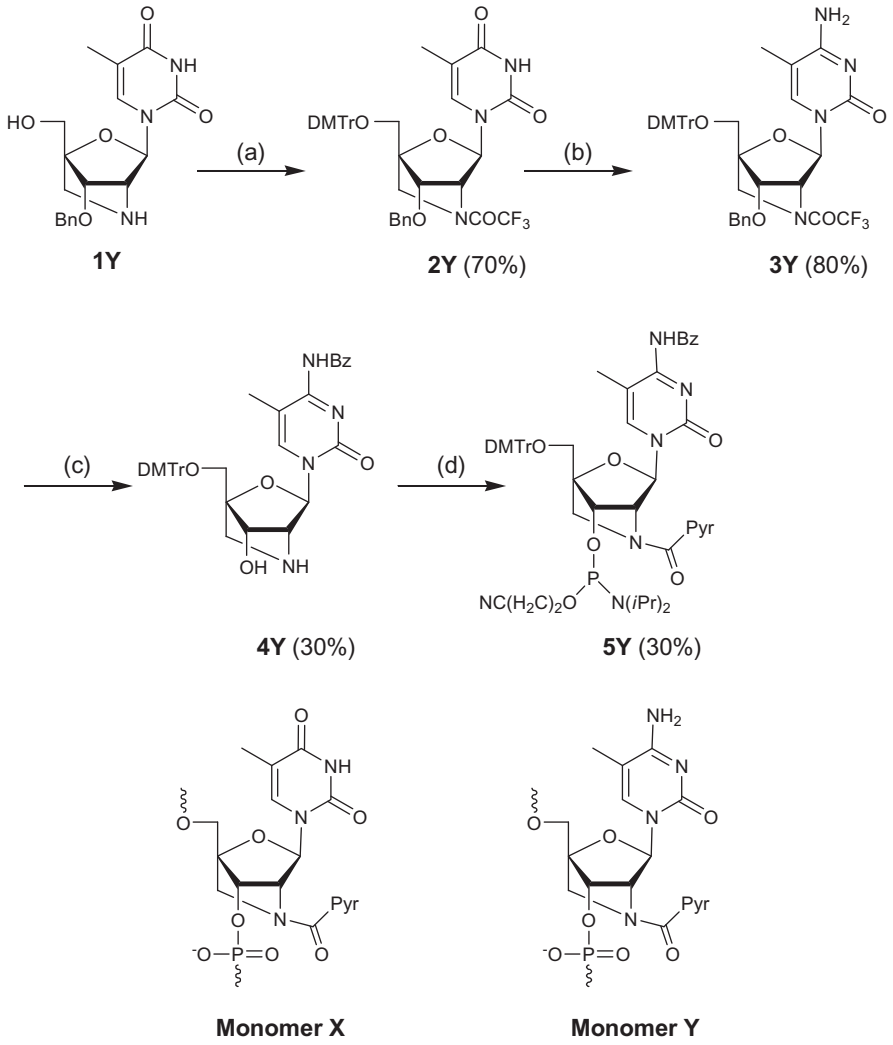

Scheme 1. Structures of monomer **X** and **Y** and synthetic outline for 5-methylcytosine phosphoramidite **5Y**. Reagents and conditions: (a) (i) 4,4′-dimethoxytrityl (DMTr) chloride, pyridine, rt, 12 h; (ii) $(CF_3CO)_2O$, pyridine, rt, 1 h; (b) (i) 1,2,4-triazole, $POCl_3$, Et_3N, CH_3CN, 0 °C to rt, 4 h; (ii) 32% aq NH_3, THF, rt, 3.5 h; (c) (i) 10% Pd/C, HCO_2NH_4, MeOH, reflux, 4 h; (ii) $(CF_3CO)_2O$, pyridine, 0 °C, 3 h; (iii) Bz_2O, DMF, rt, 16 h; (iv) 2 N aq NaOH, pyridine, EtOH, 0 °C to rt, 30 min; (d) (i) 1-pyrenecarboxylic acid, EDC·HCl, CH_2Cl_2, rt, 12 h; (ii) $NC(CH_2)_2OPN(i\text{-}Pr)_2Cl$, $EtN(i\text{-}Pr)_2$, CH_2Cl_2. Pyr = pyren-1-yl.

the 1,2,4-triazole protocol to give **3Y** in high yield. Direct O3′-debenzylation of **3Y** resulted in concomitant N2′-deacetylation (results not shown). All efforts to selectively benzoylate the exocyclic N4-amino moiety of **3Y** failed. Therefore the required key intermediate **4Y**‡ was obtained by O3′-debenzylation, N2-reacylation, N4-benzoylation and chemoselective N2′-deacylation. This nucleoside was then reacted with 1-pyrenecarboxylic acid in an EDC-mediated coupling and followed by O3′-phosphitylation to afford phosphoramidite **5Y**‡. The identity of reported compounds was ascertained by 1D and 2D NMR experiments and high-resolution MS, while purity was established by 1D NMR.

Synthesis of ONs was performed on automated DNA synthesizers (0.2 μmol scale) using standard conditions except for extended coupling times during incorporation of monomers **X** (10 min, 1*H*-tetrazole)[8a] and **Y** (15 min, pyridinium hydrochloride), which resulted in stepwise coupling yields of >97%. The composition and purity (>80%) of all modified ONs was verified by MALDI–MS (Table S1) and ion-exchange HPLC, respectively.[17]

Our initial study of 2′-*N*-(pyren-1-yl)carbonyl-2′-amino-LNA revealed that the fluorescence intensity of single stranded probes (SSPs) is high for singly modified probes but markedly lower upon incorporation of at least two **X** monomers as next-nearest neighbors.[8a] These trends presumably reflect increased quenching interactions between two pyrene moieties and/or between pyrene and nucleobase moieties in the flexible multilabeled probes. However, the influence of labeling density (i.e., inter-monomer distance) on the fluorescence intensity of SSPs was not systematically studied. Three doubly modified 13-mer ONs (**ON1**–**ON3**) with variable inter-monomer distances were therefore synthesized (Table 1).

Thermal denaturation temperatures (T_m-values) of duplexes between modified ONs and complementary DNA/RNA targets were determined at medium salt conditions ($[Na^+]$ = 110 mM).[17] Doubly modified **ON1**–**ON3** display markedly increased thermal affinity toward complementary DNA/RNA targets relative to unmodified reference strand **ON4** and irrespective of inter-monomer distance (ΔT_m = +4.0 to +8.0 °C per modification, Table 1). More thermostable duplexes are formed upon hybridization with RNA targets.

Steady-state fluorescence emission spectra (λ_{ex} = 340 nm, T = 5 °C) were recorded for single stranded ONs and the

‡ *Selected experimental data:* **4Y**: ^{13}C NMR (DMSO-d_6) δ 160.2, 158.2, 148.1, 144.8, 138.3, 136.1, 135.4, 135.2, 132.4, 129.81, 129.75, 129.2, 128.3, 127.9, 127.7, 126.8, 113.1, 108.8, 88.8, 88.3, 85.6, 69.3, 61.5, 59.6, 55.0, 49.9, 13.4; quarternary COPh carbon could not be detected; MALDI-HRMS: m/z 697.2617 ($[M+Na]^+$, $C_{39}H_{38}N_4O_7{\cdot}Na^+$ calcd 697.2633). Selected experimental data **5Y**: ^{31}P NMR ($CDCl_3$) δ 152.2, 151.7, 151.4, 150.2; MALDI-HRMS: m/z 1125.4288 ($[M+Na]^+$, $C_{65}H_{63}N_6O_9P{\cdot}Na^+$ calcd 1125.4286).

Table 1
Thermal denaturation temperatures (T_m-values) and fluorescence emission quantum yields (T = 5 °C) of **ON1**–**ON3** in the absence (SSP) and presence of complementary DNA/RNA targets[a]

ON	Sequence	T_m (ΔT_m/mod) (°C)		Φ_F		
		DNA[b]	RNA[b]	SSP	DNA[b]	RNA[b]
1	5′-TT**X** A**X**A TAT CAC G	46.5 (+6.5)	48.0 (+8.0)	0.28	0.93	0.86
2	5′-TT**X** ATA **X**AT CAC G	45.5 (+6.0)	46.5 (+7.0)	0.34	0.92	0.95
3	5′-TT**X** ATA TA**X** CAC G	41.5 (+4.0)	44.5 (+6.0)	0.30	0.94	0.89
4	5′-TTT ATA TAT CAC G	33.5	32.5	—	—	—

[a] Each strand used at 1.0 μM concentration. Thermal denaturation buffer: 110 mM NaCl, 0.1 mM EDTA, pH 7.0 adjusted with 10 mM NaH_2PO_4/5 mM Na_2HPO_4.
[b] DNA target: 3′-AAA TAT ATA GTG C; RNA target: 3′-AAA UAU AUA GUG C.

corresponding duplexes with DNA/RNA targets. Deoxygenation was deliberately not applied to mimic aerated conditions prevailing in bioassays. Cross-calibrated fluorescence emission quantum yields (Φ_F) were determined relative to pyrenebutanoic acid in methanol (Φ_F = 0.065)[18] and 9,10-diphenylanthracene in cyclohexane (Φ_F = 0.95).[19] Single stranded **ON1**–**ON3** and the corresponding duplexes with DNA/RNA complements, display two vibronic bands at λ_{max} ~382 nm and ~399 nm, which are characteristic of pyrene monomer fluorescence (Figs. 1, S3 and S4).[17] Single stranded probes **ON1**–**ON3** exhibit modest quantum yields while duplexes with DNA/RNA complements exhibit extraordinarily high quantum yields, regardless of labeling density (Φ_F ~0.9, Table 1). Accordingly, ~3.5-fold increases in fluorescence intensity are observed upon hybridization of **ON1**–**ON3** to DNA/RNA targets (Figs. 1 and S5).[17] These results suggest that the fluorophore moiety of monomer **X** is well-accommodated in the non-quenching environment of the minor groove upon hybridization. The observed hybridization-induced increases are at the lower end of typical values observed with appropriately designed multilabeled 2′-*N*-(pyren-1-yl)carbonyl-2′-amino-LNA probes.[8a] This most likely reflects the fact that AT-rich regions are particularly challenging sequence contexts due to the low inherent pyrene fluorescence quenching capacity of adenine and thymine moieties,[9] which results in higher intensity levels of SSPs. Importantly, the photophysical properties of **ON1**–**ON3** and their corresponding duplexes with DNA/RNA targets are virtually identical, which demonstrates that two **X** monomers can be separated by at least five nucleotides to afford Glowing LNA probes with desirable diagnostic properties. Greater freedom in target sequence selection can be expected since probes with 5′-T(B)$_n$T-3′ segments for n = 1–5 can be used.

Nucleobase moieties are known to quench pyrene fluorescence to different degrees depending on their electronic nature.[9] Fluorescence quantum yields of duplexes between ONs modified with

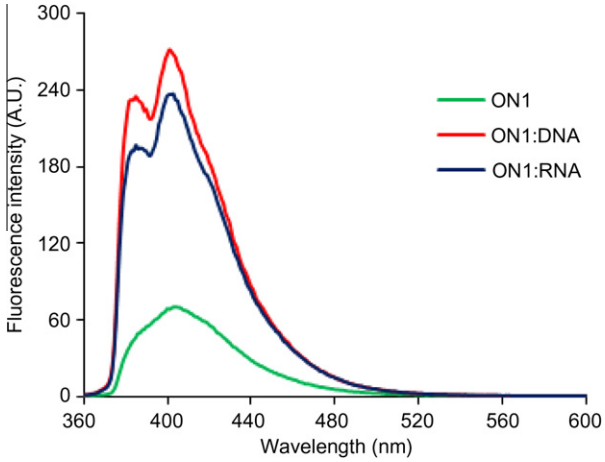

Figure 1. Steady-state fluorescence emission spectra of single stranded **ON1** and of the corresponding duplexes with complementary DNA and RNA (T = 5 °C).

monomer **X** and DNA/RNA targets display relatively little sequence dependent variation.[8a] This is attributed to precise positional control of the fluorophore in the minor groove of duplexes, which is mediated by the conformationally rigid bicyclic skeleton and short amide linker of thymine monomer **X**, which reduces interactions between pyrene and nucleobase moieties.[8a] To challenge this hypothesis, novel 5-methylcytosine monomer **Y** (Scheme 1) was incorporated into mixed sequence 9-mer ONs (**ON5**–**ON10**, Table 2).

Single incorporation of monomer **Y** into ONs results in pronounced increases in thermal stability toward DNA/RNA targets relative to unmodified strands (ΔT_m/mod = +2.5 to +10.0 °C, Table 2). As with monomer **X**, hybridization with RNA targets results in formation of more thermostable duplexes. Incorporation of additional **Y** monomers results in progressive increases in duplex thermostability, for example, the duplex between triply modified **ON10** and complementary RNA displays a T_m-value of 62.0 °C. Importantly, excellent discrimination of DNA or RNA targets with a single centrally positioned mismatch is observed for ONs modified with monomer **Y** relative to the unmodified reference strand (Table 2).

Steady-state fluorescence emission spectra of **ON5**–**ON10** and their corresponding duplexes with DNA/RNA exhibit similar emission profiles as probes modified with monomer **X** (Figs. S6–S11).[17] Singly modified SSPs exhibit intense fluorescence with quantum yields Φ_F ~0.5, while lower quantum yields are observed for multilabeled SSPs (Φ_F = 0.17–0.35, Table 2). All duplexes between **ON5**–**ON10** and complementary DNA/RNA exhibit high fluorescence

Table 2
Thermal denaturation temperatures and fluorescence emission quantum yields of **ON5**–**ON10** in the absence (SSP) and presence of complementary or mismatched DNA/RNA targets (T = 5 °C)[a]

ON	Sequence	T_m (ΔT_m/mod) (°C)		ΔT_m (°C)[c]	Φ_F		
		DNA[b]	RNA[b]	Mismatched DNA	SSP	DNA[b]	RNA[b]
5	5′-G**Y**A TCT CAC	40.5 (+6.0)	46.5 (+10.0)	—	0.48	0.58	0.61
6	5′-GCA T**Y**T CAC	39.5 (+5.0)	40.5 (+4.0)	<−29.5/<−29.5/<−29.5	0.48	0.62	0.69
7	5′-GCA TCT **Y**AC	37.0 (+2.5)	40.0 (+3.5)	—	0.52	0.60	0.67
8	5′-G**Y**A T**Y**T CAC	45.0 (+5.5)	47.0 (+5.5)	−27.0/−30.0/−32.5	0.17	0.58	0.64
9	5′-GCA T**Y**T **Y**AC	43.0 (+4.5)	52.5 (+8.0)	−27.0/−30.0/−30.0	0.35	0.58	0.59
10	5′-G**Y**A T**Y**T **Y**AC	49.5 (+5.0)	62.0 (+8.5)	−27.0/−31.0/−31.5	0.21	0.50	0.60
11	5′-GCA TCT CAC	34.5	36.5	<−24.5/<−24.5/−21.5	—	—	—

[a] For experimental details see Table 1.
[b] DNA target: 3′-CGT AGA GTG; RNA target: 3′-CGU AGA GUG.
[c] ΔT_m = change in T_m for mismatched duplexes relative to fully complementary duplexes. Mismatch sequences: 3′-CGT A**M**A GTG where ΔT_m is shown in the order M = A/C/T. ΔT_m toward the RNA mismatch 3′-CGU A**M**A GUG: **ON6**: A/C/U: −27.5/<−30.5/<−30.5; **ON11**: A/C/U: −23.5/<−26.5/<−26.5.

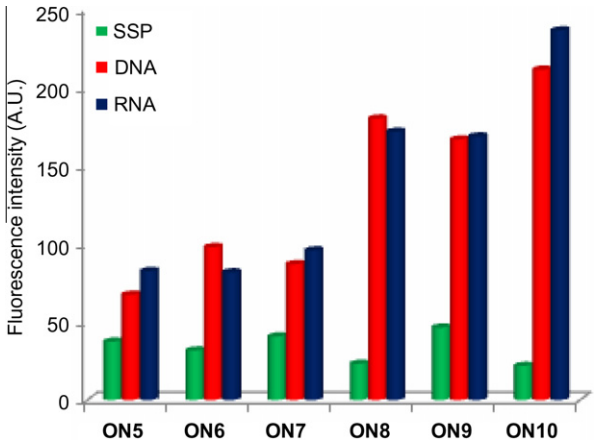

Figure 2. Fluorescence intensity of single stranded probes (SSPs) and of the corresponding duplexes with complementary DNA/RNA. Intensity recorded at λ_{em} = 382 nm at T = 5 °C.

emission quantum yields (Φ_F = 0.50–0.69). The slightly lower quantum yields compared to duplexes between **ON1** and **ON3** and DNA/RNA targets (Table 1), may reflect the fact that cytosine moieties are effective quenchers of pyrene fluorescence.[9,20] Nonetheless, appropriately designed probes **ON8**–**ON10** display 3.6- to 10.7-fold increases in fluorescence intensity upon hybridization with DNA/RNA complements (Fig. 2).

The results suggest that the pyrene moiety only interacts with the nucleobase moiety of the Glowing LNA monomer and/or the base-pairing partner to a minor degree. This supports the hypothesis that the pyrene moiety points into the minor groove of nucleic acid duplexes where it is shielded from interactions with nucleobase moieties resulting in quenching of fluorescence.[8a] An important ramification hereof is that different nucleobase analogs of 2′-*N*-(pyren-1-yl)carbonyl-2′-amino-LNA monomers can be used to generate diagnostically useful Glowing LNA probes for nucleic acid detection, which further increases the number of targetable sequences. Development of other nucleobase variants of 2′-*N*-(pyren-1-yl)carbonyl-2′-amino-LNA monomers is therefore warranted.

In summary, novel insight into the design principles and mechanism of 2′-*N*-(pyren-1-yl)carbonyl-2′-amino-LNA as nucleic acid hybridization probes has been obtained. The labeling density and nature of the monomer nucleobase only exert minor influences on the main characteristics of Glowing LNA probes which include: (a) high target affinity, (b) high target specificity, and (c) pronounced hybridization-induced increases in fluorescence intensity leading to formation of duplexes with extraordinary fluorescence emission quantum yields. Glowing LNA probes are therefore likely to enable detection of single stranded DNA/RNA targets of a variety of sequence contexts. These characteristics, in concert with recent observations demonstrating optimized nucleic acid detection by Glowing LNA composed of different probe backbones (PS-DNA, RNA O2′-Me RNA) and architectures,[8b] render Glowing LNA as promising diagnostic probes.

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Supplementary data

Supplementary data (protocols for synthesis, purification and characterization of ONs, thermal denaturation studies and fluorescence experiments; representative thermal denaturation profiles and additional fluorescence emission spectra) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.10.089.

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