# FLUORESCENT OLIGONUCLEOTIDES CONTAINING A NOVEL PERYLENE 2′-AMINO-α-L-LNA MONOMER: SYNTHESIS AND ANALYTICAL POTENTIAL

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*Dedicated to Professor Antonín Holý on the occasion of his 75th birthday in recognition of his outstanding contributions to the area of nucleic acid chemistry.*

Herein, a novel fluorescent nucleotide analogue, perylene-2′-amino-α-L-LNA, has been prepared and studied within synthetic oligonucleotides of different sequences. The phosphoramidite reagent was synthesized in 85% overall yield starting from 2′-amino-α-L-LNA nucleoside. Incorporation efficiency of the resulting perylene-2′-amino-α-L-LNA monomer (**T***) into synthetic oligonucleotides was significantly improved by replacement of the typically used 1*H*-tetrazole activator with pyridine hydrochloride. Generally, oligonucleotides containing monomer **T*** showed high binding affinity towards complementary DNA and RNA targets, batochromically shifted excitation/emission wavelengths with respect to the often applied polyaromatic hydrocarbon pyrene, high fluorescent quantum yields and very low target detection limits (5–10 nM). Fluorescence of single stranded LNA/DNA mixmer oligonucleotide having two incorporations of monomers **T*** was quenched (quantum yield $\Phi_F$ = 0.21) relative to duplexes of this probe with complementary DNA and RNA ($\Phi_F$ = 0.42 and 0.35, respectively). On the contrary, a strong fluorescence quenching upon target binding was demonstrated by two short oligonucleotides of analogues sequences containing monomers **T*** at 5′- and 3′-terminal positions. We explain the hybridization-induced light-up effect observed for double-labeled probe by a reduction of fluorescence quenching due to precise positioning of the fluorophores within the double-stranded complexes. Furthermore, we propose that a covalent link between two **T*** monomers in the double-labeled probe provides a remarkable degree of rigidity in the double helix which enforces positioning of the bulky perylene moieties in the nonpolar groove resulting in reduced fluorescence quenching.

**Keywords**: DNA; Fluorescence; Fluorescent probes; Oligonucleotides; Nucleosides; Nucleotides; LNA; Molecular diagnostics.



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Detection of nucleic acids is an extremely important task both in research and industry. This includes in applications relating to diagnostics of genetic and infectious diseases[1], antisense technology[2], fluorescent imaging[3] and other biomedical studies. After remarkable progress in automatic oligonucleotide synthesis achieved in the last three decades, fluorescently labeled oligonucleotides have become readily available as useful tools for nucleic acid detection and analysis. In order to be an effective nucleic acid diagnostic probe, the labeled oligonucleotide has to display high affinity and selectivity of target binding and considerable change of fluorescence upon formation of complexes with complementary strands[4]. Continuous development of such oligonucleotide probes has led to several formats of fluorescence nucleic acid assays, e.g. detection by fluorescence polarization[5] or fluorescence resonance energy transfer (FRET)[6], Taqman probes (also known as 5′-nuclease probes)[7], sunrise primers[8], molecular beacons[9], duplex scorpion primers[10], and oligonucleotides labeled with a single kind of a hybridization-sensitive substance[11–17]. Each of these methods and constructs has its own specific strengths and weaknesses, but there is still no universal approach of choice[18]. Moreover, in order to simultaneously analyze multiple targets it is desirable to have various fluorescent dyes displaying different excitation/emission wavelengths[19].

Among polyaromatic hydrocarbons, pyrene has long been the most popular dye for nucleic acid labeling (Fig. 1). However another polyaromatic molecule, perylene, is currently an object of increasing interest as a highly sensitive dye to measure local microenvironment changes within nucleic acids, e.g. those caused by hybridization[20–22]. Fluorescence of perylene is typically observed at 450–500 nm and its excited lifetime is relatively short (4–5 ns) while the fluorescence quantum yield is very high (0.5–1.0)[23]. Furthermore, perylene displays a stronger fluorescence anisotropy effect than pyrene[24]. High fluorescence quantum yield, red-shifted emission compared to pyrene (350–500 nm and 420–620 nm for pyrene and perylene in cyclohexane, respectively[23]), and the remarkable photostability of perylene makes it an attractive label for fluorescence-based detection both *in vitro* and *in vivo*. We previously introduced 2′-*N*-(perylen-3-yl)carbonyl-2′-amino-LNA modification which induced high thermal stability of DNA:DNA and DNA:RNA duplexes, high Watson–Crick mismatch selectivity, red shifted fluorescence emission compared to pyrene, and high fluorescence quantum yields[25]. Additionally, the perylene-LNA probe was successfully applied for detection of mRNA *in vivo* providing an excitation wavelength which completely eliminated cell autofluorescence[25].

Besides insertion of fluorescent dyes, chemical modification of oligonucleotides allows improvement of their binding affinity and selectivity towards complementary DNA and RNA targets. These properties have in particular been reported for *N*-functionalized 2′-amino locked nucleic acids (2′-amino-LNA [26] and isomeric 2′-amino-α-L-LNA [27]), also containing

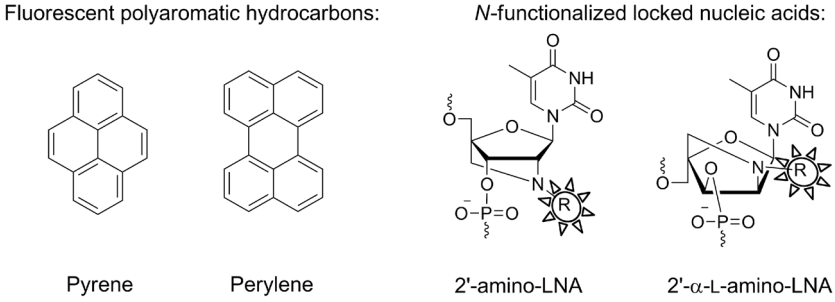



FIG. 1
Chemical structures of the fluorescent polyaromatic hydrocarbons pyrene and perylene, which are often used for oligonucleotide labeling, and of *N*-functionalized fluorescent locked nucleic acid analogues. R = polyaromatic hydrocarbon residue, also indicated with rays

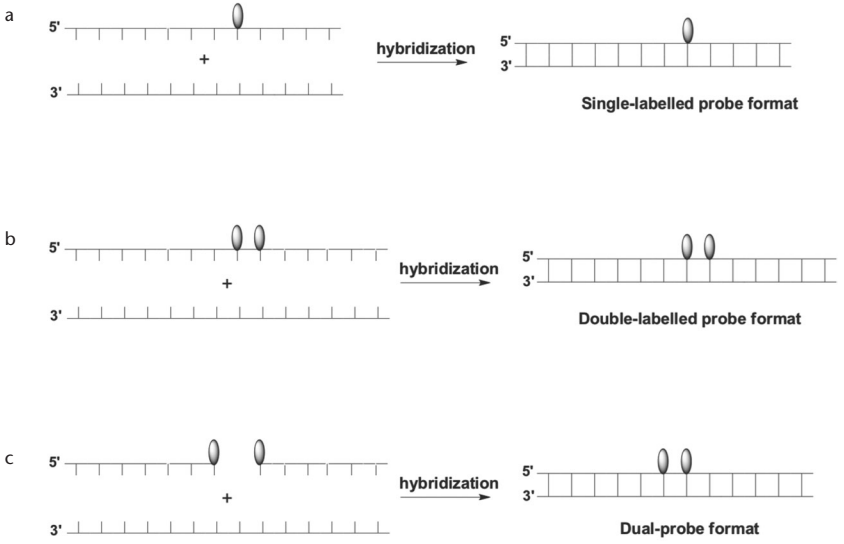



FIG. 2
Representative structures of the perylene labeled oligonucleotides prepared in this study and their hybridization with DNA/RNA targets: (a) 12mer single-labeled probes, (b) 17mer double-labeled probes and (c) 8mer+9mer dual-probes. Black droplets indicate monomer **T***

polyaromatic hydrocarbons (Fig. 1). Stimulated by recently described high affinity and selectivity of hybridization provided by 2′-amino-α-L-LNA derivatives[27], sensitivity of pyrene-functionalized 2′-amino-α-L-LNA and perylene-functionalized 2′-amino-LNA probes to hybridization[25,28], and taking the high fluorescence quantum yields and red-shifted fluorescence emission of perylene into account, we have prepared a novel perylene-modified 2′-amino-α-L-LNA derivative. In this paper, we describe synthesis of 2′-*N*-(perylen-3-yl)methyl-2′-amino-α-L-LNA monomer **T*** and its incorporation into synthetic oligonucleotides, as well as thermal denaturation studies and spectral properties of the resulting oligonucleotide probes of different sequences containing either single or double insertions of monomer **T*** (Fig. 2).

## EXPERIMENTAL

### Synthesis of 2′-*N*-(Perylen-3-yl)methyl-2′-amino-α-L-LNA Phosphoramidite Reagent

Reagents obtained from commercial suppliers were used as received; 3-perylenecarbaldehyde[29] and diisopropylammonium tetrazolide[30] were synthesized as described. Perylene used as standard for fluorescence quantum yield measurements was recrystallized. HPLC grade MeOH, EtOAc and petroleum ether were stored over activated 4Å molecular sieves. DCM was always used freshly distilled over $CaH_2$. Other solvents were used as received. Photochemical studies were performed using spectroquality cyclohexane. NMR spectra were recorded at 303 K on a Varian Gemini 2000 300 MHz instrument. Chemical shifts (δ-scale) are reported in ppm relative to solvents peaks (DMSO-$d_6$: 2.50 ppm for $^1$H and 39.5 ppm for $^{13}$C; 85% aq. $H_3PO_4$: 0.00 ppm for $^{31}$P). $^1$H NMR coupling constants (*J*) are reported in Hz and refer to apparent multiplicities. High resolution ESI-mass spectra were recorded in positive ion mode using a PE SCIEX QSTAR pulsar mass spectrometer. Analytical thin-layer chromatography was performed on Kieselgel 60 $F_{254}$ precoated aluminium plates (Merck). Silica gel column chromatography was performed using Merck Kieselgel 60 0.040–0.063 mm.

(1*S*,3*R*,4*S*,7*R*)-1-(4,4′-Dimethoxytrityloxymethyl)-7-hydroxy-5-(perylen-3-yl)methyl-3-(thymin-1-yl)-2-oxa-5-azabicyclo[2.2.1]heptane (**2**)

Nucleoside **1** [31] (200 mg, 0.35 mmol) was dried by coevaporation with anhydrous DCM (2 × 10 ml) and dissolved in anhydrous 1,2-dichloroethane (5 ml). To this solution, 3-perylenecarbaldehyde[29] (145 mg, 0.54 mmol) and $NaBH(OAc)_3$ (112 mg, 0.54 mmol) were subsequently added, and the reaction mixture was stirred at r.t. for 12 h. Absolute EtOH–toluene–$Et_3N$ mixture (9:9:2, v/v/v) was then added, and the resulting material was evaporated to dryness under reduced pressure. The residue obtained was purified by silica gel column chromatography (0–5% MeOH in DCM, v/v) to afford the *N*-alkylated product **2** (264 mg, 90%) as a white solid material (a mixture of rotamers according to NMR). $R_F$ 0.47 (5% MeOH, 1% $NEt_3$, DCM, v/v/v). $^1$H NMR (300 MHz, DMSO-$d_6$; the signals are given for the major rotamer): 11.16 (s, 1 H, NH); 8.37–6.85 (m, 26 H, Ar); 6.00 (d, 1 H, *J* = 3.6, 3′-OH); 5.90 (d, 1 H, *J* = 1.8, H-1′); 4.45 (d, 1 H, *J* = 3.6, H-3′); 4.40 (d, 1 H, *J* = 13.2, $CH_2$Ar); 4.20 (d,

1 H, *J* = 13.1, $CH_2Ar$); 3.72 (s, 6 H, 2 × $CH_3O$); 3.47 (s, 1 H, H-2′); 3.35 (d, 1 H, *J* = 10.6, H-5′a); 3.30 (d, 1 H, *J* = 10.6, H-5′b); 3.25 (d, 1 H, *J* = 9.8, H-5″a); 3.00 (d, 1 H, *J* = 9.8, H-5″b); 1.63 (s, 3 H, $CH_3$). $^{13}$C NMR (300 MHz, DMSO-$d_6$): 163.8, 158.0, 150.1 (Ar), 144.7 (Ar), 136.6 (Ar), 135.4 (Ar), 135.3 (Ar), 134.1 (Ar), 132.6 (Ar), 130.5 (Ar), 130.4 (Ar), 130.3 (Ar), 129.8 (Ar), 129.6 (Ar), 128.1 (Ar), 127.8 (Ar), 127.6 (Ar), 126.8 (Ar), 126.6 (Ar), 126.0 (Ar), 124.3 (Ar), 123.7 (Ar), 120.5 (Ar), 120.3 (Ar), 119.7 (Ar), 113.1 (Ar), 106.9, 90.4, 85.3 (C-1′), 85.1, 74.9 (C-3′), 65.4 (C-2′), 60.9 (C-5′), 58.8 (C-5″), 58.6 ($CH_2Ar$), 54.9 ($CH_3O$), 54.8 ($CH_3O$), 12.3 ($CH_3$). ESI-HRMS: *m/z* 836.3342 ([M + Na]$^+$, $C_{53}H_{45}N_3O_7Na^+$ calculated 836.3330).

(1*S*,3*R*,4*S*,7*R*)-7-(2-Cyanoethoxy(diisopropylamino)phosphinoxy)-1-(4,4′-dimethoxytrityloxymethyl)-7-hydroxy-5-(perylen-3-yl)methyl-3-(thymin-1-yl)-2-oxa-5-azabicyclo[2.2.1]heptane (**3**)

The LNA nucleoside derivative **2** (150 mg, 0.18 mmol) was coevaporated with anhydrous DCM (2 × 5 ml) and dissolved in anhydrous DCM (5 ml). To this solution, diisopropylammonium tetrazolide (62 mg, 0.36 mmol) and bis(*N*,*N*-diisopropylamino)-2-cyanoethoxyphosphine (115 µl, 0.36 mmol) were added under argon, and the resulting mixture was stirred at r.t. for 12 h. The mixture was diluted with DCM (50 ml) and washed consecutively with a saturated solution of $NaHCO_3$ (2 × 50 ml) and brine (50 ml). The organic layer was dried over $Na_2SO_4$, evaporated to dryness under reduced pressure and the residue was purified by column chromatography on silica gel (30–50% EtOAc in petroleum ether, containing 1% $NEt_3$, v/v/v) to give phosphoramidite **3** as a white solid material. Yield 176 mg (94%). $R_F$ 0.45, 0.43 (50% EtOAc, 1% $NEt_3$, petroleum ether, v/v/v). $^{31}$P NMR (300 MHz, DMSO-$d_6$): 149.9, 146.6 (1:1.7) (main diastereomer). ESI-HRMS: *m/z* 1058.4250 ([M + Na]$^+$, $C_{62}H_{62}N_5O_8PNa^+$ calculated 1058.4228).

Synthesis and Purification of Modified Oligonucleotides

LNA phosphoramidites were obtained from commercial supplier (Exiqon). Oligonucleotide synthesis was carried out on a PerSpective Biosystems Expedite 8909 instrument in 200 nmol scale using standard manufacturer's protocols, except for increased coupling time for modified phosphoramidite **3** and LNA [32]. 3′-Labeled oligonucleotide **ON6** was prepared on CPG universal support III (Glen Research) using manufacturer's protocol. In case of **3**, 30 min coupling time using 0.6 M pyridine hydrochloride in MeCN as an activator was applied, while for LNA phosphoramidites 10 min coupling time and 1*H*-tetrazole solution in MeCN were applied. Coupling yields based on the absorbance of the dimethoxytrityl cation released after each coupling were approximately 95–99% for modified phosphoramidite **3**, 98–99% for LNA, and 99–100% for unmodified DNA phosphoramidites. Cleavage from solid support and removal of nucleobase protecting groups were performed using standard conditions (32% aqueous ammonia at 55 °C for 12 h). In case of **ON6** cleavage from the universal solid support was performed using 3.5 M solution of ammonia in methanol (1 h) followed by removal of nucleobase protecting groups with 32% aqueous ammonia at r.t. for 12 h. Unmodified DNA/RNA strands were obtained from commercial suppliers and used without further purification, while all the modified oligonucleotides were purified by DMT-ON 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 5 min followed by a linear gradient to 55% B-buffer at a flow rate of 1.0 ml/min over

75 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% aqueous AcOH, 30 min), precipitation (acetone, –18 °C, 12 h) and washing with acetone twice. The identity and purity of oligonucleotides were verified by MALDI-TOF mass spectrometry (recorded on an Applied Biosystems Voyager-DE STR spectrometer) and ion-exchange HPLC (purities > 90%), respectively. Measured masses of the oligonucleotides (calculated masses *m/z* for $[M - H]^-$): **ON1**: 3730 (3733); **ON2**: 3728 (3733); **ON3**: 3732 (3733); **ON5**: 5305 (5309); **ON6**: 2455 (2455), **ON7**: 2790 (2792). For sequences of oligonucleotides prepared in this study, see Tables I, II.

### UV-Visible Absorption and Thermal Denaturation Studies

UV-Visible absorption and thermal denaturation studies were performed on a Perkin–Elmer Lambda 35 UV/VIS Spectrometer equipped with PTP 6 (Peltier Temperature Programmer) in a medium of salt phosphate buffer (100 mM sodium chloride, 10 mM sodium phosphate, 0.1 mM EDTA, pH 7.0). Concentrations of oligonucleotides were calculated using the following extinction coefficients ($OD_{260}$/μmol): G, 10.5; A, 13.9; T/U, 7.9; C, 6.6; **T***, 33.2. Modified oligonucleotides and target strands (0.5–1.0 μM each strand) were thoroughly mixed, denaturated by heating and subsequently cooled to the starting temperature of experiment. Thermal denaturation temperatures ($T_m$ values, °C) 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 °C.

### Fluorescence Steady-State Emission Studies, Quantum Yield and Limits of Target Detection Determinations

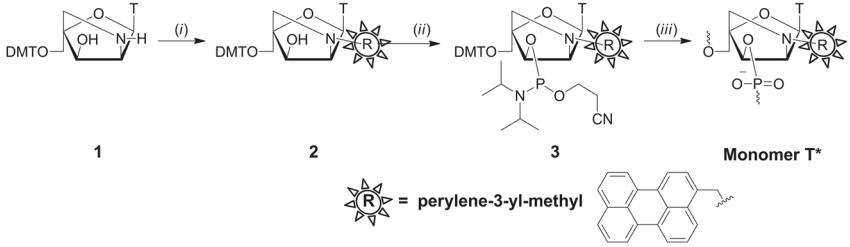
Fluorescence spectra were obtained at 19 °C in a medium of salt phosphate buffer (see above) using a Perkin–Elmer LS 55 luminescence spectrometer and an excitation wavelength of 425 nm, excitation slit of 4.0 nm, emission slit of 2.5 nm, scan speed of 120 nm/min, and 0.1–0.5 μM concentrations of the single-stranded probes or the corresponding duplexes. The fluorescence quantum yields ($\Phi_f$) were measured by the relative method using perylene ($\Phi_f$ = 0.93) in cyclohexane as a standard[33]. The optical density of the solutions in a 1-cm quartz cuvette at the excitation wavelength did not exceed 0.1. The values of $\Phi_f$ were corrected with the refractive index of the solvent; the measured refractive indexes of cyclohexane and phosphate buffer were 1.426 and 1.334 at 19 °C, respectively. For target titration fluorescence measurement in limit of target detection (LOD) determinations[34], the relevant fluorescent probe (500 nM each strand) was mixed with a series of solutions containing the corresponding DNA/RNA target at concentrations 1000, 800, 600, 400, 200, 100, 50, 10, 5, and 1 nM.

## RESULTS AND DISCUSSION

### *Synthesis of Modified Phosphoramidite Reagent*

The modified phosphoramidite reagent **3** was synthesized as shown in Scheme 1. 3-Perylenecarbaldehyde[29] was reacted by reductive amination with

the DMT-protected 2′-amino-α-L-LNA monomer **1** [31] to give nucleoside **2**. Subsequent standard phosphitylation afforded the phosphoramidite derivative **3** (85% overall yield) which was used in automated oligonucleotide synthesis to prepare series of modified oligonucleotides (Tables I, II). The sequences of the 12mer oligonucleotides were similar to those used for studies of pyrene–perylene 2′-amino-LNA FRET pair[35]. LNA/DNA mixmer 8mer, 9mer and 17mer sequences were adopted from previous studies on pyrene 2′-amino-LNA [17].




SCHEME 1
Reagents, conditions and yields: (i) 3-perylenecarbaldehyde, $NaBH(OAc)_3$, 1,2-dichloroethane (90%); (ii) $NC(CH_2)_2OP(N(i\text{-}Pr)_2)_2$, diisopropylammonium tetrazolide, DCM (94%); (iii) DNA synthesizer (95–99%). R = perylene-3-yl-methyl; fluorescent dye R is also indicated with rays; DMT = 4,4′-dimethoxytrityl; T = thymin-1-yl

Synthesis of modified oligonucleotides was performed following standard protocols, except for incorporation of monomer **T*** and LNA monomers. The coupling efficiencies of standard amidites based on the absorbance of the dimethoxytrityl cation released after each coupling varied between 98 and 100%. While incorporating LNA monomers extended coupling time (10 min) using 1*H*-tetrazole as activator was applied giving high coupling yields (98–99%). However using this procedure we obtained only 78–80% coupling yield for modified monomer **T***. We solved this problem by additional extension of monomer **T*** coupling time to 30 min and using of pyridine hydrochloride as an activator. This resulted in 95–99% stepwise coupling yields of monomer **T***. All synthesized oligonucleotides were purified by RP-HPLC and their identity confirmed by MALDI-TOF mass spectrometry and their purity by ion-exchange HPLC (see Experimental).

Hybridization and UV-Vis absorption properties of 2′-*N*-(perylen-3-yl)-methyl-2′-amino-α-L-LNA monomer **T*** within 12mer synthetic oligonucleotides were studied in a medium salt phosphate buffer (100 mM sodium chloride, 10 mM sodium phosphate, 0.1 mM EDTA, pH 7.0). In all

cases, the denaturation curves displayed sigmoidal monophasic transitions with a shape similar to that observed for unmodified reference duplexes. Thermal denaturation and UV-visible absorption data of 12mer probes and their duplexes with complementary DNA are shown in Table I (spectra not shown). The perylene-modified oligonucleotides displayed higher affinity towards complementary DNA than complementary RNA. As can be seen from data presented in Table I, single incorporation of monomer **T*** results in remarkable stabilization of duplexes with DNA targets ($\Delta T_m$ +5.5, +8.0 and +7.0 °C). Moreover, attachment of perylene to 2′-amino-α-L-LNA results in significantly higher binding affinity than previously introduced diastereoisomeric 2′-amino-LNA [35]. On the other hand, similar stabilization effect to modification **T*** was previously reported for pyrene 2′-amino-α-L-LNA analogue which was proposed to intercalate into the double helix[36]. This was also confirmed by red-shifted pyrene absorbance upon hybridization with complementary DNA. However we did not observe any significant changes in absorbance spectra of **ON1–ON3** upon their binding to DNA (Table I)[37]. The perylene fluorophore for both single strands and duplexes absorbs with a maximum at 454 nm, an additional band at 419 nm and a shoulder at 396 nm. We speculate that in case of monomer **T*** perylene moiety can be placed in the major groove of the double helix

TABLE I
Thermal denaturation temperatures and absorption maxima of the single-labeled oligonucleotides **ON1–ON3** and their duplexes with complementary DNA

| Oligonucleotide | Sequence, 5′→3′ | $T_m$ ($\Delta T_m$),°C Duplex with complementary | | $\lambda^{abs}_{max}$ SSP/Duplex with complementary DNA/RNA |
|---|---|---|---|---|
| | | DNA | RNA | |
| **ON1** | CTA G**T***A TAG TAC | 38.0 (+5.5) | 33.0 (+2.0) | 454/454/454 |
| **ON2** | CTA GTA **T***AG TAC | 40.5 (+8.0) | 34.5 (+3.5) | 454/454/454 |
| **ON3** | CTA GTA TAG **T***AC | 39.5 (+7.0) | 33.5 (+2.5) | 454/454/454 |
| **ON4**[a] | CTA GTA TAG TAC | 32.5 | 31.0 | – |

[a] Unmodified reference oligonucleotide. Thermal denaturation temperatures $T_m$ (°C) (change in $T_m$ per modification relative to corresponding reference duplex, $\Delta T_m$/mod (°C)). $T_m$ values measured as the maximum of the first derivatives of the melting curves ($A_{260}$ vs temperature). All $T_m$ and absorbance maxima ($\lambda^{abs}_{max}$) values were recorded in medium salt phosphate buffer. Reported $T_m$ values and absorbance maxima are averages of at least two measurements. **T*** = 2′-*N*-(perylen-3-yl)methyl-2′-amino-α-L-LNA monomer; SSP = single stranded probe

since this type of orientation has been also previously proposed for *N*-functionalized 2′-amino-α-L-LNA [36].

Next, we investigated fluorescent properties of monomer **T*** in dual-probe and double-labeled probe formats which are often applied in nucleic acid diagnostic settings (Fig. 2). In a dual-probe format signal generation requires simultaneous hybridization of two 5′- and 3′-labeled probes in neighboring regions on a nucleic acid target, whereas in a double-labeled

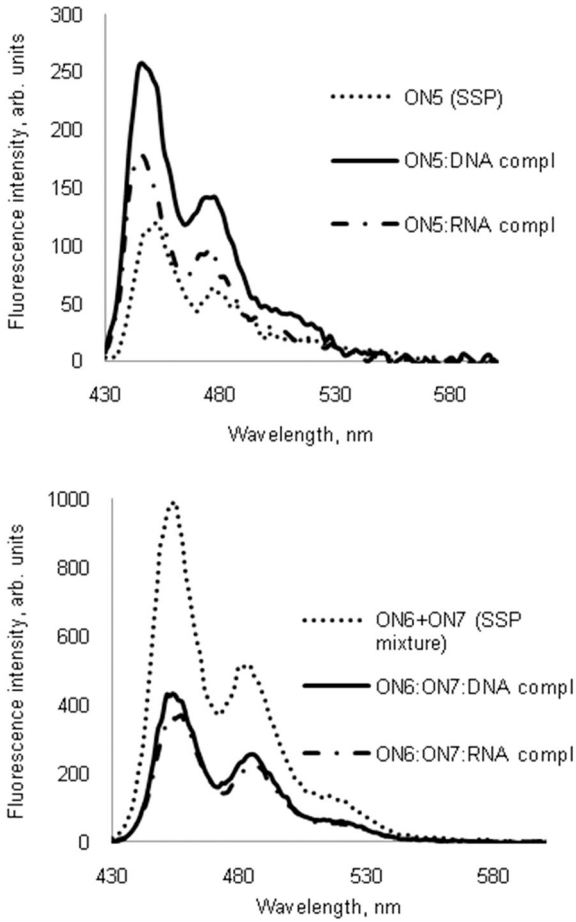



FIG. 3

Steady-state fluorescence emission spectra of a **ON5** and b **ON6+ON7** and the corresponding duplexes with complementary DNA/RNA. Spectra were obtained in a medium salt phosphate buffer at 19 °C using an excitation wavelength of 425 nm and 0.1 μM concentration of oligonucleotides and target strands

probe which is a covalently linked analogue of the dual-probe fluorescence detection of hybridization is provided by interactions of two consequently incorporated reporting groups[38,39].

Steady-state fluorescence emission spectra of oligonucleotides **ON5–ON7** and their duplexes with DNA/RNA targets were obtained in medium salt phosphate buffer using 0.1–0.5 μM concentration of the single stranded probes or the two complementary strands (Fig. 3). Table II lists the resulting spectral and photophysical properties of single stranded LNA/DNA mixmer oligonucleotides (SSP) and their duplexes with complementary DNA and RNA strands.

For the recording of fluorescence spectra an excitation wavelength of 425 nm corresponding to the second perylene absorption maximum was used. As one can see, the emission peaks of perylene 2′-amino-α-L-LNA probes lie within the 440–590 nm region, and all the spectra exhibit significant vibronic features (Fig. 3). Single stranded probes in both dual-probe and double-labeled formats display emission bands at fluorescence maxima $\lambda^{fl} \approx 453$ nm, which is approx. 60 nm red shifted relative to the corresponding probes containing pyrene 2′-amino-α-L-LNA [27]. Upon duplex formation the fluorescence maximum of **ON5** shifts 9–10 nm to shorter wavelengths which points on direction of the two fluorophores to the less polar microenvironment compared to the aqueous buffer[25,40,41]. Emission maxima of the dual-probe system **ON6+ON7** on the contrary are only slightly

TABLE II
Spectroscopic and photophysical properties of modified double-labeled and dual-probe oligonucleotides and their duplexes with complementary DNA and RNA targets

| Oligonucleotide | $\lambda^{fl}_{max}$, nm | | | $\Phi_F$ | | | FB | | |
|---|---|---|---|---|---|---|---|---|---|
| | SSP | DNA | RNA | SSP | DNA | RNA | SSP | DNA | RNA |
| **ON5** | 454 | 444 | 443 | 0.21 | 0.42 | 0.35 | 12.8 | 33.1 | 24.3 |
| **ON6+ON7** | 452 | 451 | 451 | 0.91 | 0.45 | 0.47 | 39.8 | 16.5 | 17.3 |

Modified LNA/DNA mixmer oligonucleotides prepared in this study: **ON5**: 5′-d(CA$^L$C CAA C**T*T*** CT$^L$T CCA C$^L$A), **ON6**: 5′-d(CA$^L$C CAA C**T***), **ON7**: 3′-d(AC$^L$A CCT T$^L$C**T***), where A$^L$, T$^L$ and C$^L$ are adenine-9-yl, thymin-1-yl and 5-methylcytosin-1-yl LNA monomers, respectively. $\lambda^{fl}_{max}$, $\Phi_F$ and FB are fluorescence maximum, fluorescence quantum yield and fluorescence brightness values, respectively (FB = $\Phi_F \times \varepsilon_{max}$, where $\varepsilon_{max}$ is a molar absorption coefficient measured at the corresponding absorption maximum). SSP = single stranded probe

affected by hybridization. Based on these results we propose that a covalent link between two **T*** monomers in the double-labeled probe provides a remarkable degree of rigidity in the double helix which enforces positioning of the bulky perylene moieties in the groove.

Fluorescence emission quantum yields of oligonucleotides and duplexes were determined by the relative method using highly diluted solution of perylene ($\Phi_F = 0.93$) as a reference standard[42]. Interestingly, quantum yields of modified oligonucleotides and duplexes were not affected by presence of air in the examined samples, since series of degassed probes gave exactly the same $\Phi_F$ values as those in aerated buffer solutions. This is most likely caused by short fluorescence lifetime of perylene decreasing the possibility of quenching by solvent, nucleobases or oxygen in the sample solutions. Superiority of perylene relative to pyrene with respect to quantum yield becomes clear if fluorescence rate constants are compared ($K_f = 0.25 \times 10^{-7}$ $s^{-1}$ for pyrene and $14.62 \times 10^{-7}$ $s^{-1}$ for perylene in cyclohexane, respectively[42]).

Generally, fluorescence quantum yields of **ON5–ON7** and their duplexes are remarkably high (Table II). Usually, polyaromatic hydrocarbons, e.g. pyrene and its derivatives incorporated into nucleic acids, display low quantum yields due to interactions with quenchers of fluorescence, e.g. solvent molecules and nucleobases[43]. However, if the sequence can adopt

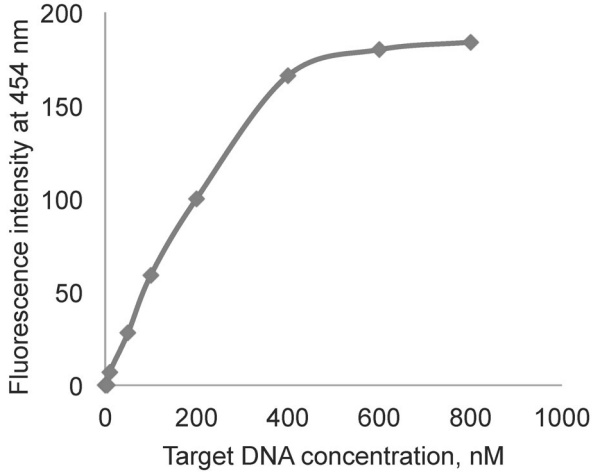



FIG. 4
Representative target titration curve for duplex of **ON5** and complementary DNA. Spectra were obtained in a medium salt phosphate buffer at 19 °C using an excitation wavelength of 425 nm and 500 nM concentration of the probe

a structure shielding the fluorophore from the quenchers (i.e. oxygen, nucleobases), quantum yields of oligonucleotides become very high[26]. Direction of the fluorophore into the groove upon hybridization can also reduce fluorescence quenching[26,44]. The higher quantum yield of single stranded **ON6+ON7** relative to **ON5** might result from an interaction between the two perylene moieties in the latter case which quenches fluorescence. Furthermore, single-stranded probe **ON5** having two incorporations of monomer **T*** exhibit lower quantum yield compared to its duplexes with DNA and RNA targets (Table II). The bright green fluorescence exhibited by the duplex **ON5**:DNA can be detected by the naked eye at concentrations below 0.1 μM. Interestingly, incorporation of perylene modifications **T*** into a dual-probe format did not result in high values of the quantum yields for the corresponding probe **ON5**. This stresses that upon attachment to 2′-amino-α-L-LNA, the fluorescence properties of perylene are as sensitive to the probe design as those of pyrene[44], and that the optimal probe design needs to be investigated for each particular nucleotide analogue containing perylene.

Finally, we determined the limit of target detection[34], a crucial parameter of any diagnostic method, for the LNA/DNA probes containing monomer **T***. The LOD value was determined as a lowest concentration of target in a dilution series detected by fluorescence at the corresponding $\lambda^{fl}_{max}$ such that the fluorescence signal to noise ratio (S/N) (relative to the blank solution of a medium salt phosphate buffer) was minimum three. As a result of the high quantum yields of the perylene 2′-amino-α-L-LNA probes, LOD values for the probes **ON5–ON7** are very low (5–10 nM) (Fig. 4). Such limits of target detection are comparable with those of molecular beacons containing bright xanthene dyes[45] and are satisfactory for direct diagnostics of PCR amplicons.

## CONCLUSIONS

Herein, we have reported the synthesis of 2′-*N*-(perylen-3-yl)methyl-2′-amino-α-L-LNA monomer **T*** and its incorporation into synthetic oligonucleotides, Generally, oligonucleotides containing monomer **T*** exhibited high-affinity hybridization towards complementary DNA and RNA with the highest affinity towards DNA. A double-labeled LNA/DNA probe containing monomers **T*** showed considerably quenched fluorescence as a single stranded probe, and an evident increase of fluorescence quantum yield (up to 2.5-fold) upon hybridization with complementary DNA and RNA targets. On the contrary, in the alternative dual-probe format, fluorescence was

remarkably quenched upon binding of complementary DNA and RNA. Notably, oligonucleotide probes containing the novel perylene 2′-amino-α-L-LNA monomer displayed approximately 60 nm red shifted absorption/emission maxima compared to pyrene, high fluorescence quantum yields and fluorescence brightnesses. Target titration experiments showed that targets in concentrations of 5–10 nM target can be effectively detected using the probes containing monomer **T***. The attractive spectral characteristics reported herein, together with high-affinity binding to complementary targets, make probes containing *2′-N*-(perylen-3-yl)methyl-2′-amino-α-L-LNA monomer potentially useful for nucleic acid targeting both *in vitro* and *in vivo*.

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