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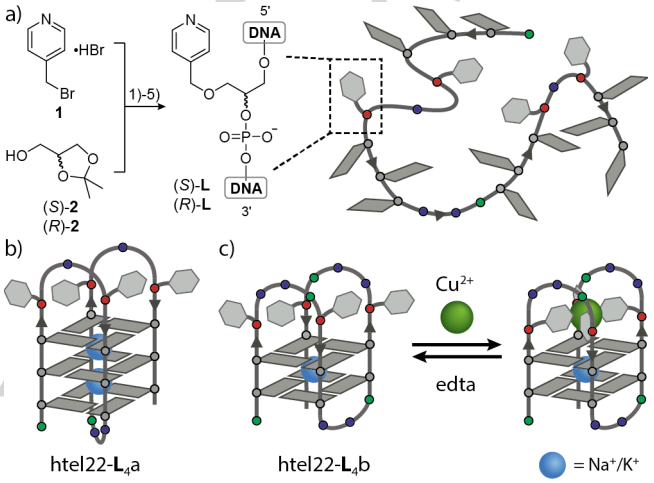
Copper-induced Topology Switching and Thrombin Inhibition with Telomeric DNA G-Quadruplexes

David M. Engelhard,[a] Julia Nowack,[a] and Guido H. Clever*[a]

Abstract: The topological diversity of DNA G-quadruplexes may play a crucial role in its biological function. Reversible control over a specific folding topology was achieved by the synthesis of a chiral, glycol-based pyridine ligand, and its fourfold incorporation into human telomeric DNA via solid-phase synthesis. Square-planar coordination to a Cu^{II} ion led to the formation of a highly stabilizing intramolecular metal-base tetrad, thus substituting one G-tetrad in the parent unimolecular G-quadruplex, as characterized by UV-Vis and circular dichroism (CD) spectroscopy, and thermal denaturation experiments (ΔT_m = +23 °C). Structural models with and without a bound Cu^{II} cation were derived from molecular dynamics (MD) simulations. For the tetrahymena telomeric repeat, Cu^{II}-triggered switching from a hybrid-dominated conformer mixture to an antiparallel topology was observed. Cu^{II}-dependent control over a protein–G-quadruplex interaction was shown for the thrombin–tba pair (tba = thrombin binding aptamer). Besides controlling protein function, this bio-hybrid system might find application in switchable DNAzymes as it allows for the construction of highly customizable coordination environments.

Since the discovery of the double-helical nature of DNA, several other DNA secondary structures such as triplexes, the four stranded i-motif and G-quadruplexes have been described.[1] The latter assemble from guanine-rich oligonucleotides to form stacked G-tetrads, held together by Hoogsteen base-pairing, π-π stacking and cations in the central channel.[2] Early studies investigated their role in inhibiting telomere elongation and oncogene expression, but in recent years, more and more potential implications of their *in vivo* formation throughout the whole genome have been discovered.[3,4] Furthermore, G-quadruplex folding was also found in RNA.[5]

Studying G-quadruplex interactions with biologically relevant compounds such as proteins or small-molecule drugs[3b,6] bears an inherent complexity due to the ability of G-quadruplexes to form a large variety of topologies. These arise mainly from variations of the loop sequence and position, strand molecularity (uni-, bi-, or tetramolecular) and the glycosidic torsion angle (*syn*/*anti* conformation of guanosines), leading to differently stacked G-tetrads and strand orientations.[7,8] This polymorphism motivated us and others to explore the construction of natural as well as biomimetic G-quadruplexes with a desired topology[9ab] or functionality such as switchable stability and control over protein binding.[10] Diagnostically exploitable properties such as fluorescence[11] or paramagnetism can be introduced by chemical modification.[12] Also, DNA nanotechnology may benefit from adjustable quadruplexes.[9,13] Furthermore, research in bioinorganic chemistry aiming at the combinatorial construction of novel coordination environments, the imitation of enzyme active centres and biological redox systems will be spurred by these developments.[14]



d)

DNA sequence (5′ → 3′)			T_m[°C]	+Cu^{II}	ΔT_m[°C]
htel22	AGGGTTAGGGTTAGGGTTAGGG		63.9	63.1	–0.8
htel22-L_4a	AGGGLTLGGGTTAGGGLTLGGG	(*S*)-L	62.1	64.1	2.0
htel22-L_4b	AGGLTTALGGTTAGGLTTALGG	(*R*)-L	18.1	33.0	14.9
		(*S*)-L	12.5	35.5	23.0
ttel24	TTGGGGTTGGGGTTGGGGTTGGGG				
ttel24-T_4	TTGGGTTTTGGGTTGGGTTTTGGG		54.6	54.8	0.2
ttel24-L_4	TTGGGLTTLGGGTTGGGLTTLGGG	(*S*)-L	32.8 50.8*	40.1 52.4*	7.3 1.6
tba15	GGTTGGTGTGGTTGG				
tba19-L_4	GGLTTLGGTGTGGLTTLGG	(*S*)-L	28.2	49.2	21.0
tba20-L_4	LGGTTGGLTGTLGGTTGGLT	(*S*)-L	12.6	34.4	21.8

Figure 1. a) Synthesis of chiral ligand (*R*/*S*)-**L** and incorporation into oligonucleotides; 1) NaH, DMF; 2) CF_3COOH, H_2O/THF; 3) DMT-Cl, DIPEA, DMAP; 4) CEP-Cl, DIPEA, THF; 5) automated solid-phase DNA synthesis. G-quadruplex topology of b) sequence htel22-L_4a and c) sequence htel22-L_4b with incorporation of Cu^{II} ions and removal of Cu^{II} with H_2EDTA^{2-} (edta); grey circles and tiles: guanosine, red circles: ligand **L**, green: adenosine, blue: thymine. d) Overview of sequences used in this work and their thermal stabilities measured in 10 mM lithium cacodylate buffer (pH 7.3) with 100 mM KCl and 4 μM of each strand (1 equiv. Cu^{II}). * second melting transition.

As a promising way to introduce such functionality, we suggest to combine the polymorphism of G-quadruplexes with the concept of metal-base pairing in which artificial ligands coordinate transition metals inside DNA. Numerous reports describe this phenomenon in duplex DNA,[15,16] but only few examples exist for other DNA secondary structures such as triplexes[17] and three-way junctions.[18,19] For G-quadruplexes, the few known examples include Hg^{II}-thymine interaction within a loop,[20] a chelated Ce^{IV} complex[21] and inversion of strand orientation upon metal ion binding to bipyridine units inserted into the loops of a bimolecular quadruplex.[22]

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Previously, we have reported the first metal-base tetrad and its incorporation into tetramolecular G-quadruplexes,[23] based on the square-planar coordination of four monodentate pyridine donors to divalent metals such as Cu^{II} and Ni^{II}. Although this system showed a remarkable increase in thermal stability upon copper binding, it was limited to 5'-modified tetramolecular G-quadruplexes, i.e. four identical DNA strands with the pyridine moiety attached to the 5'-end of each strand.

In order to pass these limitations we designed a new pyridine ligand, whose backbone architecture is similar to the glycol-based DNA (termed GNA) introduced by Meggers,[24] thus enabling incorporation of the ligand at any position during the DNA synthesis (5', 3' or internal). The synthesis of modified oligonucleotides containing ligand **L** either in the (*R*) or (*S*)-form proceeds in five steps (Supporting Information).

In a first design, the human telomeric sequence (repeating unit TTAGGG) was used to investigate the incorporation of several instances of the ligand in a single DNA strand, as most of the available structural data for G-quadruplexes are based on this sequence. Therefore, four natural nucleosides were substituted for the ligand **L** inside sequence htel22 in the following way: In strand htel22-$\mathbf{L}_4$a, two nucleosides of each edge-wise loop were exchanged; in strand htel22-$\mathbf{L}_4$b, one G-tetrad was substituted (Figure 1b and c). Despite the incorporation of four artificial nucleobases and the shortening of the backbone from three carbon atoms in the native sugar to only two in the glycol-based ligand **L,** the circular dichroism (CD) spectra (Figure 2b solid lines) and the thermal difference spectra (Supporting Information) showed that all modified oligonucleotides can form G-quadruplexes.

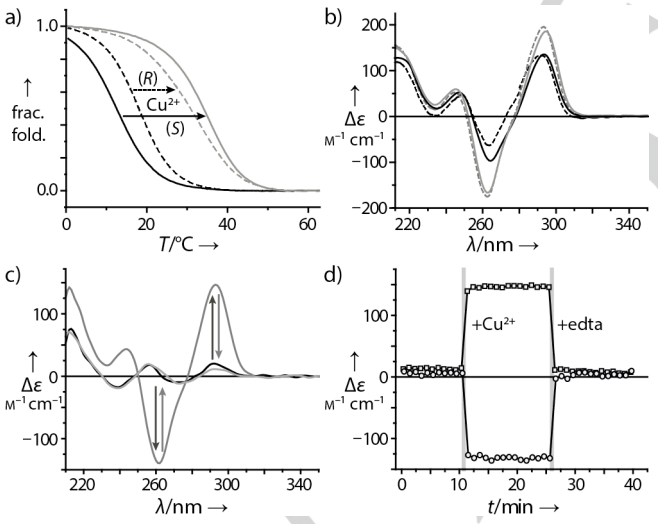

Figure 2. a) UV-VIS fraction-folded denaturation and b) CD spectra of htel22-$\mathbf{L}_4$b at 4 °C in the absence (black) or presence (grey) of Cu^{II} (ligand (*R*)-**L** dotted line, (*S*)-**L** solid line); c) CD spectra of htel22-$\mathbf{L}_4$b (*S*)-**L** at 25 °C before (black solid), right after addition of 1 equiv. Cu^{II} (dark grey) and after addition of 1.2 equiv. edta (light grey); d) time course of the same experiment (squares 293 nm, circles 262 nm). All experiments were measured in 10 mM lithium cacodylate buffer (pH 7.3), 100 mM KCl and with 4 μM of each DNA strand.

First, we investigated strand htel22-$\mathbf{L}_4$a, in which all G-tetrads remain in their wild-type state but four of the loop nucleosides are exchanged for the ligand (*S*)-**L** (Figure 1b). The melting temperatures in Na^+ and K^+ containing solutions proved to be only slightly lower compared to those of the reference strand htel22, indicating that the ligand does not influence the stability to a great extent. Furthermore, the CD spectra revealed the same polymorphism as htel22 in the Na^+ as compared to the K^+ solutions. In the Na^+ solution, the CD spectrum was identical to a purely antiparallel arrangement, whereas in K^+ solution, the mixed spectrum typical of hybrid structures was observable (Supporting Information). However, the strand showed no noteworthy effect upon addition of a $CuSO_4$ solution. From these findings we conclude that due to the close proximity of the ligands in the sequence (separated by only one nucleotide), the ligands cannot coordinate to the copper ion.

For strand htel22-$\mathbf{L}_4$b, the CD spectrum reveals two positive bands at 246 nm and 293 nm and one negative band at 263 nm, which is typical for an antiparallel arrangement of the interacting DNA strand sections (Figure 2b).[25] As four guanosines have been replaced in the modified strand by four ligands, the resulting G-quadruplex can only form two stacked G-tetrads. The CD curve closely resembles the one found for the two G-quartet chair-type unimolecular G-quadruplex formed by the *B. mori* telomeric repeat and the thrombin-binding aptamer, so that an identical folding behavior can be inferred for the modified strands (Figure 1c).[26] This assumption is supported by molecular dynamics (MD, GROMACS) simulations, where the basket-type topology with two lateral and one diagonal loop showed to be less stable than the chair-type folding pattern (see the Supporting Information).[27] Interestingly, incorporation of the two enantiomers of the ligand was found to have a substantial impact on the melting profiles of the diastereomeric sequences: While the strand with four (*R*)-**L** ligands has a melting temperature of T_m = 18.1 °C, the (*S*)-**L** form shows a lower T_m = 12.5 °C under the same experimental conditions. It is likely that, in contrast to ligand (*S*)-**L**, ligand (*R*)-**L** prefers to form attractive π-π-interactions with the top G-quartet guanine nucleobases, which is in accordance with the molecular models and their relative energies obtained from MD simulations of the (*R*)-**L** and (*S*)-**L** containing quadruplexes (Figure 3 and Supporting Information).

Upon addition of 1 equiv. $CuSO_4$ to the quadruplex solution, a significant increase in thermodynamic stability could be observed. The melting temperatures increased by ΔT_m = 23.0 °C and 15.0 °C for the (*S*)-**L** and the (*R*)-**L** form, respectively (Figure 2a). For all transitions, the heating and cooling profiles are superimposable. Remarkably, the quadruplex that is less stable without metal ((*S*)-**L**) experiences a higher stabilization in presence of the metal as compared to the quadruplex containing (*R*)-**L** (Figure 1a). A deeper investigation of the system allowed us to deduce that the overall topology of the G-quadruplex does not change upon Cu^{II} binding and that intermolecular coordination does not play any role: Concomitant with the increase in thermodynamic stability, the CD spectra reveal that once the Cu^{II} is added, all bands increase in intensity, but no significant change of their overall shape occurs (Figure 2b). Metal binding is quite fast: No further changes are observable in the CD spectra 5 minutes after copper addition at 4 °C. At 25 °C,

the equilibrium is reached already within 1 min (Figure 2c). Furthermore, addition of copper ions can induce G-quadruplex formation from the unfolded strand. To demonstrate this, time-dependant CD spectra were measured at 25 °C, monitoring the rapid folding of the denatured single strand into the copper-containing G-quadruplex upon addition of 1 equiv. of Cu^{II} (Figure 2d). Immediately after addition of the metal, the system jumps into the fully annealed G-quadruplex structure. Addition of edta removes the copper from the oligonucleotide, which leads to a fast denaturation of the quadruplex topology. A similar phenomenon of metal directed folding and edta unfolding was reported for a copper complex binding to htel22.[28]

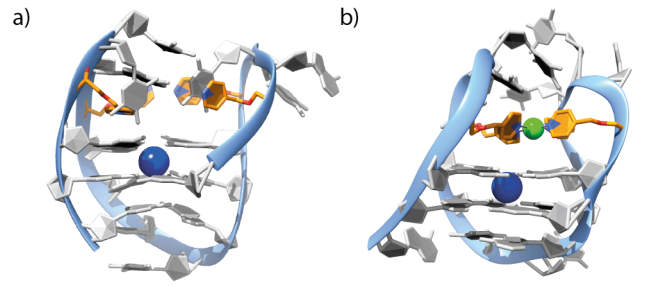

Figure 3. Chair-type models for htel22-$\mathbf{L}_4$b (*S*)-**L** quadruplex derived from the MD simulations a) without and b) with coordinated Cu^{II}. The phosphate backbone is represented as a blue ribbon, K^+ and Cu^{II} as blue and green spheres, respectively; the ligand **L** is highlighted in orange. Hydrogen atoms have been omitted for clarity.

We next devised a system based on the tetrahymena telomeric repeat $(TTGGGG)_4$, in which four guanosines are replaced by ligand (*S*)-**L**. The loop lengths between the ligands were chosen as a compromise between the flexibility in strand htel22-$\mathbf{L}_4$b and the strain in htel22-$\mathbf{L}_4$a. The native strand is known to adopt a hybrid structure in Na^+ solution;[29] no NMR or crystal structures are available for the K^+ form. As detailed in the Supporting Information, a combination of CD, thermal denaturation, and ^{1}H-NMR experiments, as well as porphyrin binding indicate that in K^+ solution, ttel24-$\mathbf{L}_4$ forms a mixture of discrete conformers. Based on topological considerations, none of them can coordinate a copper ion, as the two ligand-containing loops should be positioned on opposite sides of the G-quadruplex stem (Figure 4b, left).

When Cu^{II} is added to the G-quadruplex solution, the CD spectrum gradually changes: The bands at 240 nm and 263 nm are nearly inverted, whereas the band at 293 nm intensifies (Figure 4a). This process is quite slow at 4 °C (equilibrium after ~7h) and slightly faster at 25 °C (equilibrium after 2 h), which is in pronounced contrast to the copper coordination kinetics of htel22-$\mathbf{L}_4$b (equilibrium after ~5 min at 4 °C, <1 min at 25 °C). The slow but distinct changes indicate a change of topology induced by the copper ion,[30] resulting in a CD spectrum characteristic of a group 3 G-quadruplex.[25] The most reasonable topology of the resulting structure is that of an antiparallel G-quadruplex with three lateral loops, one TT and two **L**TT**L** which could easily accommodate the square planar copper-pyridine complex formed by the four ligands and one copper ion (Figure 4b, middle).[26] The similarity between the CD spectra of the copper bound htel22-$\mathbf{L}_4$b and ttel24-$\mathbf{L}_4$ G-quadruplexes further supports this assumption. Additionally, the thermal denaturation profile considerably sharpens with an increase of the low temperature transition by 7 °C. Apparently, the copper ion can induce a topological change within the G-quadruplex structure from a mixture of conformers to an antiparallel folding. This is further supported by the rapid removal of Cu^{II} by edta, yielding a metastable intermediate according to the CD spectral changes (Figure 4b, right). The original state is reformed after a de-/renaturation cycle (Supporting Information).

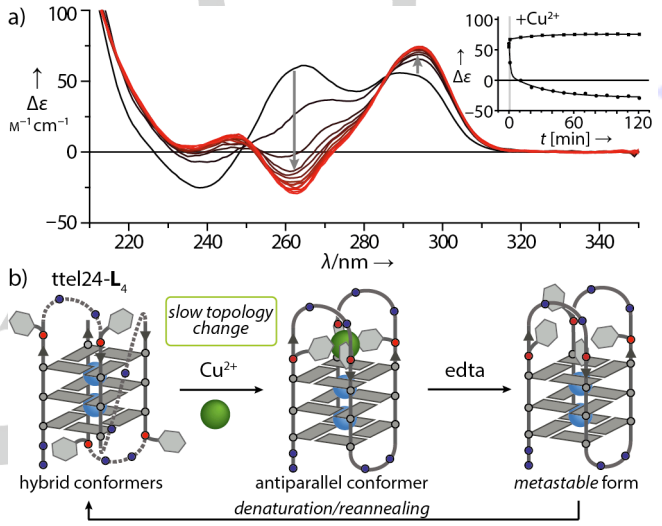

Figure 4. a) Time dependent CD spectra of ttel24-$\mathbf{L}_4$ (black line) at 25 °C upon addition of 2 equiv. Cu^{II} (red lines, 10 min between each spectrum). The inset shows the time-dependant CD signals at 294 nm (squares) and 262 nm (circles). b) Proposed topology change of ttel24-$\mathbf{L}_4$ after Cu^{II}/edta addition.

To test whether pyridine-modification interferes with protein interaction of the known thrombin binding aptamer (tba), four ligands (*S*)-**L** were introduced into its 15 nt long sequence in addition to the two G-tetrads.[32] Ligands were placed either on the same side as the two TT loops (tba19-$\mathbf{L}_4$) or opposite (tba20-$\mathbf{L}_4$) in the antiparallel chair-like G-quadruplex (Figure 1d).[33] According to CD and thermal denaturation experiments, both ligand-modified sequences fold into stable antiparallel G-quadruplexes in the presence of potassium and copper ions, similar to the unmodified thrombin binding aptamer (Supporting Information). In contrast to tba19-$\mathbf{L}_4$, tba20-$\mathbf{L}_4$ is able to slow down the thrombin catalyzed proteolysis of fibrinogen only in the presence of a square-planar coordinating metal ion such as Cu^{II} (Figure 5) or Ni^{II} (Supporting Information). Taking into account the known binding mode of the aptamer to its target protein,[34] the obtained results suggest that protein binding is precluded when the metal tetrad points towards the protein surface, whereas it is allowed when the metal site is oriented in the opposite direction, away from the protein.

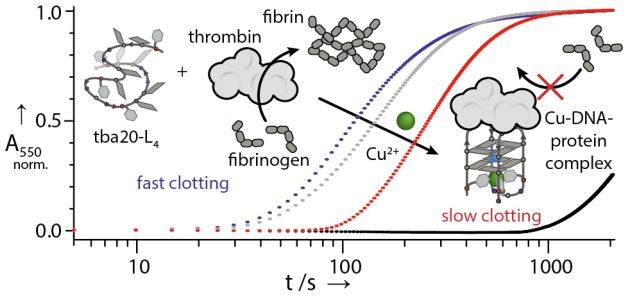

Figure 5. Time dependent absorption change monitored at 550 nm and 30 °C as a result of thrombin-catalyzed hydrolysis of fibrinogen. The effect of tba20-$\mathbf{L}_4$ on the clotting time is compared in the presence (red circles) and absence (blue circles) of Cu^{II}. Black circles: tba15; grey circles: control without DNA (both in the presence of Cu^{II}; [DNA] = 1.1 µM in all experiments).

In conclusion, we reported the synthesis of a new chiral ligand (*R*/*S*)-**L**, with a pyridine donor functionality and a glycol backbone, which can be incorporated internally into oligonucleotide strands. In a unimolecular human telomeric G-quadruplex, one G-tetrad can be replaced by four ligands, preorganized to coordinate to a copper ion, thus stabilizing the structure. Using the metal-base tetrad within a tetrahymena telomeric G-quadruplex enables switching of topologies from a hybrid-dominated conformer mixture to an antiparallel strand arrangement, as evidenced by UV melting curves and CD analysis. Incorporation of four pyridine ligands into the 15mer thrombin binding aptamer permits copper-triggered control over thrombin-catalyzed fibrinogen hydrolysis, as demonstrated by a clotting assay. Switchable G-quadruplex folding may be of value in protein affinity assays,[10,31] where paramagnetic metal ions will enable EPR-based readout. In addition, we currently extend this concept to systems containing other metals and ligands. The herein introduced incorporation of several ligands into a single-stranded G-quadruplex allows for heteroleptic ligand combinations aimed at bio-hybrid coordination environments with tunable catalytic function and redox properties.

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Keywords: bioinorganic chemistry • G-quadruplexes • metal base-pair • supramolecular chemistry • telomeric DNA

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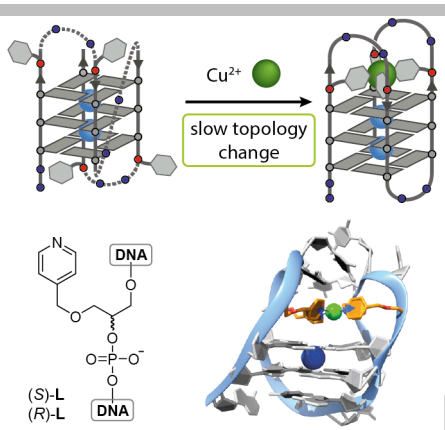
Copper is all it needs! A glycol-based pyridine ligand is incorporated four times into human and tetrahymena telomeric repeat DNA sequences. Cu^{II} coordination either highly stabilizes the unimolecular G-quadruplex or induces a topology change from a conformer mixture to an antiparallel strand orientation as evidenced by thermal denaturation, CD studies, as well as MD simulations.



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