Improved DNA Clamps by Stacking to Adjacent Nucleobases

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Three or four aromatic rings interconnected by acetylene bridges form a stiff conjugated system with sufficient conformational freedom to make it useful to link together the two strands of a DNA clamp. Upon targeting a ssDNA, the conformational flexibility allows better stacking of the linker to the underlying non-planar base triplet in the formed triplex. This type of triplexes has a substantially higher thermal melting temperature which can be further improved by inserting locked nucleic acids (LNAs) in the *Hoogsteen* part of the clamp. An extremely high sensitivity to mismatches is observed in an octamer triplex when placed in the middle of the sequence.

Introduction. – The formation of a three-stranded, or triple-helical, nucleic acid structures in which the triplex-forming oligonucleotide (TFO) binds to a polypurine-polypyrimidine region of dsDNA in the major groove through specific H-bonds is described in several reviews [1]. Triple helices have potential applications in diagnosis, gene analysis, and therapy [2]. Triplex formation can be used to extract and isolate specific nucleotide sequences [3], inhibit DNA transcription and replication [4], generate site-directed mutations [5], cleave DNA [6], or induce homologous recombination [7].

It is possible to design a single oligonucleotide capable of forming both *Watson–Crick* and *Hoogsteen* H-bonds with a homopurine sequence [8]. After forming the *Watson–Crick* bonds, the oligonucleotides fold back on the duplex as an oligonucleotide clamp to make *Hoogsteen* base pairing to form a triplex structure [9]. This is easily formed when the *Watson–Crick* and *Hoogsteen* parts are connected *via* a hexaethylene glycol linker or an oligonucleotide sequence [10]. The triple-helical complex formed by such an oligonucleotide clamp is more stable than either the corresponding trimolecular triple helix or the double helix formed upon binding of the oligopyrimidine complement to the same oligopurine target [11]. Thermal denaturing studies reveal that triple helices based on a pyrimidine oligonucleotide clamp has a single melting transition which is several degrees higher than that observed for duplex alone, reflecting the all-or-none association of the oligonucleotide clamp to its single-stranded target. In contrast, a triple helix composed of two unlinked oligonucleotides and a single-stranded target undergoes two distinct melting transitions. The first occurs at low temperatures and represents the dissociation of the TFO from the duplex. The second transition represents melting of the duplex, which was essentially unchanged from that observed for the duplex itself [12]. This distinction in stability can be ascribed

to the linker that connects the duplex- and triplex-forming regions of the oligonucleotide [13]. Because of the formation of *Watson–Crick* and *Hoogsteen* bonds, the clamps afford a highly specific binding to the oligopurine target and are extremely sensitive to mismatches [12]. Various synthetic modifications of clamp-type oligonucleotides have been made to enhance the binding affinity and/or to improve specificity to homopurine target sequences *via* different linker types [14]. Fluorescence-resonance energy transfer [15], and excimer [16] and exciplex [17] formation have been recently used for the investigation of the thermodynamic and kinetic rules of the formed triplex [18], in addition to their application in disease diagnosis [19]. Insertion of psoralen at three different positions of the DNA clamp, generating cross-linking reactions, is used to arrest DNA replication in a better manner than standard antisense *Watson–Crick* oligomers [11][20].

Hybridization affinity of peptide nucleic acid (PNA) with complementary DNA is much stronger than the affinity of the corresponding DNA-DNA hybrid. Therefore, when two identical PNA sequences joined by a flexible hairpin linker is mixed with a complementary DNA target sequence, a highly stable PNA-DNA-PNA triplex hybrid is formed, which is called a PNA clamp. Chemical modification of the PNA clamp allows a variety of characteristics to be imparted to the target DNA in an essentially irreversible fashion [21]. Recently, PNA clamps were used as a quick, easy, and reliable way for accurate detection of epidermal growth factor receptor (EGFR) and KRAS gene mutations in DNA [22]. Also PNA clamps could be used as an artificial site-specific DNA-nicking system mimicking natural nickases [23]. Fluorescently labeled PNA clamps (fluorescein, rhodamine, Cy3) can be triplexed with plasmid DNA coding for reporter genes to allow direct, simultaneous determinations of gene delivery, intracellular localization, and gene expression inside live cells [24].

Locked nucleic acids (LNAs), which are conformationally restricted nucleotide analogs [25], reduce the flexibility of the ribose and increase the local organization of the phosphate backbone. This pre-organization is assumed to increase the strength of base-stacking interaction [26]. Several studies revealed that LNAs have greater affinity for complementary DNA and RNA sequences [27].

Here, we report the effect of two linkers, **M** and **N**, inserted in the middle of 16-mer oligopyrimidine, on the stability of clamp-type oligonucleotides when hybridized with target oligopurine single strand (*Fig. 1*). It is noteworthy to mention that the two linkers have the same molecular weight. We used an octamer target which has been previously used by *Häner* and co-workers [17][19]. This target sequence was compared with a sequence having dangling ends to both sides of the same target sequence. In addition, we investigated the effect of LNA when three cytosine bases in the *Hoogsteen* part were replaced by three LNA 5-methylcytosines ($^{Me}\mathbf{C}^{L}$).

Results and Discussion. – The synthesis of phosphoramidites **M** and **N** (*Fig. 1*) has previously been described, and they were used as linkers in alternate-strand triplexes [28]. Here, the amidites are used for automated oligonucleotide synthesis in the preparation of oligodeoxynucleotides **ON3**–**ON6** (*Table 1*). Extended coupling time (15 min) for the incorporation of **M** and **N** monomers gives coupling yields comparable to those of the unmodified nucleotide phosphoramidites. All modified oligodeoxynucleotides (ONs) were purified by reversed-phase HPLC, and their molecular weights

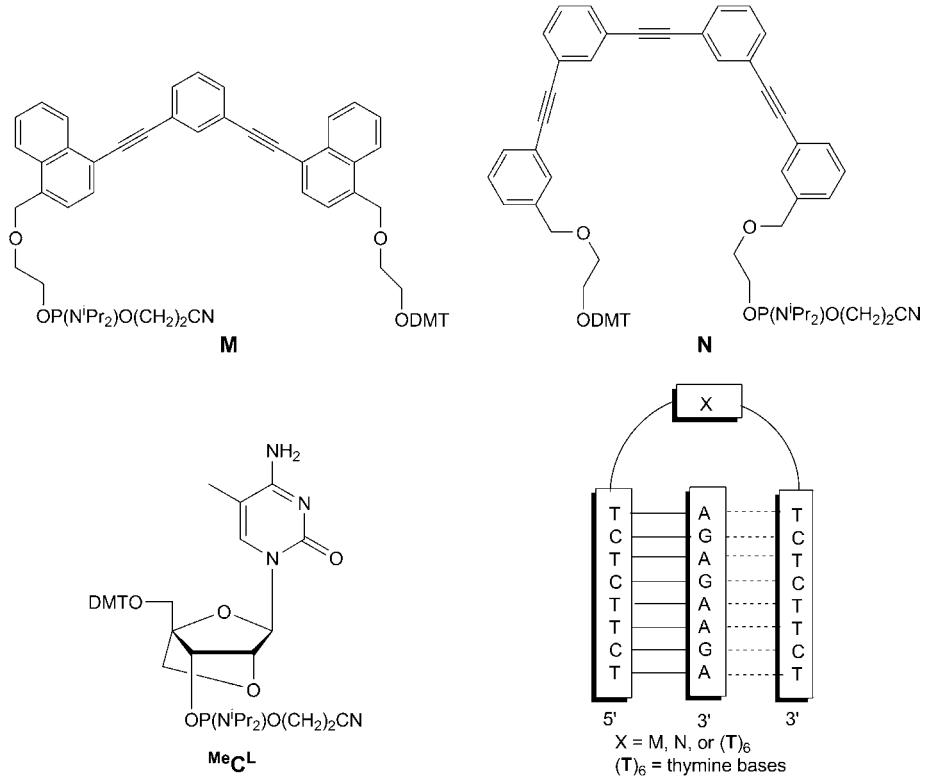

Fig. 1. *Schematic representation of triplex formation with clamp-type oligonucleotide between clamps* **ON1** – **ON6** *and target* **ON7**. **M** and **N** are phosphoramidites used as the modified linker, whereas (**T**)$_6$ are thymine bases used as a wild-type linker. LNA 5-methylcytosine (^{Me}**C**^{L}) phosphoramidite was used as LNA modification. Straight and dashed lines indicate *Watson–Crick* and *Hoogsteen* base pairing, respectively.

were confirmed by MALDI-TOF-MS analyses as reported in the *Exper. Part*. The purity of the final sequences was determined by ion-exchange HPLC (IE-HPLC) as 100%.

To compare with previously reported clamp stabilities, we used the homopurine **ON7** as the target sequence and the clamp **ON1** with six thymidines in the linker as the reference system. To illustrate the effect of LNA incorporation in the clamp, we replaced three cytosine bases in **ON1** with three LNA 5-methylcytosine bases (^{Me}**C**^{L}) to obtain **ON2**. Hybridization of the clamp oligonucleotides **ON1** – **ON6** with the homopurine strands **ON7** results in triple helix formation as illustrated in *Fig. 1*.

Thermal denaturation experiments showed a strong influence of the modifications on the stability of the triple helical complex at both pH 5.0 and 7.2 (*Table 1*). The clamps formed from **ON3**/**ON7** and **ON4**/**ON7** show thermal stability with ΔT_m values of 10.0 and 7.5°, respectively, at pH 5.0 compared to wild-type clamp **ON1**/**ON7**. Also, at pH 7.2, the two clamps show almost the same thermal stability though at lower temperature. When the hairpins contain a combination of both the linker **M** or **N**, and

Table 1. T_m *Data for Melting of Clamp-Type Oligonucleotide Triplexes Evaluated from UV Melting Curves* (λ 260 nm)

	Sequence	3′-AGAAGAGA-5′ (**ON7**)		3′-CTTAGAAGAGACTC-5′ (**ON8**)	
		T_m (ΔT_m) [°]		T_m (ΔT_m) [°]	
		pH 5	pH 7.2	pH 5	pH 7.2
ON1	5′-TCTTCTCT-**T**$_6$-TCTCTTCT-3′	44.0	23.0	37.5	< 10.0
ON2	5′-TCTTCTCT-**T**$_6$-T **^{Me}C^{L}** T **^{Me}C^{L}** TT **^{Me}C^{L}** T-3′	60.5 (16.5)	34.5 (11.5)	53.0 (15.5)	25.0 (> 15.0)
ON3	5′-TCTTCTCT**N**TCTCTTCT-3′	54.0 (10.0)	32.0 (9.0)	57.0 (19.5)	32.5 (> 22.5)
ON4	5′-TCTTCTCT**M**TCTCTTCT-3′	51.5 (7.5)	30.0 (7.0)	53.0 (15.5)	28.0 (> 18.0)
ON5	5′-TCTTCTCT**N**T **^{Me}C^{L}** T **^{Me}C^{L}** TT **^{Me}C^{L}** T-3′	67.5 (23.5)	43.0 (20.0)	68.0 (30.5)	42.5 (> 32.5)
ON6	5′-TCTTCTCT**M**T **^{Me}C^{L}** T **^{Me}C^{L}** TT **^{Me}C^{L}** T-3′	69.0 (25.0)	46.0 (23.0)	71.0 (33.5)	46.0 (> 36.0)

[a]) Conditions: oligomer concentration 1.0 μM for each strand, buffer: 20 mM sodium cacodylate, 100 mM NaCl, 10 mM $MgCl_2$, at pH 5.0 and 7.2. [b]) For the structures of **M**, **N**, and **^{Me}C^{L}**, see *Fig. 1*.

LNA replacements, extremely stable clamps are evolved. The clamp **ON5**/**ON7** is thermally stabilized by ΔT_m of 23.5°, while **ON6**/**ON7** is stabilized by ΔT_m of 25.0° at pH 5.0 when compared to wild-type **ON1**/**ON7**. At pH 7.2, **ON5**/**ON7** is stabilized by ΔT_m of 20.0° and **ON6**/**ON7** is stabilized by ΔT_m of 23.0° compared to wild-type **ON1**/**ON7**. In this way, LNA enhances the thermal stability of both **ON5**/**ON7** and **ON6**/**ON7** clamps by 13.5 – 17.5°.

To investigate the effect of dangling ends on the stability of the formed clamp, we decided to extend the target with three bases at both ends. The target **ON8** is thus used to measure the melting temperature of its corresponding triplexes under neutral and acidic conditions (*Table 1*). The measured thermal stability of **ON3** – **ON6**/**ON8** at pH 5.0, when compared to the wild-type clamp **ON1**/**ON8**, are stabilized by ΔT_m of 19.5, 15.5, 30.5, and 33.5°, respectively. An unexpected highly increased stability was observed at pH 7.2, as we obtained $>36.0°$ higher melting temperature when **ON6**/**ON8** is compared to wild-type clamp **ON1**/**ON8**. Destabilization of formed wild-type clamps **ON1**,**ON2**/**ON8** at both pH values, when compared to **ON1**,**ON2**/**ON7** is assumed to be due to steric clashes between thymidine nucleotides in the linker and in the dangling end of the target. It is interesting to note that the dangling end is not disturbing the stability, when **M** or **N** is used as the linker in the clamp systems **ON3** – **ON6**/**ON8**, when compared to the target **ON7**. The extra stability of **M**- or **N**-containing clamps is ascribed to π–π-stacking interactions between terminal thymidines and the conjugated aromatic linker system, which also can intercalate through the dangling end. Extra stabilization of the dangling end reflected by a higher melting temperature is due to the interaction between the dangling end and the **M** or **N** molecules at pH 5.0 when compared to the short target **ON7**. At both pH values and both target lengths, the **N**-containing clamp shows higher thermal stability than that containing **M** when LNA is not present, while the **M**-containing clamp shows higher thermal stability than that containing **N** when LNA is comprised in the clamp.

We considered it interesting to compare the sensitivity to mismatches, and we chose **ON1** and **ON6** as wild-type and modified clamps, respectively, where the latter with the linker **M** displayed the better thermal stability at pH 5.0 when compared to that with **N**. The short target was mutated either at the 5′-end next to the linker in the clamp or in the middle of the formed triple helix (*Table 2*). The wild-type **ON1** was able to discriminate the 5′-end mismatch slightly better than the modification **ON6**. However, when the mutation was in the middle of the modified clamp, **ON6** was better in discriminating the mismatch. The highest mismatch discrimination was obtained when guanine is replaced by thymine, where the wild-type clamp is destabilized by 34° and the modified clamp with no less than 37°. We assume that the clamp system reported here is one of the most sensitive systems to mismatches ever reported.

Molecular Modeling. To understand the capability of the clamp linker to undergo π–π stacking with adjacent nucleobases, and implicitly to interpret the T_m values observed, we performed molecular-modeling studies using a modified AMBER* force field to generate representative low-energy structures with the linker **M**. Because of possible rotation around the C≡C bond, four possible conformations should be considered. Two of those are shown in *Fig. 2* which indicates that the selected linker fits well for linking the pyrimidine duplex strand with the triplex-forming strand. The top views confirm a substantial π–π stacking of the aromatic linker with the underlying

Table 2. T_m *Data for Melting of Mismatched Clamp-Type Oligonucleotide Triplexes Evaluated from UV Melting Curves* (λ 260 nm) *at pH 5.0*

	Sequence	5′-TCTTCTCT-**T**$_6$-TCTCTTCT-3′ (**ON1**) T_m (ΔT_m) [°]	5′-TCTTCTCT**M**T ^{Me}**C**^{L}T ^{Me}**C**^{L} TT ^{Me}**C**^{L} T-3′ (**ON6**) T_m (ΔT_m) [°]
ON7	3′-AGAAGAGA-5′	44.0	69.0
ON9	3′-AGAAGAG<u>C</u>-5′	32.0 (− 12.0)	59.5 (− 9.5)
ON10	3′-AGAAGAG<u>T</u>-5′	33.5 (− 10.5)	62.0 (− 7.0)
ON11	3′-AGAAGAG<u>G</u>-5′	34.5 (− 9.5)	62.0 (− 7.0)
ON12	3′-AGAA<u>C</u>AGA-5′	18.5 (− 25.5)	40.0 (− 29.0)
ON13	3′-AGAA<u>T</u>AGA-5′	< 10.0 (> − 34.0)	32.0 (− 37.0)
ON14	3′-AGAA<u>A</u>AGA-5′	22.5 (− 21.5)	40.0 (− 29.0)

[a]) Conditions: oligomer concentration 1.0 μM for each strand, used buffer: 20 mM sodium cacodylate, 100 mM NaCl, 10 mM $MgCl_2$, pH 5.0. [b]) For the structures of **M** and ^{Me}**C**^{L}, see *Fig. 1*.

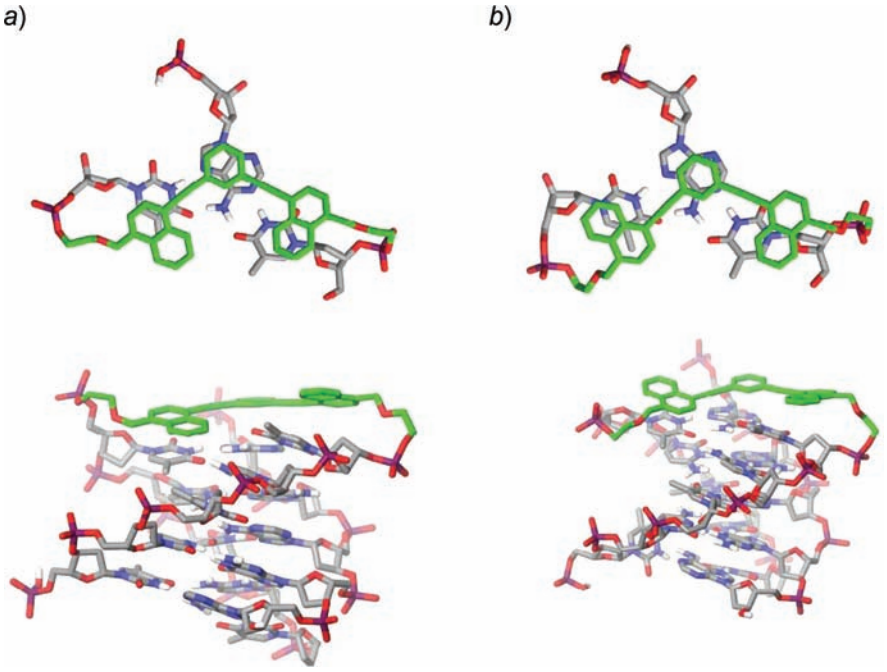

Fig. 2. *Top and side views from molecular modeling of a truncated triplex comprising* **M** *in the clamp. a*) *endo-endo*-conformation. *b*) *exo-endo*-conformation.

bases. The side views also confirm that the linker stiffness ensures a favorable structure that is most likely favored by an entropy effect. Furthermore, the side views displays a flexibility around the C≡C bonds that allows better stacking to a non-planar base triplet. This property has previously been used successfully when linking two triplex-forming oligonucleotides in an alternate-strand triplex [28][29].

Conclusions. – Thermally stable triplexes are formed upon targeting ssDNA at acidic and physiological condition (pH 7.2) with clamp-type oligonucleotides comprising non-nucleosidic linkers **M** or **N**. When LNA 5-methylcytosine bases replace three cytosine bases in the *Hoogsteen* base-pair part of the clamp, the triplex stability is even further improved as determined by increased melting temperatures up to 36° above the one observed for the corresponding wild-type clamp with a hexamer thymine linker. Molecular modeling can explain the improved binding affinity of **M**-containing clamp by π–π-stacking interactions between the terminal nucleobase and the linkers **M**. In addition to high thermal stability, the so-formed triplexes are also extremely sensitive to properly placed mismatches, and clamps comprising **M** or **N** are, therefore, considered candidates for DNA-disease recognition and therapy.

Experimental Part

General. Unmodified oligonucleotides were purchased from *Sigma* in scale of 0.2 μmol; the purity is checked by ion-exchange chromatography and MALDI-TOF mass spectrometry.

Oligonucleotide Synthesis. Modified oligonucleotides were synthesized in 0.2-μmol scale on 500-Å CPG supports using *Expedite™ Nucleic Acid Synthesis System* model *8909* from *Applied Biosystems.* Standard procedures were used for the coupling of commercial phosphoramidites, whereas modified phosphoramidites, **M** and **N**, were coupled with 1*H*-tetrazole in CH_2Cl_2 as an activator and an extended coupling time (15 min). In case of LNA phosphoramidite (LNA 5-methylcytosine monomer), the coupling-step time was 10 min. ONs were cleaved from the CPG support with 32% aq. $NH_3 \cdot H_2O$ (1 ml) and left at r.t. for 2 h, then deprotected at 55° overnight in case of non-nucleosidic linkers **M** and **N** incorporation, and directly deprotected at 55° overnight when only LNA is inserted. **ON2** was purified as DMT-on ONs, whereas **ON3** – **ON6** were purified as DMT-off by reversed-phase (RP) semi-prep. HPLC on a *Waters Xterra MS* C_{18}, 5 μm, 7.8 × 150 mm column with a *Waters Xterra MS* C_{18}, 5 μm, 7.8 × 10 mm chromatography system (buffer *A* (0.05M triethylammonium acetate (pH 7.4)) and buffer B (75% MeCN/25% buffer *A*). Flow rate 2.5 ml/min. Gradients: 2 min 100% *A*, linear gradient to 70% *B* in 38 min, linear gradient to 100% *B* in 7 min, and then 100% *A* in 10 min. **ON2** was submitted to DMT-deprotection with 80% aq. AcOH (100 μl) for 20 min, followed by addition of doubly filtered H_2O (100 μl), aq. AcONa (3M, 15 μl), and aq. $NaClO_4$ (5M, 15 μl) before precipitation from pure acetone (1 ml). **ON3** – **ON6** were submitted to direct precipitation by addition of doubly filtered H_2O (100 μl) and aq. $NaClO_4$ (5M, 15 μl), followed by addition of pure acetone (1 ml). All modified ODNs were confirmed by MALDI-TOF analysis on a *Ultraflex II* TOF/TOF system from *Bruker* (a MALDI-LIFT system) with HPA matrix (10 mg 3-hydroxypicolinic acid, in 50 mM ammonium citrate/70% MeCN). ODN found *m*/*z* (calc. *m*/*z*): **ON2** 6663.1 (6663.43), **ON3** 5310.6 (5303.8), **ON4** 5306.9 (5303.8), **ON5** 5432.3 (5426.8), and **ON6** 5420.1 (5426.8). The purity of the final clamps were found to be 100% when checked by ion-exchange chromatography using *La-Chrom* system from *Merck Hitachi* on *Dionex DNAPac Pa-100*, 4 × 250 mm anal. column.

Thermal Denaturation Studies. T_m Measurements were performed on a *Perkin–Elmer* UV/VIS spectrometer *Lambda 35* fitted with a *PTP-6* temp. programmer. **ON1** – **ON6** were first added to the buffer soln. consisting of sodium cacodylate (20 mM), NaCl (100 mM), and $MgCl_2$ (10 mM) at pH 5.0 or 7.2, followed by target strand at a concentration of 1.0 μM each. The solns. were heated to 80° then cooled down to 10°, and were then kept at this temp. for 30 min. The absorbance of the formed triplexes was measured at 260 nm from 10 to 80° with a heating rate of 1.0°/min. The melting temp., T_m, was determined as the maximum of the first derivative plots of the melting curves. All T_m values are confirmed by repetitive experiments.

Molecular Modelling. Molecular modelling was performed with Macro Model v9.1 from *Schrödinger*. All calculations were conducted with AMBER* force field and the GB/SA H_2O model. The dynamic simulations were performed with stochastic dynamics, a SHAKE algorithm to constrain bonds to H-atoms, time step of 1.5 fs, and simulation temp. of 300 K. Simulation for 0.5 ns with an equilibration time of 150 ps generated 250 structures, which were minimized using the PRCG method with convergence threshold of 0.05 kJ/mol. The minimized structures were examined with Xcluster from *Schrödinger*, and representative low-energy structures were selected. The starting triplex structure was generated with Insight II v97.2 from MSI, followed by incorporation of the linker **M**.

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