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Synthesis of an Unlocked Nucleic Acid Terpyridine Monomer and Binding of Divalent Metal Ion in Nucleic Acid Duplexes

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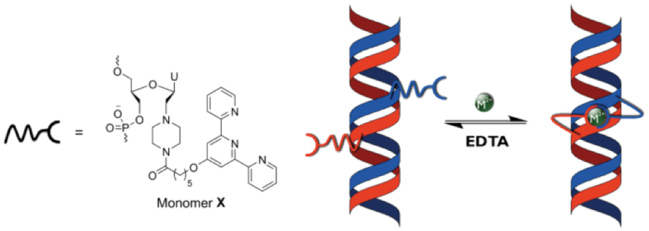

Herein we present the synthesis and thermal stability studies of modified oligonucleotides containing an unlocked nucleic acid (UNA) terpyridine monomer. Incorporation of this monomer into both strands of a DNA duplex allowed reversible thermal stability modulation upon addition or withdrawal of divalent metal ions. A likely explanation of this phenomenon is interstrand complexation between two terpyridine units and a metal ion. This system could be useful in the development of nanoscale devices based on DNA hybridization.

The unique properties of DNA regarding specific recognition and self-assembly offers researchers a significant tool for the construction of nanoscale devices.[1−5] Often, these devices assemble into structures incapable of further transitions. In order to expand the repertoire within DNA-based nanotechnology, it is important to investigate novel adjustable systems. This issue has previously been addressed, e.g., using photoreactive caged oligonucleotides (ONs) which are capable of duplex formation only after photoirradiation,[6] macromolecular folding constraints based on DNA duplex formation,[7,8] or terpyridinyl 2′-amino-LNA-modified oligonucleotides with the capability of reversibly modulating DNA duplex thermal stability in the presence or absence of divalent transition metal ions.[9]

Several examples of highly defined, self-assembled structures based on the interactions between transition metals and specific organic compounds are known.[10] In addition, interactions between ONs and transition metals have been extensively explored. Research has been published by numerous groups during the past decade dealing with DNA base pairing mediated by transition metals.[11−16] Even though metal-mediated base pairing does affect duplex stability, the technologies described were primarily aimed at constructing synthetic nanowires rather than modulating duplex stability. Furthermore, a number of modified nucleotide monomers have been prepared containing metal chelators directed toward the major or minor groove in a duplex, mostly aiming at artificial RNA cleavage.[17,18]

Modulation of duplex stability depending on the presence or absence of transition metals involving modified monomers containing metal chelators situated outside the DNA helix core has previously been reported.[9,19,20] Duplex stabilization has been achieved with modified nucleotide monomers placed in the central part of the ON[9,19] as well as with non-nucleotide chelators situated at the ON termini.[20] Kalek et al. used modified nucleosides based on N2′-substituted 2′-amino LNA[21] (locked nucleic acid) or 2′-amino-2′-deoxyuridine monomers functionalized with a terpyridine unit as metal chelator.[9] It was shown that metal chelators incorporated into both strands of a duplex in what was termed a "+1 zipper" arrangement,[22] in the presence of 1 equiv of a metal ion, generated dramatic effects on duplex thermal stability. Interestingly, reduced thermal stability was induced upon

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(22) The "zipper" nomenclature for naming different arrangements of modified monomers in the two strands of duplexes will be used in this article. An "*n* zipper" describes the arrangement of two modified nucleosides of interest, positioned in opposite strands of a DNA duplex. The number *n* indicates the distance in base pairs between the two nucleosides. 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′-ends of the two strands. The two X monomers in the duplex 5′-d(GTG AXA TGC):3′-d(CAC TAX ACG) are as an example positioned in a "+1 zipper" constitution.

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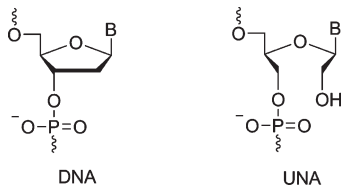
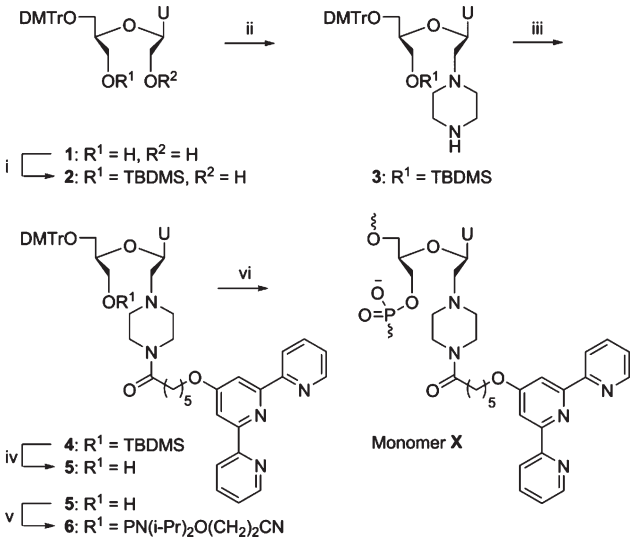


FIGURE 1. Structure of DNA and UNA (unlocked nucleic acid) monomers.

SCHEME 1. Synthetic Route to Monomer X[a]



[a]Key: (i) (a) BzCl, anhyd DCM, −78 °C, 1 h, (b) TBDMSCl, anhyd pyridine, rt, 19 h, (c) NaOH, MeOH, rt, 2.5 h (62%, three steps); (ii) (a) MsCl, anhyd pyridine, rt, 2 h, (b) piperazine, anhyd pyridine, 100 °C, 2 h (70%, two steps); (iii) 6-(2,2′:6′,2″-terpyridin-4′-yloxy)hexanoic acid, HATU, DMF, DIPEA, rt, 1.5 h (83%); (iv) TBAF, THF, rt, 24 h (88%); (v) NC-$(CH_2)_2$OP(Cl)N(i-Pr)$_2$, DCM, DIPEA, rt, 4.5 h (94%); (vi) DNA synthesizer.

addition of ethylenediamine tetraacetic acid (EDTA), which binds strongly to divalent metal ions rendering them unavailable for interstrand complexation.[9]

Herein we explore a nucleic acid modification based on unlocked nucleic acid (UNA, Figure 1)[23,24] for metal ion binding, namely 2′-deoxy-2′-piperazino-2′,3′-secouridine conjugated with a terpyridine unit (monomer **X**, Scheme 1). Our focus is on interstrand complexation involving two **X** monomers, one in each strand. Due to the high flexibility of the UNA monomer, interstrand complexation is expected to be less position dependent than with the previously studied system based on 2′-amino-LNA monomers having a locked furanose ring.[9] Furthermore, the synthesis of the UNA terpyridine monomer is reported herein and involves significantly fewer steps than synthesis of the 2′-amino-LNA terpyridine monomer.[9,21,25,26] Furthermore, in contrast to 2′-amino-LNA derivatives, the synthesis of A-, C-, G-, and U-UNA monomers all follow the same procedure, underlining that UNA monomers in general are easier to obtain and less expensive to use for functionalizing ONs than 2′-amino-LNA monomers. If the reduction of duplex stability caused by insertion of UNA monomers is found to be problematic, we envision that LNA monomers can be added to increase duplex stability.

Synthesis. 5′-*O*-4,4′-Dimethoxytrityluridine was subjected to oxidative cleavage of the vicinal diol function using $NaIO_4$ in 1,4-dioxane and water followed by $NaBH_4$ reduction[27] affording 2′,3′-seconucleoside derivative **1** as described earlier.[24,27,28] The next conversion involved three consecutive reactions. First benzoylation of the 2′-hydroxy group carried out at −78 °C using benzoyl chloride in a mixture of DCM and pyridine, then O3′-silylation using TBDMSCl in pyridine, and finally hydrolysis under basic condition of the O2′-benzoyl group to give derivative **2** in 62% yield. To install the piperazino group, the 2′-hydroxy group was mesylated using mesyl chloride in pyridine, whereupon this intermediate was treated with a large excess of piperazine affording compound **3** in 70% yield. Compound **3** thus obtained was treated with 6-(2,2′:6′,2″-terpyridin-4′-yloxy) hexanoic acid and 2-(7-aza-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) as coupling reagent to give compound **4** in 83% yield. Desilylation using TBAF in THF (to give compound **5**, 88% yield) followed by O3′-phophitylation by reaction with 2-cyanoethyl-*N*,*N*-diisopropylphosphoramidochlorodite afforded phosphoramidite **6** (94% yield) suitable for incorporation of monomer **X** into ONs on an automated DNA synthesizer (**ON1**-**ON6**; Table 1). This was accomplished using 1*H*-tetrazole as activator and 15 min coupling time. The stepwise coupling yield for the phosphoramidite **6** was ∼80% and ∼99% for unmodified DNA amidites (2 min coupling time). **ON1**−**ON4** were purified using RP-HPLC while **ON5** and **ON6** were purified by precipitation only. The composition of all ONs were confirmed by MALDI-MS analysis (Supporting Information) and their purity confirmed as >80% by ion-exchange HPLC.

Thermal Denaturation Studies. Initially, 9-mers **ON1**−**ON3** were synthesized to evaluate the effect on thermal stability of monomers **X** in "+1 and −1 zipper"[22] arrangements with and without metal ions added. As we discovered (vide infra) that the "−1 zipper" arrangement with 1 equiv of metal ion displayed a positive effect on thermal stability, we decided to also synthesize **ON4**−**ON6** to evaluate if a similar effect would be observed also for "−2 and −3 zipper" arrangements. Thermal denaturation studies were carried out for all duplexes with and without metal ions added (Table 1). Incorporation of monomer **X** in one strand only, with or without metal ions added, significantly reduces thermal duplex stability relative to the stability of the unmodified DNA control duplex (T_m = 28 °C). This decrease was expected from previous reports on ONs modified with UNA monomers.[24,27] When comparing the results for duplexes containing one **X** monomer with or without metal ions it is clear that addition of metal ions in some cases increases the melting temperature (**ON3**:DNA, **ON4**:DNA, **ON6**:DNA),

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TABLE 1. Thermal Denaturation Temperatures (T_m Values) of Duplexes Containing Monomer X Measured at Different Concentrations of Divalent Metal Ions[a]

			+ Ni^{2+}		+ Cu^{2+}		+ Zn^{2+}	
code	sequence	+ EDTA[b]	1 equiv[c]	5 equiv[c]	1 equiv[c]	5 equiv[c]	1 equiv[c]	5 equiv[c]
ON1 **DNA**	5′-d(GTG A**X**A TGC) 3′-d(CAC TĀT ACG)	16.5 (−11.5)	12.5 (−15.5)	13.5 (−14.5)	11.0 (−17.0)	9.5 (−18.5)	11.5 (−16.5)	11.5 (−16.5)
DNA **ON2**	5′-d(GTG ATA TGC) 3′-d(CAC TA**X** ACG)	16.5 (−11.5)	n.t.	13.0 (−15.0)	9.5 (−18.5)	9.0 (−19.0)	12.5 (−15.5)	13.0 (−15.0)
DNA **ON3**	5′-d(GTG ATA TGC) 3′-d(CAC **X**AT ACG)	n.t.	n.t.	n.t.	11.0 (−17.0)	11.5 (−16.5)	12.5 (−15.5)	12.0 (−16.0)
ON4 **DNA**	5′-d(GTG ATA **X**GC) 3′-d(CAC TAT ĀCG)	13.0 (−15.0)	n.t.	15.0 (−13.0)	14.0 (−14.0)	14.0 (−14.0)	14.0 (−14.0)	15.0 (−13.0)
ON5 **DNA**	5′-d(GTG AA**X**TGC) 3′-d(CAC TTĀ ACG)	13.0 (−15.0)	n.t.	10.5 (−17.5)	10.0 (−18.0)	10.0 (−18.0)	10.0 (−18.0)	10.0 (−18.0)
DNA **ON6**	5′-d(GTG AAT TGC) 3′-d(CAC **X**TA ACG)	11.0 (−17.0)	n.t.	14.0 (−14.0)	12.5 (−15.5)	12.0 (−16.0)	14.0 (−14.0)	13.5 (−14.5)
ON1 **ON2**	5′-d(GTG A**X**A TGC) 3′-d(CAC TĀ**X** ACG)	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	14.0 (−14.0)
ON1 **ON3**	5′-d(GTG A**X**A TGC) 3′-d(CAC **X**ĀT ACG)	14.0 (−14.0)	38.0 (+10.0)	n.t.	34.5 (+6.5)	n.t.	34.5 (+6.5)	18.0 (−10.0)
ON5 **ON6**	5′-d(GTG AA**X** TGC) 3′-d(CAC **X**TĀ ACG)	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
ON4 **ON3**	5′-d(GTG ATA **X**GC) 3′-d(CAC **X**AT ĀCG)	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.

[a]Thermal denaturation temperatures (T_m values, °C). ΔT_m values (°C) are shown in parentheses, i.e., the change in T_m value relative to the T_m values of the unmodified DNA:DNA duplex of the same sequence (28.0 °C). T_m values were measured as the maximum of the first derivatives of the melting curve (A_{260} vs temperature) recorded in medium salt buffer (100 mM NaCl, adjusted to pH 7.0 with 10 mM NaH_2PO_4/5 mM Na_2HPO_4), using 1 μM concentration of the two complementary strands; T_m values are averages of at least two measurements. The structure of monomer **X** is shown in Scheme 1. All samples were denatured for 15 min at 80 °C before measurement. [b][EDTA] = 0.1 mM. [c]M^{2+} equivalents are relative to duplex. n.t. = no transition.

while addition of metal ions in other cases lowers the melting temperatures (**ON1**:DNA, **ON2**:DNA, **ON5**:DNA). These data indicate a sequence dependence which might be of steric origin, i.e., caused by different possibilities of structurally encompassing the bulky terpyridine unit complexed to a metal ion. The duplexes with two modifications, one in each strand, generally show either no melting transition or strongly increased melting temperatures. This depends on the concentration of the metal ions, on the nature of the metal ion (Zn^{2+}, Cu^{2+}, or Ni^{2+}) and, most prominently, on the positioning of the monomers in the two strands. The monomers are positioned either in a "+1 zipper" (**ON1**:**ON2**), a "−1 zipper" (**ON1**:**ON3**), a "−2 zipper" (**ON5**:**ON6**), or a "−3 zipper" (**ON4**:**ON3**) constitution. Upon addition of 1 equiv of metal ion, a large thermal stabilization was induced exclusively in the "−1 zipper" arrangement. On the basis of previous[9] and all current results, the most plausible explanation of the large stabilization induced by addition of 1 equiv of metal ion is the joint coordination from both terpyridine moieties of **ON1** and **ON3** to the metal ion. No thermal stabilization was observed upon addition of 5 equiv of metal ions. We explain this by the likely scenario that both terpyridine units coordinate a metal ion which disables interstrand complexation. These data thus add support to the theory that the large thermal stability of the "−1 zipper" arrangement with 1 equiv of metal ion is caused by interstrand complexation. Despite the flexible structure of monomer **X**, our data indicate that interstrand coordination is impossible for the "+1, −2, or −3 zipper" arrangements. Additionally, the large thermal stability of "−1 zipper" (**ON1**:**ON3**) with 1 equiv of metal ion was reversed upon addition of 5 equiv of EDTA and denaturation for 15 min. The structure of the "−1 zipper" (**ON1**:**ON3**) duplex was studied using circular dichroism (CD) spectroscopy. CD spectra were recorded for the "−1 zipper" duplex in the presence (1 equiv) and absence of Ni^{2+} and compared with the spectrum obtained for the unmodified reference duplex (Figure 2). All three curves display similar overall characteristics which indicate that the structure of the "−1 zipper" duplex, even when interstrand complexed, is of a standard B

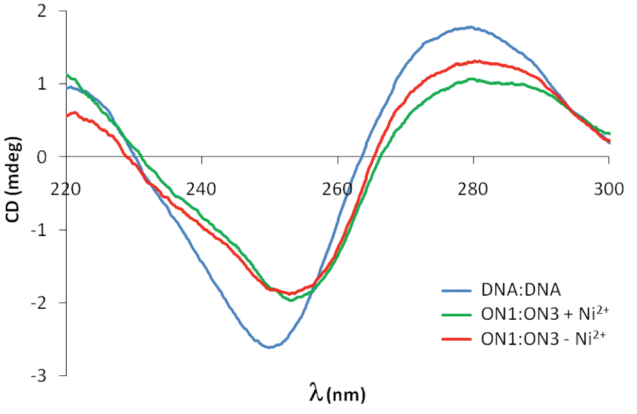

FIGURE 2. CD spectra of the **ON1**:**ON3** duplex in the presence (1 equiv) and absence of Ni^{2+} and the DNA:DNA reference duplex. The spectra were recorded at 10 °C using the same conditions as described for the thermal denaturation studies (Table 1).

TABLE 2. MALDI-MS Analysis of ON1-ON6

code	sequence	found m/z [M]	calcd m/z [M]
ON1	5′-d(GTG A**X**A TGC)	3169.7	3169.9
ON2	5′-d(GCA **X**$\overline{\text{A}}$T CAC)	3100.7	3098.9
ON3	5′-d(GCA $\overline{\text{T}}$A**X** CAC)	3098.5	3098.9
ON4	5′-d(GTG ATA $\overline{\textbf{X}}$GC)	3171.7	3169.9
ON5	5′-d(GTG AA**X** $\overline{\text{T}}$GC)	3170.7	3169.9
ON6	5′-d(GCA AT$\overline{\textbf{X}}$ CAC)	3099.0	3098.9

type. In conclusion, the UNA monomer introduced in this paper is easy to synthesize and possess the ability to reversibly change the thermal stability of a DNA duplex upon addition or withdrawal of divalent metal ions. Furthermore, two complementary ONs modified with monomer **X** in a "−1 zipper" arrangement can exist either as single strands (T_m = 14 °C, with EDTA) or as a duplex (T_m = 38 °C, with 1 equiv of Ni^{2+}) at room temperature. Therefore, the technology can be applied to switch between ordered and less ordered structures at room temperature.

Experimental Section

General Procedure for Synthesis and Purification of Oligonucleotides. Synthesis of oligonucleotides was performed in 0.2 μmol scale using an automated DNA synthesizer. Standard cycle procedures were applied for unmodified phosphoramidites using 0.45 M solution of 1*H*-tetrazole as activator. Stepwise coupling yields, as determined by a spectrophotometric DMT+ assay, were >99% for standard phosphoramidites and ~80% (15 min coupling time) for phosphoramidite **6**. Removal of the nucleobase protecting groups and cleavage from solid support was effected using standard conditions (32% aqueous ammonia for 12 h at 55 °C). Purification of oligonucleotides **ON1**−**ON4** was performed by DMT-ON RP-HPLC using a C18-column (10 μm, 300 mm × 7.8 mm). The following eluent system was used: eluent A, 95% 0.1 M Et_3NH_3 5% CH_3CN; eluent B, 25% 0.1 M $Et_3NH \cdot HCO_3$, 75% CH_3CN; gradient, 0−5 min isocratic hold of 100% eluent A followed by a linear gradient to 55% eluent B over 75 min at a flow rate of 1.0 mL/min. Fractions containing pure oligonucleotides were collected and evaporated on a speed-vac, followed by detritylation (80% aq AcOH, 20 min), precipitation (abs EtOH, −18 °C, 12 h) and washing with abs EtOH. The composition of oligonucleotides was verified by MALDI-MS analysis (Table 2), whereas the purity (>80%) was verified by ion-exchange HPLC (100 mm × 4.6 mm column size). The following eluent system was used: eluent A, 25 mM Tris-Cl, 1 mM EDTA, pH 8.0; eluent B, 1 M NaCl, gradient, 0−5 min isocratic hold of 95% eluent A followed by a linear gradient to 70% eluent B over 41 min at a flow rate of 0.75 mL/min.

General Procedure for Thermal Denaturation Studies. 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; terpyridine, 8.0.[29] Oligonucleotides (1.0 μM of each strand) were thoroughly mixed in T_m buffer (100 mM NaCl, pH 7.0 adjusted with 10 mM NaH_2PO_4/5 mM Na_2HPO_4 and 0.1 mM EDTA (EDTA was included only in experiments without divalent metal ions added)), denatured by heating at 85 °C for 15 min, and subsequently cooled to the starting temperature of the experiment. In experiments with divalent metal ions present, an appropriate amount of an aqueous solution of $CuCl_2$, $ZnCl_2$, or $NiSO_4$ (1 equiv; 10 μL, 5 equiv; 50 μL, 100 μM) was added after mixing the strands. Quartz optical cells with a path length of 1.0 cm were used. Thermal denaturation temperatures (T_m values/°C) were measured on a UV/vis spectrometer equipped with a Peltier temperature programmer and determined as the maximum of the first derivative of the thermal denaturation curve (A_{260} vs temperature). A temperature ramp of 1.0 °C/min was used in all experiments. Reported thermal denaturation temperatures are an average of two measurements within ±1.5 °C.

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Supporting Information Available: Detailed experimental procedures and characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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