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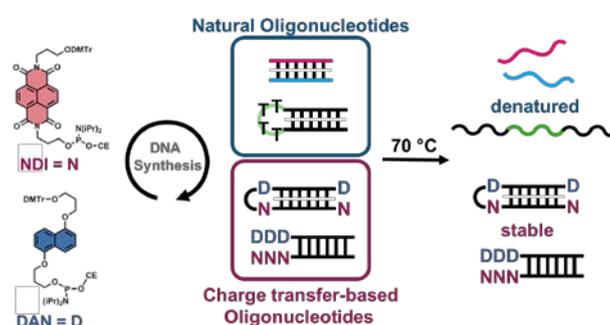
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Stabilization of DNA duplexes and hairpins by charge-transfer interactions using DAN:NDI pairs

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TOC



Abstract

Electron-rich 1,5-dialkoxynaphthalene (DAN) and electron-deficient 1,8,4,5-naphthalenetetracarboxylic diimide (NDI) are known to interact through the formation of charge-transfer complexes. The introduction of DAN and NDI into various DNA duplexes and hairpins was investigated by UV melting curve analysis. The positioning of the DAN:NDI pair was found to strongly influence the stability of DNA duplex and hairpins. In particular, while the introduction of one DAN/NDI pair in front of each other in the centre of a DNA duplex led to a decrease of the thermal stability ($\Delta T_m - 6\text{ }^\circ\text{C}$), the addition of a second pair restored or even increased the stability. In contrast, the introduction of DAN:NDI pairs at the end of a duplex always induced a strong stabilization (ΔT_m up to $+ 20\text{ }^\circ\text{C}$). Finally a DAN:NDI pair positioned in the loop of a hairpin induced a stronger stabilization than a T4-loop ($\Delta T_m + 10\text{ }^\circ\text{C}$). Based on charge-transfer interactions, the strong stabilizations observed allow the preparation of highly stabilized DNA nanostructures opening the way to numerous applications in nanotechnology.

Keywords. Oligonucleotides ; Charge-transfer ; DNA duplexes ; hairpins ; stability

Introduction

Modified oligonucleotides are commonly used for applications ranging from therapeutics,¹⁻³ diagnostics,^{4,5} to supramolecular chemistry and materials.⁶⁻⁸ Among the plethora of modifications that have been described, the replacement of nucleosides by polyaromatic compounds are of particular interest because the resulting π -stacking interactions allow the elaboration of supramolecular architectures having fluorescence or electron transfer properties.⁹ Pyrene,¹⁰⁻¹⁸ stilbene,^{19,20} anthraquinone,²¹ porphyrin²² phenanthrene^{17,23} or perylene²⁴⁻²⁶ are among the most studied aromatic modifications that have been incorporated into DNA structures to increase the thermal stability of the resulting duplexes through π - π interactions. Another possibility to increase the stability of DNA structures is to use charge-transfer (CT) interactions based on the recognition of relatively electron-rich and relatively electron-deficient polyaromatic derivatives²⁷ like the pyrene:perylene diimide²⁸⁻³⁰ or dialkoxynaphthalene (DAN):naphthalenetetracarboxylic diimide (NDI) pairs.^{31,32} The latter is able to self-assemble into alternating face-centered stacking of NDI and DAN moieties to produce foldamers and supramolecular arrangements.^{31,33-43}

Surprisingly, the use of NDI:DAN pairs as a surrogate for natural nucleoside pairs in DNA duplexes has been poorly studied. Iverson and coworkers reported the introduction of NDI and DAN derived from glycerol in the middle of a 12mer duplex. Four combinations having 3 DAN or NDI units were evaluated and among these the triad NDI:DAN:NDI led to a stabilization similar to three AT pairs.³¹ Our group was also recently interested in the incorporation of DNA:NDI pairs at the end of a thrombin binding aptamers that led to stabilizations up to 17 °C.³² This impressive result was attributed to the CT interaction combined with the solvophobic effect⁴⁴ as it has been demonstrated that in water NDI and DAN have an association constant that is 10- to 100-fold higher than their respective self-association.⁴⁴ In another study, we showed that in crystals of the aptamer:thrombin complex, NDI and DAN form an alternate stacking starting from a NDI close to the DNA.⁴⁵ These data confirmed molecular modeling studies that predicted alternate stacking.^{42,46}

Along with our previous works devoted to the synthesis of DAN-NDI-based supramolecular structures as an alternated sequence,⁴² or as a duplex formed by recognition of a hexaDAN and

a hexaNDI,⁴⁶ these results prompted us to investigate in depth the influence of DAN:NDI pairs as surrogate base pairs in DNA duplexes. We present here a comprehensive study, performed by thermal denaturation, of the effect of charge-transfer interactions induced by the incorporation of multiple DAN (D) and NDI (N) units into DNA duplexes and hairpins at different positions.

RESULTS AND DISCUSSION

Oligomers synthesis. Starting from the previously reported phosphoramidite derivatives **1** and **2**⁴² and Q-linker solid supports **3** and **4**³² (Fig. 1), DAN and NDI motifs were introduced into oligonucleotides at different positions using standard automated oligonucleotide synthesis on solid support according to phosphoramidite chemistry⁴⁷ (Table 1). NDI being sensitive to hydroxyl attacks in aqueous basic media, oligonucleotides containing this unit were synthesized with commercially available nucleosides protected with ultra-labile groups while the deprotection was performed with 7N methanolic ammonia solution. All oligonucleotides were analyzed by HPLC and characterised by MALDI-TOF mass spectrometry (see supporting information).

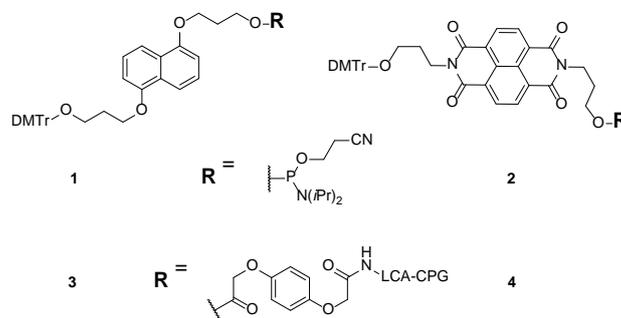


Figure 1. DAN and NDI phosphoramidites and solid supports. LCA-CPG: Long Chain Alkyl-Controlled Pore Glass.

Melting temperature studies. For the melting studies, the change in absorbance at 260 nm was measured with increasing and decreasing temperature (melting and annealing, respectively).⁴⁸ For each duplex and hairpin, a reversible transition was observed as reflected by the similar T_m value found for the melting and the annealing ($\Delta T_m < 0.5^\circ\text{C}$). The data are presented as the average curves expressed as percentage of hypochromicity for ease of comparison.

Sequences description. Two models were selected for the study, the first one being a dodecamer duplex (5'-GAC TGA CCT GCG-3'/5'-CGC AGG TCA GTC-3'),³¹ and the second a hexamer duplex corresponding to half of the dodecamer (5'-CCT GCG-3'/5'-CGC AGG-3'). DAN and NDI were introduced either at the centre (12mer) or extremities (12mer and 6mer) of duplexes and hairpins. (Table 1-3).

Melting temperature study of 12-mer duplexes and hairpins. The unmodified dodecamer duplex exhibited a T_m value of 56.8 °C (Table 1, entry 1), while the addition of a GC or AT base pair at the centre of the duplex increased the T_m by 4.7 and 3.0 °C respectively (Table 1, entries 2-3). The addition of two GC pairs led to a higher stabilization of 7.4 °C (entry 4). As a control the duplex with a propanediol linker in the middle of the sequences induced a strong destabilization of - 16.3 °C (Table 1, entry 5).

The introduction of a DAN:NDI pair in the centre of the sequence induced a significant destabilization ranging between - 3.6 and - 6.8 °C depending on the nature of the nucleobases adjacent to the DAN and NDI units (Table 1, entries 6-7 and Fig. 2). Interestingly the introduction of an additional DAN:NDI pair in the centre of the duplex (Table 1, entries 8-9) induced a small increase of the thermal stability (+ 0.8 to 6.2 °C) compared to the unmodified duplex that corresponds to a stabilization between one and two GC base pairs for the most stable. Again, neighbour effects were found to have a significant influence on stability. In contrast, the introduction of a DAN-NDI:NDI-DAN pair induced a strong destabilization of - 8.3 °C, (Table 1, entry 10) that could be explained by DAN:NDI intra-strand interactions resulting in a high duplex distortion.

For comparison, we studied the effect of one or two NDI:NDI and DAN:DAN pairs on the duplex stability (Fig. 2). The introduction of one or two NDI:NDI pair induced a destabilization of - 2.1 °C and - 3.8 °C respectively (entries 11 and 13) similar to one NDI:DAN. For the DAN:DAN pair, a very strong destabilization was observed with the introduction of one pair (- 22.5 °C, entry 12), while two pairs completely prevented the duplex formation (entry 14). These results are not surprising as it is known that DAN:DAN interaction is poor compared to NDI:NDI which is stabilised by π - π stacking.⁴⁴

Despite the destabilization observed for the different duplexes with one or two DAN:NDI pairs in the middle of the sequence (entries 6-10), the data suggest that there is still some stacking of

the DAN:NDI pairs with the adjacent DNA duplex, as a stronger destabilization was observed for the duplex with a propanediol in the middle of the duplex (entry 5, ΔT_m -16.3 °C).

Table 1. T_m value of duplexes of 12-mer model exhibiting D/N pairs in the sequence

Entry	Structure 5'-GAC TGA CCT GCG-3' 3'-CTG ACT GGA CGC-5'	T_m (°C)	ΔT_m (°C)
1		56.8±0.3	-
2		61.5±0.2	4.7
3		59.8±0.2	3.0
4		64.2±0.3	7.4
5		40.5±0.4	-16.3
6		50.0±0.5	-6.8
7		53.2±0.2	-3.6
8		57.6±0.2	0.8
9		63.0±0.2	6.2
10		48.5±0.2	-8.3
11		54.7±0.2	-2.1
12		34.3±0.2	-22.5
13		53.0±0.2	-3.8
14		NT	NA

The thermal studies were performed at 1 μ M for each strand in a 10 mM sodium phosphate buffer (pH 7) containing 100 mM NaCl.

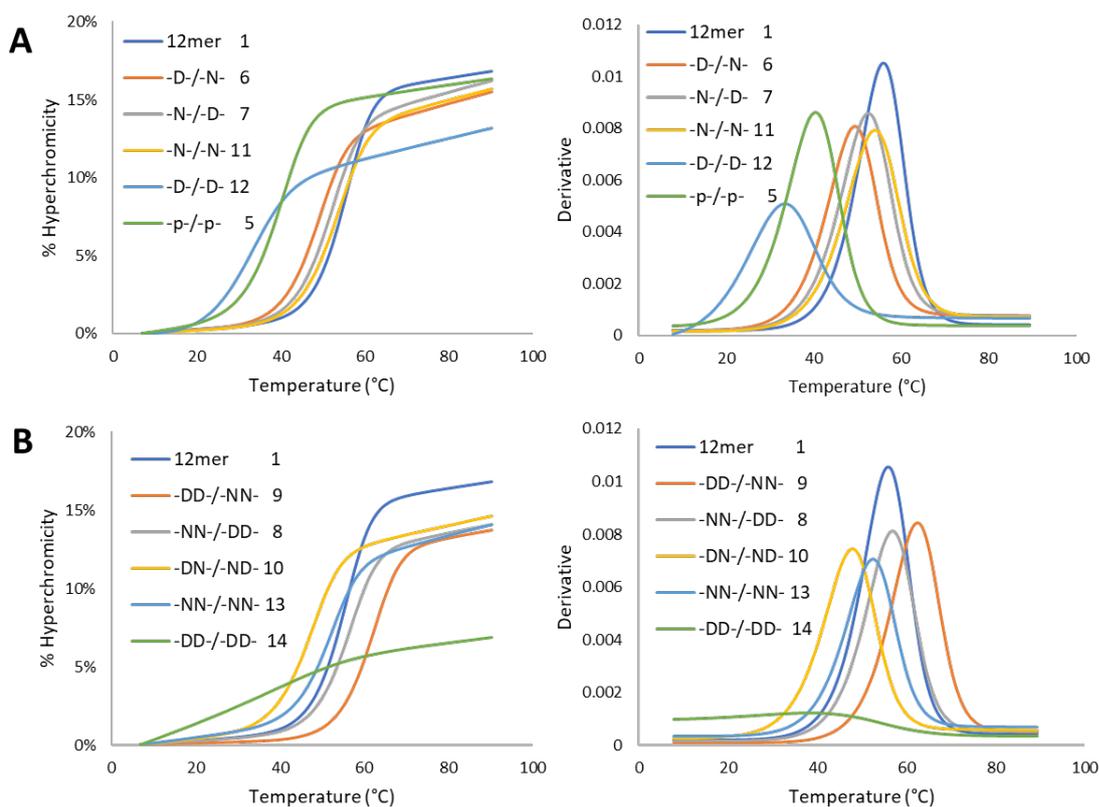


Figure 2. T_m curves of 12-mer duplexes with DAN and NDI at the centre of the sequence: (A) one pair, (B) two pairs. Numbers of the legend correspond to the entry of Table 1. Curve fits data were averaged from fits of two melting and two annealing curves.

When DAN and NDI motifs were introduced at the end of the sequences (Figs. 3 and S1), the addition of one DAN (Table 2, entries 15-16) or one NDI (Table , entries 17-18) at the 5', or 3'-end of the duplex led to slight stabilizations (+ 1.0-1.2 °C for DAN and + 1.5-4.1 °C for NDI). These results suggest that NDI is able to induce a stronger stacking with the duplex than DAN. In addition, while for DAN the nature of its neighbour is insignificant, we observed a better stabilization when DNI was close to a deoxyguanosine versus a deoxycytidine (Table 2, entries 17 and 18). The addition of a DAN:NDI pair (Table 2, entries 19-20) at the end of the sequence led to a high stabilization of 8.3 to 9.2 °C which is higher than the stabilization induced by two GC pairs. In this case, the T_m value was not influenced by the nature of the adjacent nucleosides. However, the hyperchromicity was much lower for the DAN:NDI duplex (D/N) than for the NDI:DAN duplex (N/D) analogue suggesting that stacking was weaker when NDI is close to a deoxycytidine. Interestingly, a similar low hyperchromicity was observed for the NDI:NDI duplex (N/N).

The duplex ending with a DAN:DAN pair (Table 2, entry 21) presented, as expected a low stabilization similar to that of only one DAN due to a low interaction between two DANs. In contrast, the duplex ending with a NDI:NDI pair exhibited a strong stabilization of + 7.5 °C only slightly lower than that for a DAN/NDI pair (Table 2, entry 22). This observation is surprising since Iverson previously reported, in an NMR study in D₂O, that the association constant of a NDI:NDI pair was 8-fold lower than a DAN:NDI pair.⁴⁴ This implies that when inserted into oligonucleotides π - π stacking interactions of an NDI:NDI ending-pair is almost as stabilising than a DAN:NDI pair stabilized by charge transfer interaction.

Finally, when a DAN:NDI pair was incorporated at each end of a duplex or in its hairpin analogue a very strong stabilizations was observed with T_m values above 85 °C ($\Delta T_m > 30$ °C, entries 23, 24). Due to these high T_m values we switched to a shorter model, corresponding to the half of the dodecamer, to investigate the effect of several introductions of DAN:NDI pairs in duplexes and hairpins.

Table 2. T_m value of duplexes and hairpins of 12-mer model exhibiting D/N pairs at the extremity.

Entry	Structure 5'-GAC TGA CCT GCG-3' 3'-CTG ACT GGA CGC-5'	T _m (°C)	ΔT_m (°C)
1		56.8±0.3	-
15		58.0±0.2	1.2
16		57.8±0.2	1.0
17		60.9±0.5	4.1
18		58.7±0.7	1.9
19		66.0±0.2	9.2
20		65.1±0.2	8.3
21		57.6±0.2	0.8
22		64.3±0.2	7.5
23		>85	> 30
24		>85	>30

The thermal denaturation studies were performed at 1 μ M for each strand in a 10 mM sodium phosphate buffer (pH 7) containing 100 mM NaCl.

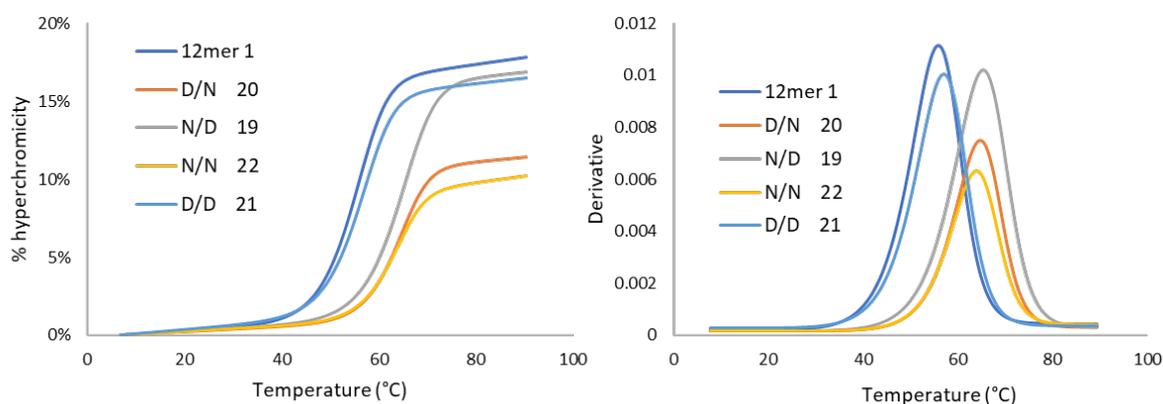


Figure 3. T_m curves of 12-mer duplexes with DAN and NDI at the end. Numbers of the legend correspond to the entry of Table 2. Curve fits data were averaged from fits of three denaturation curves.

Melting temperature study of 6-mer duplexes and hairpins. The unmodified hexamer duplex exhibited a T_m value of 23.5 °C and the addition of an AT or GC base pair increased the T_m by + 8.0 and + 14.1 °C respectively (Table 3, Fig. 4 and S3, entries 26 and 27). The addition of only one DAN or NDI induced a similar increase in stability (+ ~6 °C), more pronounced than in the dodecamer duplex (Table 3, entries 28-29). Similarly, the addition of a DAN:NDI pair at the end of the duplex resulted in a strong stabilization ($\Delta T_m = 20.9$ °C entry 30), higher than the corresponding duplex with an additional GC base pair. The further addition of DAN:NDI pairs leads to a continuous increase in stability until a plateau is reached with at three pairs ($\Delta T_m + 54$ °C, Table 3, entries 31-33). This stabilization is similar to that found for a 6-mer duplex terminated by four pyrene : perylenediimide pairs which shows an increase in stabilization from 51.5 to 53.5 °C.²⁹

Interestingly, the formation of DAN:NDI pairs can be monitored at 363 nm during the thermal denaturation experiments. Indeed, a slight hypochromicity was observed as the temperature increased (Fig. S2). The T_m values determined at 363 nm were slightly lower for two and three pairs but higher for four pairs (63.5 °C, 74.5 °C and > 84 °C respectively). This result suggests that the chimeric duplexes with two or three DAN:NDI pairs start melting from dissociation of the DAN:NDI pairs whereas the chimeric duplex with four DAN:NDI pairs starts melting from dissociation of the DNA base pairs.

The addition of a DAN:NDI pair at the other end of the duplex resulted in a further stabilization of +12 °C leading to a strong stabilization of the initial duplex ($\Delta T_m + 32.7$ °C, Table 3, entry 34). Note that the addition of two DAN:NDI pairs at one extremity is more stabilising than one at each end ($+\Delta T_m 8.8$ °C). This suggests that adjacent DAN:NDI pairs have a synergic effect on the stabilization.

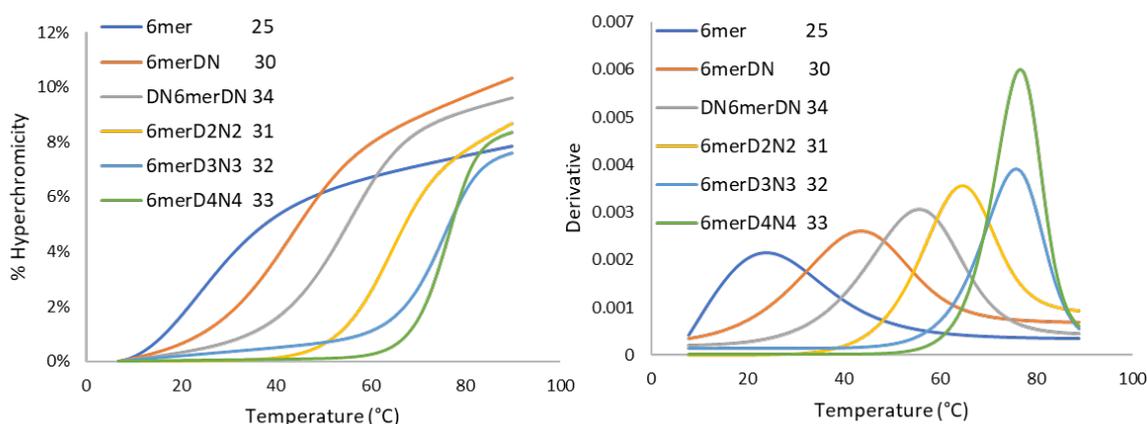
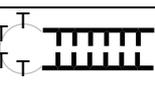


Figure 4. T_m curves of 6-mer duplexes with DAN and NDI at the end. Numbers of the legend correspond to the entry of Table 3. Curve fits data were averaged from fits of three denaturation curves.

Table 3. T_m value of 6-mer duplexes and hairpins.

Entry	Structure	T_m (°C)	ΔT_m (°C)
	5'-CCT GCG-3' 3'-GGA CGC-5'		
25		23.5±0.6	-
26	A	31.5±0.5	8.0
27	G	37.6±0.3	14.1
28	D	29.6±0.6	6.1
29	N	29.3±0.6	5.8
30	D	44.4±0.5	20.9

31		65.0±0.4	41.5
32		77.3±0.7	53.8
33		77.8±0.7	54.3
34		56.2±0.4	32.7
35		71.6±0.3	48.1
36		81.3±0.4	57.8
37		>85	> 61
38		>90	> 66

The thermal denaturation studies were performed at 1 μ M for each strand in a 10 mM sodium phosphate buffer (pH 7) containing 100 mM NaCl.

The effect of a DAN:NDI pair was then studied in hairpin structures (Fig. 5). When the duplex was closed by a T4 loop a T_m of 71.6 $^{\circ}$ C was observed corresponding to an increase of 48.1 $^{\circ}$ C (Table 3, entry 35). The analogue hairpin closed with a DAN-NDI loop showed an impressive T_m of 81.3 $^{\circ}$ C, a stabilization of 9.7 $^{\circ}$ C compared to the T4 loop hairpin and 57.8 $^{\circ}$ C compared to the unmodified duplex (Table 3, entry 36). The introduction of a DAN:NDI pair at the end of the hairpins induced a stabilization of more than 10 $^{\circ}$ C (Table 3, entries 37, 38), resulting in very stable structures.

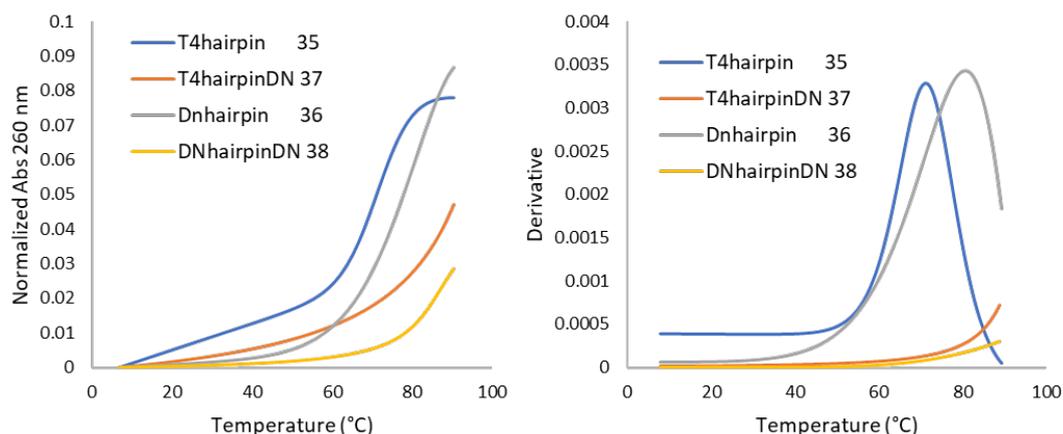


Figure 5. T_m curves of 6-mer hairpins with DAN and NDI. Numbers of the legend correspond to the entry of Table 3. Curve fits data were averaged from fits of three denaturation curves.

As complementary experiment, we studied by circular dichroism (CD) the 12-mer duplexes **6**, **9** having respectively one and two DAN/NDI pairs in the middle; the duplex **20** which has one pair at the end, and the hairpin **36** featuring a DAN:NDI pair in the loop. For duplexes **6** and **9** the CD spectra showed a characteristic spectrum of a B-DNA with a positive band around 280 nm and a negative band around 245 nm. Interestingly for duplex **20** and hairpin **36**, we observed an offset with a positive band at 275 nm and a negative band at 252 and the appearance of a positive band at 245 nm for the duplex and 240 nm for the hairpin (Fig. S4).

CONCLUSION

The introduction of DAN:NDI pairs to surrogate base pairs in DNA leads to weak stabilization or destabilization when they are inserted in the centres of duplexes. In contrast, when these pairs are introduced at the ends strong stabilizations are observed from + 9 °C for a dodecamer to up to + 21 °C for a hexamer. This phenomenon is even more important when several pairs are added at one of the extremities. In addition, a hairpin with a DAN:NDI loop and a DAN:NDI pair at the end exhibits a very stable structure with T_m values above 90 °C. It has been shown that in crystals of an aptamer closed by two NDIs and two DANs, DANs and NDIs form an alternate stacking starting from an NDI close to the DNA⁴⁵, so we can assume that a similar arrangement occurred in the current duplexes with DANs and NDIs at the end. These results highlight the importance of charge-transfer interactions for the stabilization of DNA duplexes.

DAN:NDI pairs thus appear as an efficient tool to stabilize short sequences that could be used for nanomaterials,⁴⁹ DNA origami,⁵⁰ decoy,⁵¹ or new PCR primers.⁵²

METHODS

The solid-supported synthesis of oligonucleotides was performed on a 394 ABI DNA synthesizer. All conventional CPG columns, reagents and solvents for DNA synthesis were purchased from Link© technologies, ChemGenes© Corporation and Biosolve© Chimie. Water came from a milli-Q water system from MilliporeSigma.

Oligonucleotides synthesis

The DAN, NDI phosphoramidites⁴² and their solid supports³² with a Q-linker were synthesized according to our previously reported procedures. The oligonucleotides were elongated from the corresponding supports on the DNA synthesizer, at 1 μ mol scale, according to standard phosphoramidite chemistry protocols using fast-labile protecting groups (tBu-Pac-dG). The detritylation step was performed for 65 s using 3 % TCA in CH₂Cl₂. For the coupling step, benzylmercaptotetrazole (0.3 M in anhydrous CH₃CN) was used as the activator with a concentration of 0.075 M in dry CH₃CN for commercially available phosphoramidites for 60 s and 0.075 M for DAN and NDI phosphoramidites for 3 min. The capping step was performed with phenoxyacetic anhydride using commercially available solutions (Cap A: phenoxyacetic anhydride:pyridine:THF 10:10:80 v/v/v; Cap B: 10% N-methylimidazole in THF) for 60 s. The oxidation step was performed with a standard, diluted iodine solution (0.1 M I₂, THF:pyridine:water 90:5:5, v/v/v) for 15 s.

Oligonucleotides deprotection and cleavage

The CPG beads were washed with anhydrous acetonitrile, then flushed with argon, the beads were then treated with a 7N MeOH/NH₃ solution on a thermoshaker for 4 hours at 55 °C. **WARNING:** Treatment with concentrated aqueous ammonia leads to degradation due to the opening of the imides of NDI. Ammonia was removed by evaporation on a speed vacuum and beads were washed with water.

Oligonucleotides purification, characterization and quantification

For purification, each synthesized oligonucleotide was dissolved in Milli-Q water and purified by reverse phase C₁₈ HPLC (Macherey-Nagel, Nucleodur 250 \times 10 mm, 5 μ m) on a Dionex Ultimate 3000 system with an automated injector with a detector UV DAD 3000 at a flow rate

of 4.5 mL/min. Analytical reverse phase C₁₈ HPLC (Macherey-Nagel, Nucleodur 75x4,6 mm, 5 μm) analyses were performed on a Dionex Ultimate 3000 system with a detector UV DAD 3000 at a flow rate of 1 mL/min, with a gradient of 1% to 40% acetonitrile with 50 mM of TEAAc over 20 minutes. MALDI-TOF mass spectra were recorded on Axima© Assurance (Kyoto, Japan) in negative mode, using anthranilic acid as a matrix in presence of ammonium citrate.

Thermal denaturation study (T_m)

Quantification of oligonucleotides was performed in 1 mL of water in a quartz UV cuvette (Hellma) with 1 cm of path-length, the oligonucleotides were heated for 15 min at 90 °C before measure of the absorbance at 260 nm. Melting temperature studies were performed at a concentration of 1 μM for each oligonucleotide in 1 mL of 10 mM NaH₂PO₄/Na₂HPO₄, 100 mM NaCl pH 7 buffer in a quartz UV cuvette (Hellma) with 1 cm of path-length. The melting and annealing transitions were performed twice at a slope of 0.5 °C/min from 3 °C to 90 °C. The curves showed reversible transitions. The T_m value corresponds to the average of the temperatures at the inflection point of each transition with a standard error around 0.5°C. Percentage of hyperchromicity (H%) was calculated as following:

$$H\% = \frac{Abs(t) - Abs(t_{mini})}{Abs(t)} \times 100$$

where Abs(t) is the absorbance at t temperature, Abs(t_{mini}) is the absorbance at the minimal temperature.

ASSOCIATED CONTENT

The Supporting Information is available free of charge at <https://>

Additional T_m curves, CD spectra of 6, 9, 20 and 36, raw data of T_ms, MALDI-Tof data and HPLC profiles of oligonucleotides.

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Conflicts of interest

There are no conflicts to declare.

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NDI is not significant compared to that of the oligonucleotides. As previously reported, DAN and NDI fluoresce alone, but the formation of a DAN:NDI pair induces a very strong inhibition of both fluorophores. Similarly, loss of fluorescence emission was observed for DAN or NDI single-stranded oligonucleotides. It was therefore not possible to monitor the formation of these modified duplexes by means of fluorescence.

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