Novel interstrand communication systems within DNA duplexes based on 1-, 2- and 4-(phenylethynyl)pyrenes attached to 2′-amino-LNA: high-affinity hybridization and fluorescence sensing†

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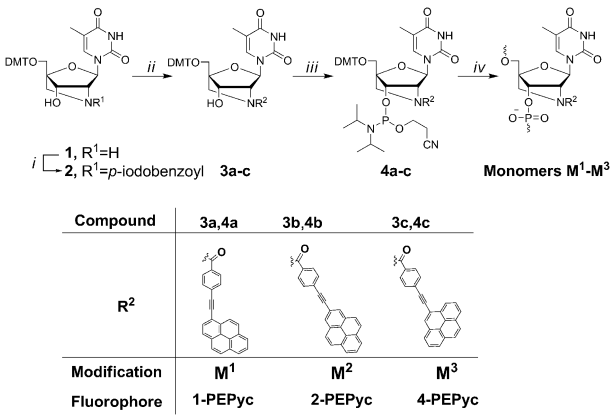
Functionalisation of 2′-amino-LNA oligonucleotides with 1-, 2- and 4-(phenylethynyl)pyrene fluorophores *via* a carbonyl linker (PEPyc) resulted in efficient interstrand communication systems in nucleic acid duplexes, providing effective tools for stabilization of nanostructures and fluorescence monitoring of DNA self-assembly.

Nucleic acids, best known as the carriers of genetic information, are demonstrated to be highly useful materials for designing nanometre-scale structures (Ångström-scale chemical engineering).[1] Oligonucleotides (ONs) have unique and predictable recognition capabilities due to the specificity of Watson–Crick base-pairing, making DNA a perfect tool for growing well-defined nanostructures. Potential applications of the latter include nanoelectronics, biosensors and various molecular machines. For the preparation of such DNA nanostructures it should be possible to form stable constructs following the process of self-assembly by a sensing method of choice. Stimulated by high-affinity hybridization of LNA (locked nucleic acid),[2] 2′-amino-LNA[3] and its 2′-*N*-derivatives,[4] Hrdlicka *et al.* introduced 2′-*N*-(pyren-1-yl)-methyl-2′-amino-LNA as a key element in interstrand communication systems in dsDNA.[5] Being incorporated into short ONs, this monomer provides two effects: stabilization of duplexes and fluorescence signaling of hybridization. We have attached various (phenylethynyl)pyrenes (PEPys)[6] to 2′-amino-LNA[7a] as improved fluorescent monomers for nucleic acid labeling. Among other interesting properties, these conjugates displayed higher fluorescent quantum yields and long-wave shifted absorption and emission maxima (by 25–100 nm) compared to the parent pyrene, which makes them perspective dyes for nucleic acids labeling and diagnostics.

Inspired by this work, and by recent research on 2′-*N*-(pyren-1-yl)methyl-2′-amino-LNA[5] and other fluorescent derivatives of 2′-amino-LNA,[7] we introduce herein novel



Scheme 1 Synthesis of modified monomers **M[1]–M[3]** (for details see ESI†).

2′-amino-LNAs containing 1-, 2- and 4-PEPy fluorophores linked *via* a carbonyl linker (PEPyc-LNA) as improved fluorescent labels for double-stranded DNA interstrand communication systems (Scheme 1). Herein, we report the synthesis, thermal denaturation experiments and fluorescence properties of ONs containing 1-, 2- and 4-PEPyc-2′-amino-LNAs.

In order to prepare PEPyc functionalized oligonucleotide building blocks, phosphoramidite reagents **4a–c** were prepared (Scheme 1). Synthesis of the compounds is described in ESI.†

Attachment of fluorescent dyes to the 2′-position of 2′-amino-LNA nucleoside was performed by acylation of **1**[8] with *p*-iodobenzoic acid in the presence of HATU (*N*,*N*,*N*′,*N*′-tetramethyl-*O*-(7-azabenzotriazol-1-yl)uronium hexafluorophosphate)[9] followed by Sonogashira coupling[10] with 1-, 2- or 4-ethynylpyrenes[11] (Scheme 1). The yields of Sonogashira couplings correlate with the sterical accessibility of the ethynyl group in the pyrene derivative: the less hindered 2-ethynylpyrene gave the highest coupling yield (89%), while the most hindered 4-ethynylpyrene gave only 71% of product **3c**. The nucleoside derivatives **3a–c** were then phosphitylated with bis(*N*,*N*-diisopropylamino)-2-cyanoethoxyphosphine in DCM in the presence of diisopropylammonium tetrazolide to give phosphoramidites **4a–c**, which were used in automated oligonucleotide synthesis to prepare series of modified ONs. The sequences of mixed base 9-mer ONs were similar to those previously designed for studies of 2′-*N*-(pyren-1-yl)methyl-2′-amino-LNA.[5]

The effect of modifications **M[1]–M[3]** on duplex stability was evaluated by UV thermal denaturation experiments using medium salt buffer ([Na^+] = 110 mM)† and compared to

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† Electronic supplementary information (ESI) available: Details of synthesis and purification of modified phosphoramidites and ONs; MALDI-MS data of modified ONs (Table S1); selected thermal denaturation curves, absorption spectra, fluorescence emission spectra, and quantum yields measurements. See DOI: 10.1039/c0cc03026k

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Table 1 ONs used in this study and representative thermal denaturation data for **ON5–ON13** towards unmodified DNA and RNA complements (**ON1–ON4**)[a]

#	Sequence, 5′ → 3′	#	Sequence, 5′ → 3′
ON1	GTG ATA TGC	**ON8**	GTG A**M²**A TGC
ON2	r(GUG AUA UGC)	**ON9**	GCA **M²**AT CAC
ON3	GCA TAT CAC	**ON10**	GCA TA**M²** CAC
ON4	r(GCA UAU CAC)	**ON11**	GTG A**M³**A TGC
ON5	GTG A**M¹**A TGC	**ON12**	GCA **M³**AT CAC
ON6	GCA **M¹**AT CAC	**ON13**	GCA TA**M³** CAC
ON7	GCA TA**M¹** CAC	—	—

#	Schematic structure	T_m, °C	ΔT_m,°C
ON6:ON1		37.5	+7.0
ON6:ON2		33.5	+4.0
ON7:ON1		29.5	−1.0
ON7:ON2		29.0	−0.5
ON9:ON1		37.0	+6.5
ON9:ON2		33.0	+3.5
ON10:ON1		38.0	+7.5
ON10:ON2		36.5	+7.0
ON12:ON1		40.5	+10.0
ON12:ON2		39.0	+9.5
ON13:ON1		37.0	+6.5
ON13:ON2		36.0	+6.5

[a] Melting temperatures were measured in a medium salt buffer using 1.0 μM concentrations of ONs.† The droplets represent PEPyc-LNA monomers **M¹** (blue), **M²** (red) and **M³** (green). ΔT_ms are calculated relative to T_m values of unmodified DNA:DNA (T_m = 30.5 °C) and DNA:RNA (T_m = 29.5 °C) duplexes.

the stability of the corresponding unmodified reference duplexes (Table 1). Incorporation of a single PEPyc-2′-amino-LNA modification **M¹–M³** into a mixed 9-mer DNA sequence results in high thermal stabilities against both complementary DNA and RNA (Table 1, ΔT_m values = +3.5 to +9.5 °C) except for 1-PEPyc containing **ON7** (ΔT_m = −0.5 to −1 °C). The resulting T_m values are much higher than those observed previously for 2′-*N*-(pyren-1-yl)methyl-2′-amino-LNA in analogous constructs, for which molecular modelling showed positioning of the pyrene units along the minor groove of the duplexes.[5] Thermal stabilities of the duplexes containing monomers **M¹–M³** are also much higher, than for previously reported mono-PEPyc-labeled 2′-amino-LNA nucleotides with alternative arrangement of phenyl- and pyrene-groups.[7a] The increased stabilities of the duplexes observed herein (Table 1) are therefore most likely caused by favorable interactions between the PEPyc units and functionalities in the minor groove. In the case of monomer **M¹** the melting temperatures varied significantly upon insertion of a modification in position 6 or 4. However, this effect was not observed for the corresponding ONs containing **M²** and **M³**. The most stable duplexes were formed by the 4-PEPyc modified **ON12** showing a ΔT_m value of +9.5 °C despite the bulkiness of the appended fluorophore. We propose that the geometry of the 4-PEPyc entity linked to a 2′-amino-LNA monomer is most favorably accommodated within the minor groove of the duplexes.

Next, we examined spectral and photophysical properties of the modified ONs and their duplexes with complementary unmodified DNA and RNA (Table 2). Hybridization results in increased fluorescense quantum yields of the dyes and in slight shifts of absorption and emission maxima. Compared to analogous constructs containing single incorporations of 2′-pyrenemethyl-[5] and 2′-pyrenecarbonyl-LNA,[7c] we observe remarkable shifts of absorption and emission maxima, and dramatical increases of fluorescence quantum yields and, hence, of fluorescence brightness values especially for monomer **M²**. These properties are of significant importance for fluorescent labels. Thus, controlled shifts of absorption/emission spectra allow application of monomers **M¹–M³** in multiplex formats and in living cells, while high Φ_F values lower the detection limit.

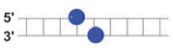
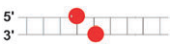
We furthermore studied +1 and −1 upstream zipper§ constructs containing modifications **M¹–M³** in both

Table 2 Representative spectroscopic and photophysical properties of **M¹–M³** modified ONs and duplexes with complementary DNA (**ON1**) and RNA (**ON2**)[a]

#	λ^{abs}_{max}, bands I–II/nm	λ^{fl}_{max}/nm	Φ_f	ε_{max}	FB
ON5	372, 395	455	0.33	38 900	12.8
ON5:ON1	370, 391	457	0.37	36 000	13.3
ON5:ON2	372, 392	459	0.42	36 800	15.5
ON8	305, 325	402, 430	0.55	51 900	28.5
ON8:ON1	303, 320	402, 431	0.72	46 000	33.8
ON8:ON2	304, 322	405, 433	0.81	45 500	36.9
ON11	351, 364	422	0.11	32 700	3.6
ON11:ON1	347, 360	424	0.16	30 000	4.8
ON11:ON2	350, 366	426	0.22	39 300	8.7

[a] λ^{abs}_{max}, λ^{fl}_{max}, Φ_f and ε_{max} are maxima of absorbance, fluorescence, fluorescence quantum yield and molar absorption coefficient, respectively; FB (fluorescence brightness) = $\Phi_f \times \varepsilon_{max}$. Φ_f values are measured by a relative method (ESI†) using 5-(pyrene-1-ylethynyl)-2′-deoxyuridine and 9,10-diphenylantracene as fluorescence standards. Φ_f values are found to be independent on the presence of oxygen in solutions of samples and all Φ_f values are therefore presented for non-degassed samples.

Table 3 Thermal denaturation studies, and spectroscopic and photophysical properties of modified double stranded DNA containing modifications **M¹–M³** in both complementary strands[a]

#	Schematic structure	Zipper[3]§	T_m/ ΔT_m °C	λ^{fl}_{max}/ nm	Φ_f
ON6:ON5		+1	64.0/ +16.8	455	0.52
ON7:ON5		−1	63.0/ +16.3	**512**	0.61
ON9:ON8		+1	60.0/ +14.8	430	0.87
ON10:ON8		−1	62.0/ +15.8	435	**0.92**
ON12:ON11		+1	61.0/ +15.3	430	0.35
ON13:ON11		−1	65.0/ +17.3	**490**	0.40

[a] For conditions see Tables 1–2. Δs are given per modification and calculated relative to the T_m value of unmodified double-stranded DNA (T_m = 30.5 °C).

complementary strands (Table 3). Previously it was shown that insertion of two pyrenylmethyl-2′-amino-LNA monomers in analogous constructs results in stable duplexes which display intensive fluorescence signals resulting from pyrene excimer formation.[5] In case of **M^1**–**M^3**, complementary pairs of modified ONs formed highly stable duplexes in both types of zipper constructs. Such high ΔT_m values imply effective stabilizing interactions between fluorophores, which is also confirmed by the appearance of an excimer signal in the fluorescence spectra for −1 upstream constructs containig 1- and 4-PEPyc-2′-amino-LNA, or strongly stabilizing interactions between fluorophores and the minor groove of the duplexes. An excimer band was not detected either for their +1 zippers or for the 2-PEPyc isomer. Similarly, an excimer signal was previously not observed neither for a +1 upstream zipper containing pyrenylmethyl-2′-amino-LNA monomers[5] nor for analogous constructs having different PEPyc-2′-amino-LNAs.[7a] A +1 zipper constitution thus seems to be unfavorable for positioning the dyes within a short distance as needed for excimer formation. As 1-, 2- and 4-PEPy fluorophores are known to display very short fluorescence lifetimes on DNA (~ 4–15 ns),[12] we propose that excimer formation depends on a preorganized structure of the duplex rather than reorganization of a structure not predisposed for excimer formation. We determined a significant increase in fluorescence quantum yields for all +1 zippers compared to their singly labeled analogues (Tables 2 and 3). We explain these data by generally short fluorescence lifetimes of PEPy dyes attached to DNA,[6b,12] and also by their precise positioning within duplexes typically resulting from their attachment to 2′-amino-LNA *via* a short linker.[7] The latter may result in decreased interaction of fluorophores with such quenchers of fluorescence as medium, DNA nucleobases and oxygen, and hence, may increase fluorescence quantum yields of the PEPy dyes.[7c]

In addition, we monitored hybridization of 1-, 2- and 4-PEPyc containing complementary ONs by changes in fluorescence colour of solutions (Fig. 1). As a result of high fluorescense brightness values, the formation of duplexes can be monitored by fluorescence change at sample concentrations down to approx. 1 nM using a fluorescense spectrometer and down to 50 nM by the nacked eye.

In conclusion, ONs containing novel PEPyc-LNA monomers **M^1**–**M^3** are demonstrated to be promising fluorescent building blocks due to significant improvement of pyrene's spectral and photophysical characteristics accompanied by increased affinity of hybridization. Upon attachment of phenylethynyl substituents, the absorption/emission maxima are drastically shifted, while fluorescence quantum yields are remarkably enhanced. Besides useful for diagnostic applications, we propose that excimer formation within 1- and 4-PEPyc labelled duplexes and the remarkable increase in fluorescence quantum yields of the 2-PEPyc isomer, makes the three PEPyc 2′-amino-LNA monomers useful as advanced signalling units to monitor self-assembly of nucleic acid nanostructures.

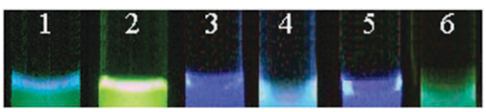


Fig. 1 Photographs showing fluorescence in medium salt buffer of the two complementary ONs before (vials 1, 3, 5) and after annealing (vials 2, 4, 6): **ON7**:**ON5** (vials 1–2); **ON10**:**ON8** (vials 3–4), **ON13**:**ON11** (vials 5–6) (0.25 μM solutions). Photographs were taken with a digital camera using a laboratory UV-lamp (λ_{ex} = 365 nm).

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Notes and references

§ The "zipper" nomenclature for defining arrangements of modified duplexes containing monomers in both complementary strands will be used in this article. An "*n* zipper" describes the arrangement of two modified nucleotides of interest, positioned in opposite strands of a DNA duplex. The number *n* indicates the distance in base pairs between the two nucleotides. If *n* is positive, the two modified monomers are positioned relatively toward the 5′-end of the two strands, and if *n* is negative, the two modified monomers are positioned relatively toward the 3′-end of the two strands.

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