Optimized DNA targeting using *N*,*N*-bis(2-pyridylmethyl)-β-alanyl 2′-amino-LNA†

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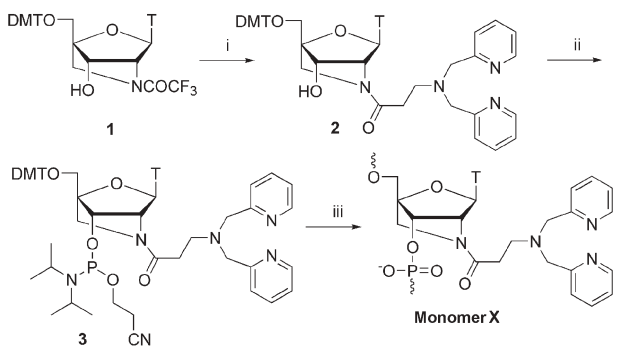
Incorporation of *N*,*N*-bis(2-pyridylmethyl)-β-alanyl 2′-amino-LNA (bipyridyl-functionalized 2′-amino locked nucleic acid) monomers into DNA strands enables high-affinity targeting of complementary DNA with excellent Watson–Crick selectivity in the presence of divalent metal ions. Positioning of bipyridyl-functionalized 2′-amino-LNA monomers in two complementary DNA strands in a "3′-end zipper" constitution allows modulation of duplex stability, *i.e.*, a strong stabilizing effect with one equivalent of divalent metal ion per bipyridyl pair, or a strong destabilizing effect with an excess of divalent metal ions.

Chemical modification of oligonucleotides (ONs) that structurally mimic RNA has enabled high-affinity targeting of RNA for diagnostic and therapeutic applications.[1,2] The development of ON analogues capable of high-affinity targeting of DNA has proven significantly more difficult. Fully or partially modified LNA (locked nucleic acid)[3–5] and pyrene-functionalized ONs[6–10] offer solutions to this challenge although their general applicability is hampered by, *e.g.*, the tendency to self-complementarity[5] or lack of sequence generality and selectivity of binding.[6,9,10] Continued efforts towards general and sequence selective high-affinity DNA targeting are therefore needed to improve the fidelity of DNA diagnostics and to enable *in vivo* targeting of DNA. We herein present an answer to this challenge employing ONs containing bipyridyl-functionalized 2′-amino-LNA monomers (**X**, Scheme 1).

We have recently reported that N-functionalized 2′-amino-LNA derivatives hybridize with complementary DNA or RNA with binding affinities as observed for LNA.[11–13] We therefore decided to study 2′-amino-LNA functionalized with metal-chelating moieties aiming at modulating hybridization processes and at making structurally well-defined metal arrays.§[14–22]

Phosphoramidite building block **3**, suitable for the incorporation of monomer **X** into ONs, was synthesized as shown in Scheme 1. Removal of the *N*-trifluoroacetyl group of 5′-*O*-dimethoxytrityl-2′-amino-LNA nucleoside **1**,[11,12] followed by treatment with *N*,*N*-bis(2-pyridylmethyl)-β-alanine[23] in the presence of EDC gave nucleoside **2** as a 1:2 mixture of rotamers.¶,‖ Subsequent phosphitylation of the 3′-hydroxy group afforded phosphoramidite **3**¶ (56% yield from **1**) which was used for standard automated solid-phase synthesis of **ON1**–**ON5** (Table 1; >80% purity by ion exchange HPLC; MALDI-MS data are shown in the caption below Table 1; the stepwise coupling yield of amidite **3** was ~98% using 1*H*-tetrazole as catalyst and 10 min coupling time).

The effect of the bipyridyl-functionalized 2′-amino-LNA monomer **X** on duplex stability was studied by UV thermal denaturation experiments (Tables 1 and 2). Single denaturation transitions with hyperchromicities >5% were observed for all duplexes involving modified ONs.† A single incorporation into a 9-mer mixed base sequence (Table 1, **ON1**–**ON3**) induced a strong increase in duplex stability towards the DNA complement (ΔT_m = +4 to +6 °C relative to the unmodified DNA:DNA reference duplex). These experiments were conducted in the presence of EDTA to remove traces of free divalent metal ions, and similar T_m values have been reported for other N-acylated 2′-amino-LNAs.[12] Identical denaturation experiments conducted in the presence of one equivalent or excess (10 equivalents) of Ni^{2+}, Cu^{2+} or Zn^{2+} ions induced further duplex stabilization against complementary DNA with metal ion induced *additional increases* in T_m values of +6 °C, +2 °C and +4/+5 °C, respectively. Addition of divalent metals ions had no effect on the stability of the reference DNA:DNA duplex (Table 1), duplexes formed between **ON1**, **ON2** or **ON3** and their RNA complements (data not shown), nor DNA:DNA duplexes containing a single *N*-benzoyl- or *N*-(1-adamantylmethyl)carbonyl-2′-amino-LNA monomer (data not shown). An additive effect on DNA binding was observed upon incorporation of two monomers **X** (**ON4** and **ON5**; overall increases in T_m values of approximately +23 °C, +13 °C and +20 °C in the presence of Ni^{2+}, Cu^{2+} or Zn^{2+}, respectively). The affinity-increase upon incorporation of monomer **X** occurs with excellent Watson–Crick base pairing selectivity (Table 2). To the



Scheme 1 *Reagents and conditions*: (i) (a) sat NH_3 in MeOH (ref. 12), (b) *N*,*N*-bis(2-pyridylmethyl)-β-alanine, EDC·HCl, CH_2Cl_2, rt (77%, two steps); (ii) 2-cyanoethyl *N*,*N*-diisopropylphosphoramidochloridite, DIPEA, CH_2Cl_2, rt (73%); (iii) DNA synthesizer. T = thymin-1-yl; DMT = 4,4′-dimethoxytrityl.

† Electronic supplementary information (ESI) available: table of synthesized 13-mer ONs and thermal denaturation studies thereof; Sample thermal denaturation curves; Procedure for molecular modelling; Modelling structures of **ON1**:DNA. See http://www.rsc.org/suppdata/cc/b4/b417101b/

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Table 1 9-mer ONs synthesized and thermal denaturation studies at different concentrations of divalent metal ions[a]

		T_m (ΔT_m)/°C						
			Ni^{2+}		Cu^{2+}		Zn^{2+}	
		+ EDTA	1 equiv.[b]	Excess[c]	1 equiv.[b]	Excess[c]	1 equiv.[b]	Excess[c]
DNA DNA	5′-GTG ATA TGC 3′-CAC TAT ACG	28 (ref)	28	28	28	28	28	28
ON1 DNA	5′-GTG A**X**A TGC 3′-CAC TAT ACG	34 (+6)	40 (+12)	40 (+12)	36 (+8)	36 (+8)	38 (+10)	39 (+11)
DNA **ON2**	5′-GTG ATA TGC 3′-CAC **X**AT ACG	32 (+4)	38 (+10)	38 (+10)	34 (+6)	34 (+6)	37 (+9)	37 (+9)
DNA **ON3**	5′-GTG ATA TGC 3′-CAC TA**X** ACG	33 (+5)	39 (+11)	40 (+12)	nd	nd	38 (+10)	38 (+10)
ON4 DNA	5′-GTG A**X**A **X**GC 3′-CAC TAT ACG	38 (+10)	51 (+23)	51 (+23)	41 (+13)	41 (+13)	48 (+20)	48 (+20)
DNA **ON5**	5′-GTG ATA TGC 3′-CAC **X**A**X** ACG	39 (+11)	51 (+23)	51 (+23)	41 (+13)	41 (+13)	48 (+20)	48 (+20)
ON1 **ON2**	5′-GTG A**X**A TGC 3′-CAC **X**AT ACG	34 (+6)	53 (+25)	24 (−4)	40 (+12)	20 (−8)	36 (+8)	31 (+3)
ON1 **ON3**	5′-GTG A**X**A TGC 3′-CAC TA**X** ACG	39 (+11)	47 (+19)	50 (+22)	42 (+14)	39 (+11)	46 (+18)	47 (+19)
ON4 **ON5**	5′-GTG A**X**A **X**GC 3′-CAC **X**A**X** ACG	47 (+19)	48 (+20)	20 (−8)	56 (+28)	<10	50 (+22)	37 (+9)

[a] Thermal denaturation temperatures [T_m values/°C (ΔT_m = change in T_m value calculated relative to the DNA:DNA reference duplex)] measured as the maximum of the first derivative of the melting curve (A_{260} *vs.* temperature) recorded in medium salt buffer ([Na^+] = 110 mM, [Cl^-] = 100 mM, pH 7.0 (NaH_2PO_4/Na_2HPO_4)), using 1.0 μM concentrations of the two complementary strands, and a micromolar extinction coefficient of 14.6 for monomer **X**; The T_m values are based on at least two independent denaturation experiments giving T_m values varying within ± 0.5 °C. A = adenin-9-yl DNA monomer, C = cytosin-1-yl DNA monomer, G = guanin-9-yl DNA monomer, T = thymin-1-yl DNA monomer; see Scheme 1 for structure of monomer **X**; "+EDTA" ~0.1 mM EDTA. [b] 1 equiv. refers to 1 equivalent of metal ion per **X** monomer for experiments with DNA complements, and to 0.5 equivalent of metal ion per **X** monomer for experiments with **ON1**:**ON2**, **ON1**:**ON3** or **ON4**:**ON5**. [c] [M^{2+}] = 10 μM; $NiSO_4 \cdot 6H_2O$, $CuCl_2 \cdot 2H_2O$ and $ZnCl_2$ were used; "nd" = not determined; MALDI-MS *m*/*z* ([M − H]$^-$; found/calc): **ON1**, 3031/3033; **ON2**, 2960/2962; **ON3**, 2956/2962; **ON4**, 3309/3313; **ON5**, 3242/3242.

Table 2 Melting temperatures (T_m values) towards matched and singly mismatched complementary DNA[a]

	DNA target (3′-CAC T**Y**T ACG)							
	+ EDTA				Ni^{2+} (10 μM)			
Y:	**A**	**T**	**G**	**C**	**A**	**T**	**G**	**C**
5′-GTG A**X**A TGC (**ON1**)	**34**	17	19	14	**40**	24	19	20
5′-GTG A**X**A **X**GC (**ON4**)	**38**	24	24	21	**51**	34	32	31

[a] T_m values for the matched sequences are shown in boldface type. See below Table 1 for details.

best of our knowledge, the approach introduced herein constitutes the most efficient way yet described of enhancing DNA binding affinity with preserved Watson–Crick selectivity.

Hybridization studies involving two complementary 9-mers each containing one or two **X** monomers revealed several interesting features. When positioned in a "1 + 1 3′-end zipper" constitution (duplex **ON1**:**ON2**), addition of one equivalent of Ni^{2+} ion leads to a remarkable increase in T_m value (+19 °C relative to **ON1**:**ON2** measured with EDTA; +25 °C relative to the reference DNA:DNA duplex). However, with an excess of Ni^{2+} ions, significant destabilization was observed, even relative to the reference DNA:DNA duplex. Similar experimental observations were made with a "2 + 2 3′-end zipper" constitution (**ON4**:**ON5**) and, to a variable degree, also with Cu^{2+} and Zn^{2+} ions (Scheme 1). Notably, no duplex formation above 10 °C was observed between **ON4** and **ON5** with excess of Cu^{2+} ions. Conversely, upon incorporation of two **X** monomers in a "1 + 1 5′-end zipper" constitution (**ON1**:**ON3**), stabilization corresponding to an additive effect of **X** monomers was induced with one equivalent as well as with an excess of divalent metal ions. A very similar affinity-enhancing effect on DNA binding and directional preference was observed upon incorporation of monomer **X** in a mixed 13-mer sequence.† A similar hybridization pattern for ONs modified with alkyl-linked terpyridine ligands at the 5′-end (or 3′-end) of duplexes in the presence of transition metals has been reported, but high-affinity targeting of DNA was not realized.[17]

Encouraged by the effect of monomer **X** on DNA binding we attempted to rationalize these results by simplified molecular modelling studies.† MMFF force field[24] calculations of 9-mer DNA-duplexes containing a single monomer **X** (**ON1**:DNA) or two monomers **X** arranged in a "1 + 1 5′-end zipper" constitution (**ON1**:**ON3**) and one Zn^{2+}-ion** indicate that the metal ion induced increased binding affinity towards DNA may arise from complexation of a divalent metal ion with the *N*,*N*-bis(2-pyridylmethyl)-β-alanyl ligand, the phosphate backbone of either of the two strands near the brim of the minor groove and solvent molecules/counter ions [Fig. 1 (left) and S1,† **ON1**:**ON3** and **ON1**:DNA, respectively].†† The lowest energy model structure of the 9-mer duplex where two monomers **X** are positioned in a "1 + 1 3′-end zipper" constitution [**ON1**:**ON2**; Fig. 1 (right)] containing a single Zn^{2+}-ion suggests formation of an interstrand

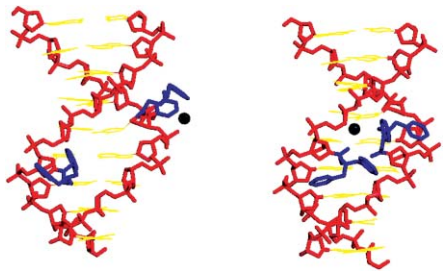

Fig. 1 Lowest energy structure of **ON1**:**ON3** ("1 + 1 5′-end zipper") including one Zn^{2+}-ion (to the left), and lowest energy structure of **ON1**:**ON2** ("1 + 1 3′-end zipper") including one Zn^{2+}-ion (to the right). For clarity hydrogens, sodium ions and bond orders have been omitted. Colouring scheme: nucleobases, yellow; sugar–phosphate backbone, red; *N*,*N*-bis(2-pyridylmethyl)-β-alanyl ligand, blue; Zn^{2+}-ion, black.

complex between a divalent metal ion and the *N*,*N*-bis(2-pyridylmethyl)-β-alanyl ligands of the two monomers **X**.‡‡ A similar interstrand complexation in the "1 + 1 5′-end zipper" constitution (**ON1**:**ON3**; see Fig. 1) seems precluded due to the spatial separation of the *N*,*N*-bis(2-pyridylmethyl)-β-alanyl ligands. Interestingly, the lowest energy structure of **ON1**:**ON2** including two Zn^{2+}-ions (results not shown) does not indicate formation of an interstrand complex but rather points towards complexation of divalent metal ions in a similar manner as described for **ON1**:DNA and **ON1**:**ON3**. Although molecular modelling does not provide compelling evidence for this, we speculate that the observed destabilization of duplexes containing monomers **X** in a "1 + 1 3′-end zipper" constitution (**ON1**:**ON2**) upon addition of excess divalent metal ions results from steric or electrostatic repulsion of two spatially close metal complexes. Additional biophysical studies have been initiated to further elucidate the structural basis of the observed effects.

A strategy for optimized high-affinity DNA targeting, using bipyridyl-functionalized 2′-amino-LNA in the presence of divalent metal ions, has been introduced. The observed metal-induced increases in duplex stability without compromising Watson–Crick base-pairing selectivity suggest bipyridyl-functionalized 2′-amino-LNA′s as candidates for optimal probes for DNA targeting. Furthermore, since positioning of bipyridyl-functionalized 2′-amino-LNA monomers in two complementary DNA strands allows engineering of duplex stability, probes with reduced (or no) tendency for self-complementarity can be designed.

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Notes and references

§ Conjugates of metal chelators have been studied for, *e.g.*, metal-mediated base pairing,[14–16] metal-mediated joining of the ends of complementary ONs,[17] modulation of DNA hairpin stability,[18] metal- and template-mediated ON assembly,[19] association of metal ions at internal positions of duplexes,[20,21] and RNA cleavage by synthetic metallonucleases.[22]

¶ ^{13}C NMR data for compound **2**: δ ($CDCl_3$, major rotamer) 172.0, 164.3, 158.7, 158.6, 158.3, 150.3, 148.9, 144.6, 137.0, 136.8, 135.7, 135.5, 134.6, 130.2, 130.1, 128.2, 128.0, 127.1, 124.0, 123.4, 122.4, 122.3, 113.3, 113.2, 110.3, 89.1, 87.6, 86.6, 70.1, 64.2, 60.1, 59.8, 55.3, 51.8, 51.6, 32.7, 12.7. ^{31}P NMR data for compound **3**: δ ($CDCl_3$) 150.6, 150.2, 149.0.

‖ The ratio between rotamers when bipyridyl-functionalized nucleotides are present in an ON is unknown. The denaturation curves display smooth single transitions (see ESI†).

** Zn^{2+} ions were used as a model divalent metal ion due to the availability of their parameters in the MMFF force field.[24] The Cu^{2+} and Ni^{2+} ions will show greater ligand field preferences in their coordination geometries compared with a Zn^{2+} ion. The consequent effect on binding modes and topology is a likely origin of the observed differences in the thermal denaturation temperatures.

†† The lowest energy structure of **ON1**:DNA (Fig. S2†) suggests that complexation of a divalent metal ion buried in the minor groove with the carbonyl functionality of the *N*,*N*-bis(2-pyridylmethyl)-β-alanyl ligand, nearby nucleobases or solvent molecules is an alternative explanation for duplex stabilization.

‡‡ The model structure does not yield a well-defined coordination complex but shows the two metal chelators and backbone phosphate oxygen atoms in proximity (approx. 3 Å) of the Zn^{2+} ion. It is clear that further refinement of the model necessitates that the double helix is not restrained, as was the case for our calculations. Also because of computing limitations, the possible role of chloride ions in the metal coordination spheres was not explored.

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