Electronic Supplementary Information

Di-Copper(II) DNA G-Quadruplexes as EPR Distance Rulers

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1 Phosphoramidite Synthesis

Ligand L phosphoramidites necessary for DNA synthesis were synthesized as previously reported.¹

2 DNA synthesis

All oligonucleotides were synthesized on a *K&A Laborgeraete GbR* Synthesizer H-8 on a 1 μ mol scale using the standard phosphoramidite methods on CPG and following previously published procedures for synthesis, work-up and purification.^{1,2}

The concentration of each DNA sample was determined via the absorbance at 260 nm at 25 °C and using revised extinction coefficients for the nucleosides.^{1,3}

2.1 Analytical RP-HPLC

Samples were measured on an *Agilent* 1260 Infinity system with a (4.6 mm) *Machery Nagel* 250 Nucleodur 100-5 C18ec column (oven temperature 60 °C, flow rate 1 mL min⁻¹, solvent A: 0.05 mol L⁻¹ TEAA pH 7, B: 70:30 MeCN/0.05 mol L⁻¹ TEAA pH 7). The following figures depict the analytical RP-HPLC traces of the synthesized oligonucleotides after RP-HPLC purification, detritylation and desalting. Purity is given in percent relative to the trace total peak areas.







2.2 Oligonucleotide ESI mass spectra

ESI (negative mode) mass spectra were measured on a *Bruker* HCT Ultra, maXis, or MicrOTOF mass spectrometer. All DNA samples were measured in $H_2O/MeOH$ 10:1 or 9:1, or in $H_2O/MeCN$ 6:4.





3 DNA sample preparation

Each DNA sample contained 10 mmol L⁻¹ lithium cacodylate buffer (pH 7.3), 100 mmol L⁻¹ NaCl, CsCl or KCl. Oligonucleotide single-strand concentration was 7.5 μ mol L⁻¹ for the UV-VIS or CD experiments and 500 μ mol L⁻¹ for the EPR measurements. For all experiments,

samples were prepared with ultrapure water (type I, $18.2 \text{ M}\Omega \text{ cm}$), obtained with a Puranity TU 3 UV *VWR International GmbH*, Darmstadt.

Samples were heated to 85 °C (or 95 °C) for 10 min, then slowly cooled to 4 °C at 0.5 °C min⁻¹ and then left at this temperature for several hours (typically over night), followed by a freeze-thaw cycle (>1 h at -25 °C or -30 °C). In case of the EPR samples, CuSO₄ (375 μ mol L⁻¹) was added before the freeze-thaw cycle. Prior to the EPR measurements, ethylene glycerol (50 % *v*/*v*) was added to the respective DNA samples.

4 UV-VIS spectroscopic thermal denaturation studies

Both UV-VIS spectra and thermal denaturation curves (melting curves) were recorded as described previously.^{1,2} For normal UV-VIS spectra the scan rate was 200 nm min⁻¹. Spectra were recorded both before and after thermal denaturation. All samples were temperature equilibrated (4 °C) for at least two minutes prior to measurement of full spectra. For the thermal difference spectra, the spectrum after denaturation (at 4 °C) was subtracted from the one before denaturation (also 4 °C). In both cases, a negative band (hypochromic shift) at approximately 295±2 nm and positive bands at 243±2 nm and 273±2 nm (hyperchromic shift) indicated G-quadruplex formation.⁴ UV-VIS spectra were background corrected (buffer and electrolyte) and zeroed using the absorption at 350 nm.

In case of the thermal denaturation curves, absorption of the sample was recorded in a 0.5 °C interval with a temperature gradient set to 0.5 °C min⁻¹, which corresponds to ~0.4 °C min⁻¹ including the measurement time. To prevent major deviations from this temperature gradient, the absorption of always three cuvettes was measured. Data points were recorded from 4 °C to 85 °C or 95 °C and recursively back. Melting curves were background corrected using the absorption at 350 nm and converted to the fraction folded values by linear fitting of the lowand high temperature baselines.⁵ Melting temperatures were determined by reading the respective value at the fraction folded value $\alpha = 0.5$.



4.1 Thermal difference spectra (TDS)

Fig. S1: TDS profiles with and without Cu^{II} addition for the G-quadruplex $[d(TG_4)LdT]_4$ with (S)-L (left) and (R)-L (right).



Fig. S2: TDS profiles with Cu^{II} addition for the G-quadruplex $[Ld(G_3)LdT]_4$ with (*S*)-L in NaCl (left) and KCl containing solution (right).



Fig. S3: TDS profiles with Cu^{II} addition for the G-quadruplex $[Ld(G_3)LdT]_4$ with (*R*)-L in NaCl (left) and KCl containing solution (right).



Fig. S4: TDS profiles with and without Cu^{II} addition for the G-quadruplex $[Ld(G_4)LdT]_4$ with (*S*)-L in NaCl (left) and CsCl containing solution (right).



Fig. S5: TDS profiles with and without Cu^{II} addition for the G-quadruplex $[Ld(G_4)LdT]_4$ with (*R*)-L in NaCl (left) and CsCl containing solution (right).



Fig. S6: TDS profiles with and without Cu^{II} addition for the G-quadruplex $[Ld(G_5)LdT]_4$ with (*S*)-L in NaCl (left) and CsCl containing solution (right).



Fig. S7: TDS profiles with and without Cu^{II} addition for the G-quadruplex $[Ld(G_5)LdT]_4$ with (*R*)-L in NaCl (left) and CsCl containing solution (right).

4.2 Thermal denaturation curves

Table 1: Overview of G-quadruplex denaturation temperatures $T_{1/2}$ with ligand (*R/S*)-L. * double melting transition; † may also be a biphasic transition, apparent melting temperature $T(\alpha=0.5)$ is given; ‡ estimate due to very broad and multiphasic transitions.

G-quadruplex	L	salt	equiv.	$T_{1/2}$	$\Delta T_{1/2}$ (±Cu ^{II})
- 1···· F·	~		CuSO ₄	[°C]	[°C]
$[d(TG_4)LdT]_4$	S	NaCl	0	56.5	
			1	63.9	+7.4
			2	66.4	+9.9
	R	NaCl	0	56.6	
			1	64.3	+6.7
			2	66.7	+10.1
$[\mathbf{Ld}(\mathbf{G}_3)\mathbf{LdT}]_4$	S	NaCl	0	≤ 0	
			1	33.8	+33.8
			2	37.5	+37.5
			3	38.3	+38.3
	R	NaCl	0	≤ 0	
			1	23.0	+23.0
			2	23.0	+23.0
			3	24.2	+24.2
	S	KCl	0	≤ 0	
			1	47.8/66.8*	+47.8/66.8
			2	66.8	+66.8
			3	70.4	+70.4
	R	KCl	0	≤ 0 .	
			1	50.6 [†]	+50.6
			2	58.2	+58.2
			3	62.5	+62.5
$[Ld(G_4)LdT]_4$	S	NaCl	0	54.6	
			1	64.4	+9.8
			2	68.6	+14.0
	R	NaCl	0	54.0	
			1	57.1	+3.1
			2	61.8	+7.8
	S	CsCl	0	20.2	
			1	40.9/57.5*	+20.7/37.3
			2	47.1/59.7*	+26.9/39.5
	R	CsCl	0	20.5	
			1	21.1/38.4*	+0.6/17.9
			2	39.1	+18.6
$[Ld(G_5)LdT]_4$	S	NaCl	0–2	≥95	
	R	NaCl	0–2	≥88	
	S	CsCl	0	50.5 [‡]	
			1	68.9 [‡]	+18.4
			2	71.6 [‡]	+21.1
	R	CsCl	0	50.2 [‡]	
			1	64.2*	+14.0
			2	65.7*	+15.5

The usage of Cs^+ as the stabilizing cation results in thermally less stable G-quadruplexes, due to the greater ionic radius compared to K^+ or Na^+ .



Fig. S8: Thermal denaturation spectra with and without Cu^{II} addition for the G-quadruplex $[d(TG_4)LdT]_4$ with (S)-L (left) and (R)-L (right).

For both ligand enantiomers the increase in stability after Cu^{II} addition was found to be around $\Delta T_{1/2}(\pm Cu^{II}) = +7$ °C, clearly lower than when the metal-base tetrad is located at the 5'-end of the G-quadruplex ($\Delta T_{1/2}(\pm Cu^{II}) = +16$ °C).² Care must be taken when correlating denaturation temperatures with actual thermodynamic stabilities, as the annealing process for tetramolecular G-quadruplexes is extremely slow.⁶ Unlike observed for the 5'-modification, however, ligand chirality does not affect the thermal stability, neither in the absence nor in the presence of Cu^{II}.



Fig. S9: Thermal denaturation spectra with Cu^{II} addition for the G-quadruplex $[Ld(G_3)LdT]_4$ with (*S*)-L in NaCl (left) and KCl containing solution (right). Without Cu^{II} addition, the G-quadruplex does not form, so no profile corresponding to a denaturation can be observed.



Fig. S10: Thermal denaturation spectra with Cu^{II} addition for the G-quadruplex $[Ld(G_3)LdT]_4$ with (*R*)-L in NaCl (left) and KCl containing solution (right). Without Cu^{II} addition, the G-quadruplex does not form, so no profile corresponding to a denaturation can be observed.



Fig. S11: Thermal denaturation spectra with and without Cu^{II} addition for the G-quadruplex $[Ld(G_4)LdT]_4$ with (S)-L in NaCl (left) and CsCl containing solution (right).



Fig. S12: Thermal denaturation spectra with and without Cu^{II} addition for the G-quadruplex $[Ld(G_4)LdT]_4$ with (*R*)-L in NaCl (left) and CsCl containing solution (right).



Fig. S13: Thermal denaturation spectra with and without Cu^{II} addition for the G-quadruplex $[Ld(G_5)LdT]_4$ with (S)-L (left) and (R)-L (right) in CsCl containing solution. In case of the NaCl containing solutions, no or only minor denaturation was observed within the temperature window.

As mentioned in the main text, the two metal-base tetrads do not lead to an additive increase in thermal denaturation temperatures. The combination of two metal-base tetrads inside one G-quadruplex leads to a smaller increase in stability compared to the G-quadruplex carrying only one ligand tetrad at the 5'-end. So the observed thermal denaturation results from a more complex interplay between stabilizing and destabilizing factors.

5 Circular dichroism spectroscopy

5.1

CD spectra

CD spectra were measured on a *Jasco* J-810 Spectropolarimeter (350 - 200 nm, 50 nm min⁻¹, 1 nm data interval, 1.0 nm bandwidth, standard sensitivity, D.I.T. 1 s, 5x data accumulation) equipped with a PTC-423s thermostat for temperature control. Data were processed as previously reported.^{1,2}

All spectra were averaged, background corrected (cuvette, buffer and electrolyte), zeroed to the average signal between 330 – 350 nm, and smoothed. Ellipticity θ in mdeg was converted to molar circular dichroism $\Delta \varepsilon$ (per strand, not per nucleotide).

For all samples, the CD data can be attributed to *group I* spectra, corresponding to purely homopolar couplings between the G-tetrads and typical for a parallel strand orientation.⁷



Fig. S14: Circular dichroism spectra with and without Cu^{II} addition for the G-quadruplex $[d(TG_4)LdT]_4$ with (S)-L (left) and (R)-L (right).



Fig. S15: Circular dichroism spectra with Cu^{II} addition for the G-quadruplex $[Ld(G_3)LdT]_4$ with (*S*)-L in NaCl (left) and KCl containing solution (right). For (*S*)-L also the spectrum without Cu^{II} addition is shown (left, black curve), typical for single-strands and thus no G-quadruplex formation.



Fig. S16: Circular dichroism spectra with Cu^{II} addition for the G-quadruplex $[Ld(G_3)LdT]_4$ with (*R*)-L in NaCl (left) and KCl containing solution (right).



Fig. S17: Circular dichroism spectra with and without Cu^{II} addition for the G-quadruplex $[Ld(G_4)LdT]_4$ in NaCl containing solution with (*S*)-L (left) and (*R*)-L (right).



Fig. S18: Circular dichroism spectra with and without Cu^{II} addition for the G-quadruplex $[Ld(G_5)LdT]_4$ with (S)-L in NaCl (left) and CsCl containing solution (right).



Fig. S19: Circular dichroism spectra with and without Cu^{II} addition for the G-quadruplex $[Ld(G_5)LdT]_4$ with (*R*)-L in NaCl (left) and CsCl containing solution (right).

6 Molecular Dynamics (MD) Simulations

MD simulations were carried out using the GROMACS 2016.1 program⁸ and mainly following literature recommendations⁹ and as described previously.^{1,2} The AMBER force field *ff99bsc1*¹⁰ was used for all simulations.

Initial models for the G-quadruplexes were obtained by using the solid state structure of a $[d(TG_4T)]_4$ G-quadruplex (PDB entry 2O4F).¹¹ All manipulations were carried out in Chimera.^{12,13} Models were prepared by deletion of all water molecules, counter cations and by removal of the respective thymine nucleotides and manual insertion of the geometry optimized copper ligand complexes.

6.1 MD simulation procedure

The respective model obtained above was first energy minimized 2000 steps steepest descent (600 kJ mol⁻¹ nm⁻¹ tolerance) in vacuum. A periodic rhombic dodecahedron box (cutoff 1.5 nm) was used, as well as PME and van-der-Waals cutoff of 1.3 nm. The system was solvated with TIP3P water molecules and the negative charge of the system was neutralized with the corresponding amount of randomly positioned Na⁺, K⁺, or Cs⁺ ions. An additional 100 mmol L⁻¹ NaCl, KCl, or CsCl was added to simulate the ionic strength of the experiments.

The system was then energy minimized in two steps, first 500 steps of steepest descent (500 kJ mol⁻¹ nm⁻¹ tolerance) and secondly 3000 steps of conjugate gradient minimization (300 kJ mol⁻¹ nm⁻¹ tolerance). The non-bonded Lennard-Jones cutoff was set to 1.3 nm, the non-bonded pair list updated every 50 steps. For the coulombic interactions, Particle-mesh Ewald summation (PME) was used.¹⁴ Next, the system was equilibrated with positional constraints on the model's heavy-atoms 100 ps in a first round (NVT ensemble, constraints 1000 kJ mol⁻¹ Å⁻², time step 2 fs; Temperature coupling modified Berendsen, 298 K); second round 100 ps with additional pressure coupling (isotropic, Berendsen, 1 bar, time constraints (100 kJ mol⁻¹ Å⁻², Nose-Hoover temperature coupling 2 ps coupling, Parinello-Rahman

isotropic pressure coupling, 2 ps coupling time). The equilibration phase was finished with 200 ps of an unconstraint DNA MD run (coupling times increased to 4 ps). A 50 ns MD production run was then performed. Coordinates were written every 10 ps. Trajectories were centered, aligned and fitted to the first frame using the built-in Gromacs tools and then analyzed and visualized with UCSF Chimera.^{12,13}

6.2 MD derived mean Cu^{\parallel} - Cu^{\parallel} distances and angles ζ and β

The average distances, angles and standard deviations were calculated based on the last 30 ns of each trajectory and using the built-in command *gmx distance* and *gmx gangle* of the GROMACS program package. The angle β is calculated from the angle between the plane normals of the Cu coordination planes, using N/Cu/N atom triplets. The angle ζ is calculated in the same way using N/Cu/N triplets and the Cu-Cu atom vector. For one G-quadruplex, two ζ values are obtained.

			•			
G-quadruplex	L	ion	<r> [nm]</r>	<ζ ₁ > [°]	<ζ ₂ > [°]	<β> [°]
$[Ld(G_3)LdT]_4$	S	Na^+	1.60 ± 0.04	9 ± 4	15 ± 5	9 ± 4
	R	Na^+	1.59 ± 0.05	13 ± 7	21 ± 5	14 ± 6
	S	K^+	1.63 ± 0.04	10 ± 5	23 ± 6	16 ± 6
	R	K^+	1.57 ± 0.03	9 ± 4	17 ± 4	12 ± 5
$[Ld(G_4)LdT]_4$	S	Na^+	1.84 ± 0.04	7 ± 4	9 ± 5	7 ± 4
	R	Na^+	1.87 ± 0.04	10 ± 4	17 ± 4	11 ± 5
	S	Cs^+	1.96 ± 0.04	10 ± 5	17 ± 5	14 ± 6
	R	Cs^+	1.95 ± 0.05	17 ± 6	15 ± 6	9 ± 5
$[Ld(G_5)LdT]_4$	S	Na^+	2.22 ± 0.05	9 ± 4	12 ± 5	8 ± 4
	R	Na^+	2.22 ± 0.04	11 ± 4	21 ± 4	13 ± 5
	S	Cs^+	2.28 ± 0.04	9 ± 4	13 ± 7	12 ± 6
	R	Cs^+	2.32 ± 0.04	14 ± 5	13 ± 5	9 ± 5

Table S2: Mean Cu^{II}–Cu^{II} distances $\langle r \rangle$ and angles ζ and β as determined by MD simulations.

6.3 MD structures and rmsd trajectories

The depicted structures are representatives generated after clustering of the MD trajectory (Chimera built-in based on rmsd deviations). A structure from the cluster with the most members was chosen. For the rmsd plots, the first frame of the trajectory was used as the reference. The highlighted solid lines are averages (50 points) of the actual data (grey). Black line: non-hydrogen DNA atoms; red line: non-hydrogen ligand atoms; blue line: non-hydrogen guanosine nucleotide atoms.

$Cu_{2}[Ld(G_{3})LdT]_{4}(R)-L(K^{+})$



 $Cu_2[Ld(G_3)LdT]_4$ (R)-L (Na⁺)



 $Cu_2[Ld(G_3)LdT]_4$ (S)-L (K⁺)



$Cu_2[Ld(G_3)LdT]_4$ (S)-L (Na⁺)



 $Cu_2[Ld(G_4)LdT]_4 (\textit{R})-L (Na^+)$



 $Cu_2[Ld(G_4)LdT]_4$ (*R*)-L (Cs⁺)



$Cu_2[Ld(G_4)LdT]_4$ (S)-L (Na⁺)



 $Cu_{2}[Ld(G_{4})LdT]_{4} (S)-L (Cs^{+})$



 $Cu_2[Ld(G_5)LdT]_4$ (*R*)-L (Na⁺)



$Cu_2[Ld(G_5)LdT]_4(R)-L(Cs^+)$



 $Cu_2[Ld(G_5)LdT]_4$ (S)-L (Na⁺)



 $Cu_2[Ld(G_5)LdT]_4$ (S)-L (Cs⁺)



6.4 Alternative G-quadruplex topologies

In the pulsed-EPR measurements, two Cu^{II} - Cu^{II} distances were obtained in some cases, indicating the presence of other, non-standard, G-quadruplex topologies. To evaluate the most likely candidates, representative alternative G-quadruplex models were analyzed in MD simulations, following the same protocol as described above. Fig. S20 shows the corresponding schematic representations for models based on the $Cu_2[Ld(G_4)LdT]_4$ G-quadruplex with (*R*)-L in Na⁺-containing solution.

The G-quadruplex models with additional ions (Fig. S20b and c) are not stable during the MD simulations, resulting in release of the added Cl⁻ or Na⁺ ions to the bulk ions. For the topology T4 with inward bent 3'-dT residues (Fig. S20g), a thymine tetrad was modeled as the MD input structure. Although the tetrad does not remain stable during the course of the MD simulation, a stable conformation is obtained with three thymines bent inward and one pointing to the outside (see also the model graphic below). The average distances calculated for models T4 and $2S_{opp}$ give the best agreement with the experimental EPR values (Table S3). Given that for model T4, no strand slipping is involved, and formation of stabilizing thymine tetrads was already reported for tetramolecular G-quadruplexes, this model represents the most likely explanation for the observed second EPR derived distances.



Fig. S20: Schematic representations of $Cu_2[Ld(G_4)LdT]_4$ G-quadruplexes. a) Original model, b) two bound chloride anions, c) four bound sodium cations, d) one slipped strand, e) two slipped strands (adjacent), f) two slipped strands (opposite), g) inward bent 3'-dT residues. Ions represented with a dotted contour do not remain inside the G-quadruplex central channel during the MD simulation and are released to the bulk.

Table S3: Mean Cu^{II} – Cu^{II} distances $\langle r \rangle$ and angles ζ and β as determined by MD simulations for the $Cu_2[Ld(G_4)LdT]_4$ G-quadruplex with (*R*)-L in Na⁺ containing solution depending on topology. * The model topology reverts back to the *native* structure during the MD simulation. *n.d.* = not determined.

model	< <i>r</i> > [nm]	<ζ ₁ > [°]	<ζ ₂ > [°]	<β> [°]
native	1.88 ± 0.04	n.d.	n.d.	n.d.
2Cl	$1.88 \pm 0.04*$	n.d.	n.d.	n.d.
4Na	$1.88 \pm 0.04*$	n.d.	n.d.	n.d.
$2S_{adj}$	2.06 ± 0.05	n.d.	n.d.	n.d.
<i>1S</i>	2.14 ± 0.05	n.d.	n.d.	n.d.
Τ4	2.20 ± 0.05	11 ± 5	11 ± 5	10 ± 5
$2S_{opp}$	2.23 ± 0.05	16 ± 7	15 ± 5	13 ± 6

Model 2Sadj



Model 1S





The topology undergoes a strand slipping during the MD simulation, resulting in the high guanosine rmsd fluctuation between 12 - 30 ns.

7 EPR distance measurements

As stated in the main text, the PELDOR and RIDME experiments are similar but still complementary in some respects. For example, the background correction of the RIDME time traces is theoretically less well understood, which might lead to artefacts in the distance distribution. Conversely, orientation selectivity, also leading to distance distribution artefacts using standard data processing implemented in the software DeerAnalysis, is less pronounced in RIDME.^{15–17}

7.1 Materials and methods

All pulsed EPR experiments were performed on a *Bruker* ELEXSYS 580 EPR spectrometer equipped with a SpecJet-II and a PatternJet-II combination. For measurements at cryogenic temperatures, a helium flow cryostat (CF935) and an ITC 502 temperature control system from Oxford Instruments was used. For pulsed Q-band measurements, an ER5106QT-2 resonator from Bruker was used together with a 150 W amplifier from Applied Systems *Engineering*. RIDME measurements were performed using the five-pulse sequence: $\pi/2 - \tau_1 - \tau_1$ $\pi - \tau_{1+t} - \pi/2 - T - \pi/2 - \tau_2 - \pi$ – echo. RIDME measurements were performed with an 8step phase cycle. For RIDME^{18,15} experiments, the refocused virtual echo was optimized and detected. The initial τ_1 -value was 350 ns, with a T value of 30000 ns. RIDME measurements were performed at 28 K. PELDOR¹⁹ measurements were performed using the four-pulse sequence: $\pi/2(\nu_A) - \tau_1 - \pi(\nu_A) - \tau_{1+t} - \pi(\nu_B) - \tau_{2-t} - \pi(\nu_A) - \tau_2$ - echo. Again, a τ_1 -value of 350 ns was used. PELDOR measurements were performed at 20 K. For all pulsed experiments, the π -pulse length is 24 ns. PELDOR experiments were performed with a frequency offset of 80 MHz. For all samples and methods, the experiment was performed at five different field positions and the acquired time traces added to account for orientation selectivity effects. The background for the PELDOR time traces was assumed to be an exponential decay function, with the exception of the example shown in Figure S24, where an empirical fitting using a third order polynomial was used, which is unusual for PELDOR data an does not agree with the model of homogenously distributed spin centers leading to an exponential background decay. The background of the RIDME time traces was fitted using a third order polynomial, which has been used successfully before to remove background decay functions from RIDME time traces.^{16,20}

The dipolar coupling frequency v_{dip} is determined by the interspin distance *r* and the angle θ between the interspin vector and the external magnetic field (Eq. S1):

$$v_{dip} = \frac{52.01 \, \text{MHz} \cdot nm^3}{r^3} (1 - 3\cos^2\theta) \tag{S1}$$

If all possible values of θ contribute to the modulation of the time trace with their appropriate statistical weight, a characteristic shape of the frequency spectrum is obtained which is called a Pake pattern. DeerAnalysis extracts distance distribution out of time traces under the assumption that each time trace is modulated by frequencies corresponding to such a Pake pattern spectrum, i.e. the data is assumed to be non-orientation selective. This assumption is often useful for the case of nitroxide-nitroxide distance measurements at X-band MW frequency, but no longer justified for Cu(II) spin centers that have a spectral width of >2000 MHz at Q-band frequency, leading to orientation selective time traces. Orientation selection in EPR based distance measurements is a result of the finite excitation bandwidth of the

microwave pulses far below the width of the Cu(II) EPR spectrum and the correlation of the relevant parameters, i.e. the interspin vector as well as the EPR tensors of the spin centers involved in the distance measurements.²¹ The limited excitation bandwidth leads to excitation of only a small subset of spin centers with EPR parameters that lead to excitation of spins with orientations leading to Larmor frequencies close to the MW frequency of the applied pulses. Since the orientation of the spin centers with respect to the external field are correlated with the orientation of the interspin vector with the external field, only a subset of angles θ is excited as well. Thus, summing up data of these subsets should remove orientation selection, if all subsets are summed up. In the case of RIDME, this would mean that the observer frequency would have to be placed at various positions of the Cu(II) spectrum in a sufficiently narrow mesh. For example, if time traces would have been obtained in 20 MHz steps along the whole spectrum, orientation selection might have been removed almost completely. However, this would require >100 measurements per sample and is therefore not feasible. Consequently, a coarser mesh is applied which only leads to partial removal of orientation selection. The situation is even worse for PELDOR, where in addition to the observer pulses also the pump pulses would need to be applied at all possible field values. Since the resonator bandwidth is limited, this would not be possible for technical reasons (aside from the huge amount of time required to do so). That orientation selection is worse in PELDOR is also reflected in the frequency spectra depicted below for the orientation averaged PELDOR and RIDME data.

As mentioned in the main text, the g_z value is of importance for the discussion of the orientation selective time traces. The g_z value is defined as the highest g value and occurs along the surface normal of the Cu(II)-pyridine quartet. This assignment is also directly evident from the experimental data, as measurements at high g values lead to a strongly over pronounced occurrence of the parallel dipolar coupling. Since the Cu(II) centers have axial EPR spectra, the orientation of the other two g axes is arbitrary. For further details concerning the EPR parameters of Cu(II) complexes see Meyer *et al.*^{16,22}

It is important to note that the position of the main distance peaks for all measured Gquadruplexes is independent of the type of experiment and data analysis, within a margin of about 0.05 nm. The width σ of the main peaks is below 0.15 nm for all samples. The lowest σ values are obtained for the shortest quadruplexes or if Cs⁺ is used as stabilizing cation. The low σ values demonstrate that the flexibility of any given conformer is rather low, in agreement with expectations based on the rigidity of the quadruplex backbone and the fourpoint-attached Cu^{II} spin label. Some low intensity peaks occur in the distance distributions of the orientation averaged *class a* data sets in addition to the main peaks given in the main text in Table 1. These vary somewhat in intensity and position, depending on the measurement and analysis method used, and are therefore less reliable and might stem from either further trapped conformers or from the aforementioned drawbacks of the fitting procedures.

Concerning the angles described in the main text, it is noteworthy to mention that two angles ζ exist in each quadruplex, i.e. one angle per Cu(II) ion. However, according to the model outlined in Figure 5 in the main text and in accordance with the values obtained by the molecular dynamics simulations, those two angles are expected to be very similar. Therefore, only one effective, average ζ value was used in the simulation of the orientation selective PELDOR time traces in order to avoid overparameterization. Since β defines the relative

orientation of the g- and hyperfine-tensors of both spin centres, only one angle β exists. Since the g tensors of the copper complexes are axially symmetric no further angles can be extracted. The need to restrict the geometrical parameters during the fitting implies that the parameters are not unique and might have a wide range of error. Indeed, when using the standard procedure to obtain error estimates, i.e. plotting the rmsd of the fitted time traces against the geometrical parameters, flat error surfaces or even minima at physically nonsensical points are obtained in some cases, especially if all parameters are varied in a wide range. Taking the distribution width σ as an example, it was observed that large values of $\sigma \approx$ 0.35 nm often achieve lower rmsd, although it is clear from inspection that such high values lead to clearly too much damping (see Fig. S49). For $\langle r \rangle$ and σ , comparison with the DeerAnalysis data allows estimating an error of ~0.05 nm for both quantities, based on the deviations between the different methods of analysis. As with σ , the parameters ζ and β are not susceptible to the standard error analysis of PeldorFit, as physically nonsensical solutions which also produce time traces which show clear systematic deviations from the experimental data lead to lower rmsd than solutions that imply the correct geometrical model (see Figures S51 and S52). Since these parameters are not obtained with the other methods and the standard methods of error analysis fails, no meaningful error estimate other than the restriction of the parameter space used during fitting can be given.

7.2 Pulsed EPR time traces, spectra and distance distributions



Fig. S21: PELDOR of $Cu_2[Ld(G_3)LdT]_4(R)$ -L (K⁺). Figure shows original time trace (a), background corrected time trace (b), the Pake pattern (c) and the resulting distance distribution (d).

Orientationally averaged RIDME data

 $Cu_{2}[Ld(G_{3})LdT]_{4}(R)-L(K^{+})$



Fig. S22: RIDME of $Cu_2[Ld(G_3)LdT]_4(R)$ -L (K⁺). Figure shows original time trace (a), background corrected time trace (b), the Pake pattern (c) and the resulting distance distribution (d).

Orientationally selective PELDOR data



Fig. S23: Orientation selective PELDOR time traces of $Cu_2[Ld(G_3)LdT]_4(R)-L(K^+)$ (full, black lines) and PeldorFit simulations thereof (dotted). The observer position is indicated, the pump position is at $\Delta v = v_{obs} - v_{pump} = +80$ MHz unless stated otherwise in the Figure.

Cu₂[Ld(G₃)LdT]₄ (S)-L (Na⁺) Orientationally averaged PELDOR data



Fig. S24: PELDOR of $Cu_2[Ld(G_3)LdT]_4$ (*S*)-L (Na⁺). Figure shows original time trace (a), background corrected time trace (b), the Pake pattern (c) and the resulting distance distribution (d).



Fig. S25: RIDME of $Cu_2[Ld(G_3)LdT]_4$ (*S*)-L (Na⁺). Figure shows original time trace (a), background corrected time trace (b), the Pake pattern (c) and the resulting distance distribution (d).

Orientationally selective PELDOR data



Fig. S26: Orientation selective PELDOR time traces $Cu_2[Ld(G_3)LdT]_4$ (*S*)-L (Na⁺) (full, black lines) and PeldorFit simulations thereof (dotted). The observer position is indicated, the pump position is at $\Delta v = v_{obs} - v_{pump} = +80$ MHz unless stated otherwise in the Figure.

Cu₂[Ld(G₄)LdT]₄ (*R*)-L (Na⁺) Orientationally averaged PELDOR data



Fig. S27: PELDOR of $Cu_2[Ld(G_4)LdT]_4$ (*R*)-L (Na⁺). Figure shows original time trace (a), background corrected time trace (b), the Pake pattern (c) and the resulting distance distribution (d).





Fig. S28: RIDME of $Cu_2[Ld(G_4)LdT]_4$ (*R*)-L (Na⁺). Figure shows original time trace (a), background corrected time trace (b), the Pake pattern (c) and the resulting distance distribution (d).

Orientationally selective PELDOR data

The orientation selective PELDOR data for this sample is shown in the main text, Figure 3.

Cu₂[Ld(G₄)LdT]₄ (S)-L (Na⁺) Orientationally averaged PELDOR data

The PELDOR data of $Cu_2[Ld(G_4)LdT]_4$ (*S*)-L (Na⁺) appears to be the only example of a quadruplex molecule stabilized by Na⁺ ions in which just one distance peak is obtained in the distance distribution. The distance peak is centered at ~1.88 nm, which is in good correspondence to the position of the singularities of the Pake pattern predicted by DeerAnalysis, which occur at ~7.2 MHz. However, on the inside flanks of these singularities, a small peak centered at ~4.6 MHz is visible, indicated by arrows in Fig. S29. This frequency corresponds to ~2.24 nm, which would be in agreement with all the other examples. The low intensity of this peak, probably in combination with the suppression of low frequency contributions in the time trace owed to orientation selection, prevents DeearAnalysis from taking this contribution into account.



Fig. S29: PELDOR of $Cu_2[Ld(G_4)LdT]_4$ (*S*)-L (Na⁺). Figure shows original time trace (a), background corrected time trace (b), the Pake pattern (c) and the resulting distance distribution (d). Arrows in (c) indicate frequencies possibly corresponding to a second distance.

Orientationally averaged RIDME data

In agreement with the above argument for PELDOR, the RIDME time traces do indeed show a second frequency component which also leads to an additional broad distance peak at \sim 2.25 nm.



Fig. S30: RIDME of $Cu_2[Ld(G_4)LdT]_4(S)-L(Na^+)$. Figure shows original time trace (a), background corrected time trace (b), the Pake pattern (c) and the resulting distance distribution (d).

Orientationally selective PELDOR data



Fig. S31: Orientation selective PELDOR time traces $Cu_2[Ld(G_4)LdT]_4(S)-L(Na^+)$ (full, black lines) and PeldorFit simulations thereof (dotted). The observer position is indicated, the pump position is at $\Delta v = v_{obs} - v_{pump} = +80$ MHz unless stated otherwise in the Figure.

Cu₂[Ld(G₄)LdT]₄ (*R*)-L (Cs⁺) Orientationally averaged PELDOR data



Fig. S32: PELDOR of $Cu_2[Ld(G_4)LdT]_4$ (*R*)-L (Cs⁺). Figure shows original time trace (a), background corrected time trace (b), the Pake pattern (c) and the resulting distance distribution (d).

Orientationally averaged RIDME data



Fig. S33: RIDME of $Cu_2[Ld(G_4)LdT]_4$ (*R*)-L (Cs⁺). Figure shows original time trace (a), background corrected time trace (b), the Pake pattern (c) and the resulting distance distribution (d).

Orientationally selective PELDOR data



Fig. S34: Orientation selective PELDOR time traces Cu₂[Ld(G₄)LdT]₄ (*R*)-L (Cs⁺) (full, black lines) and PeldorFit simulations thereof (dotted). The observer position is indicated, the pump position is at $\Delta v = v_{obs} - v_{pump} = +80$ MHz unless stated otherwise in the Figure.

Cu₂[Ld(G₄)LdT]₄ (S)-L (Cs⁺) Orientationally averaged PELDOR data



Fig. S35: PELDOR of $Cu_2[Ld(G_4)LdT]_4$ (*S*)-L (Cs⁺). Figure shows original time trace (a), background corrected time trace (b), the Pake pattern (c) and the resulting distance distribution (d).





Fig. S36: RIDME of $Cu_2[Ld(G_4)LdT]_4$ (*S*)-L (Cs⁺). Figure shows original time trace (a), background corrected time trace (b), the Pake pattern (c) and the resulting distance distribution (d).

Orientationally selective PELDOR data

The orientation selective PELDOR data for this sample is shown in the main text, Figure 4.

Cu₂[Ld(G₅)LdT]₄ (*R*)-L (Na⁺) Orientationally averaged PELDOR data



Fig. S37: PELDOR of $Cu_2[Ld(G_5)LdT]_4$ (*R*)-L (Na⁺). Figure shows original time trace (a), background corrected time trace (b), the Pake pattern (c) and the resulting distance distribution (d).

b) _{1,0} a) 1,00 0,75 0,9 Norm. Int. Norm. Int. 0,50 0,8 0,25 0,7 0,6+ 0 0,00 ò 500 1000 1500 500 1000 1500 Time [ns] Time [ns] d) c) 6 0,06 **Probablility Density** 4 Intensity [a.u.] 0,04 2 0,02 0 0,00 20 60 -60 -40 -20 Ò 40 ż 3 4 5 Distance [nm] Frequency [MHz]

Orientationally averaged RIDME data

Fig. S38: RIDME of $Cu_2[Ld(G_5)LdT]_4$ (*R*)-L (Na⁺). Figure shows original time trace (a), background corrected time trace (b), the Pake pattern (c) and the resulting distance distribution (d).

Orientationally selective PELDOR data



Fig. S39: Orientation selective PELDOR time traces Cu₂[Ld(G₅)LdT]₄ (*R*)-L (Na⁺) (full, black lines) and PeldorFit simulations thereof (dotted). The observer position is indicated, the pump position is at $\Delta v = v_{obs} - v_{pump} = +80$ MHz unless stated otherwise in the Figure.

Cu₂[Ld(G₅)LdT]₄ (S)-L (Na⁺) Orientationally averaged PELDOR data



Fig. S40: PELDOR of $Cu_2[Ld(G_5)LdT]_4$ (*S*)-L (Na⁺). Figure shows original time trace (a), background corrected time trace (b), the Pake pattern (c) and the resulting distance distribution (d).

Orientationally averaged RIDME data



Fig. S41: RIDME of $Cu_2[Ld(G_5)LdT]_4$ (*S*)-L (Na⁺). Figure shows original time trace (a), background corrected time trace (b), the Pake pattern (c) and the resulting distance distribution (d).

Orientationally selective PELDOR data



Fig. S42: Orientation selective PELDOR time traces of Cu₂[Ld(G₅)LdT]₄ (*S*)-L (Na⁺) (full, black lines) and PeldorFit simulations thereof (dotted). The observer position is indicated, the pump position is at $\Delta v = v_{obs} - v_{pump} = +80$ MHz unless stated otherwise in the Figure.

Cu₂[Ld(G₅)LdT]₄ (*R*)-L (Cs⁺) Orientationally averaged PELDOR data



Fig. S43: PELDOR of Cu₂[Ld(G₅)LdT]₄ (*R*)-L (Cs⁺). Figure shows original time trace (a), background corrected time trace (b), the Pake pattern (c) and the resulting distance distribution (d).



Orientationally averaged RIDME data

Fig. S44: RIDME of $Cu_2[Ld(G_5)LdT]_4(R)$ -L (Cs⁺). Figure shows original time trace (a), background corrected time trace (b), the Pake pattern (c) and the resulting distance distribution (d).

Orientationally selective PELDOR data



Fig. S45: Orientation selective PELDOR time traces of $Cu_2[Ld(G_5)LdT]_4$ (*R*)-L (Cs⁺) (full, black lines) and PeldorFit simulations thereof (dotted). The observer position is indicated, the pump position is at $\Delta v = v_{obs} - v_{pump} = +80$ MHz unless stated otherwise in the Figure.

Cu₂[Ld(G₅)LdT]₄ (S)-L (Cs⁺) Orientationally averaged PELDOR data



Fig. S46: PELDOR of $Cu_2[Ld(G_5)LdT]_4$ (*S*)-L (Cs⁺). Figure shows original time trace (a), background corrected time trace (b), the Pake pattern (c) and the resulting distance distribution (d).

Orientationally averaged RIDME data



Fig. S47: RIDME of $Cu_2[Ld(G_5)LdT]_4$ (*S*)-L (Cs⁺). Figure shows original time trace (a), background corrected time trace (b), the Pake pattern (c) and the resulting distance distribution (d).

Orientationally Selective PELDOR data



Fig. S48: Orientation selective PELDOR time traces of Cu₂[Ld(G₅)LdT]₄ (*S*)-L (Cs⁺) (full, black lines) and PeldorFit simulations thereof (dotted). The observer position is indicated, the pump position is at $\Delta v = v_{obs} - v_{pump} = +80$ MHz unless stated otherwise in the Figure.

7.3 PeldorFit simulations without geometrical restraints, error profiles and fits with ζ and β parameters restrained to values close to 90°.

As mentioned in the main text, using the fitting routine of PeldorFit without restraining the geometrical parameters close to those which are expected based on the geometrical model outlined in Figure 5 in the main text does not lead to satisfying fits. Typically, the simulations are much more strongly damped than the experimental data and only the first half modulation is reproduced in such simulations. One example of this observation is shown in Figure S49, which corresponds to the experimental data set for $Cu^{II}_2[Ld(G_4)LdT]_4$ ((*R*)-L, Na⁺) also shown in the main text (Figure 3).



Fig. S49: Orientation selective PELDOR time traces (full, black lines) and PeldorFit simulations thereof (dotted) without geometrical restraints. The observer position is indicated, the pump position is at $\Delta v = v_{obs} - v_{pump} = +80$ MHz unless stated otherwise in the Figure.

Inspection of Fig. S49 reveals that the coincidence of simulation and experiment is very good for high effective g values, where either heavy damping occurs or only shallow modulations are visible. In contrast, the time traces at low effective g values with intense modulations are not reproduced nicely. The good agreement between simulation and experiment of the high g value time traces appears to outweigh the bad agreement for time traces recorded at low effective g values. For the strongly modulated time traces at low effective g values, only the first half modulation is fitted nicely, but the remaining modulations are not reproduced at all. Often, this first modulation is already sufficient to get a rough estimate of the interspin distance. Inspection of the geometrical parameters obtained from the simulations shown in Figure S49 reveals, that one of the two distances is indeed more or less reproduced in this simulation ($r_1 = 2.06$ nm, this is roughly the average of the two distances obtained in the restrained fit shown in the main text in Figure 3), whereas the other distance is much too large ($r_2 > 3.0$). The widths of these two distance peaks are far too high, causing the strong damping of the simulated time traces. Fig. S50 shows error plots for the orientation selective PELDOR data on $\text{Cu}^{\text{II}_2}[\text{Ld}(\text{G}_4)\text{Ld}\text{T}]_4$ ((*R*)-L, Na⁺), which plot the rmsd of simulated an experimental time traces as a function of two geometrical parameters. The presented results are typical for the data sets obtained in this study.



Fig. S50: Error plots for the first distance r_1 vs. the width of the first distance peak σ_1 (a), for the first distance r_1 vs. the width of the first distance peak σ_1 (b) and for the first distance r_1 vs. the angle ζ_1 (c). Blue color corresponds to low deviation between simulated and experimental time traces, red color to large deviation.

Fig. S50 a) shows that the first distance has a broad minimum at 2.06 nm in the unrestrained fit shown in Fig. S49. The width σ_1 has a broad minimum at 0.36 nm, exceeding the values in the restrained fit by far, which was a typical problem for all data sets. The second distance and width are even less defined, as several minima in close proximity are observed in the error plot. Furthermore, the values in every minimum are far too high to be accounted for in the geometrical model of the DNA quadruplex molecules. With such high values for the second distance and width, it can safely be assumed that these values lead to a better agreement by improving the superposition of the unmodulated or only weakly modulated parts of the time traces. The last error profile plots the rmsd of simulated and experimental time traces as a function of the first distance r_1 and the angle ζ_1 . In agreement with the model outlined in Figure 5 in the main text, a clear minimum is observed at values below 30° and a distance r_1 of ~ 2.0 nm. However, an additional, shallower minimum at $\sim 2.3 - 2.4$ nm is observed. This does not contradict the geometrical model which has been used to explain the orientationally selective PELDOR data, as it means that a considerably larger distance than expected in the model would be necessary to allow a large value of ζ . Such a larger distance would lead to lower modulation frequencies. However, since the angle ζ would also be large, this would lead to the observation of the parallel component of the dipolar coupling tensor at low effective g values. Thus, the two effects would partially cancel out at these observer positions, leading to good agreement between simulation and experiment at these observer positions. At high effective g values, the erroneous parameters would not have a large impact on the rmsd, as the time traces are heavily damped at these positions.

Another way of demonstrating that the geometrical model agrees with the orientation selective PELDOR data is to take the values obtained from PeldorFit simulations shown above, and then change one of the angles ζ or β to a high value, which would not be

compatible with the geometrical model outlined in the main text (Figure 5). For these parameters this approach is especially useful as no other means of estimating error are available, since DeerAnalysis does not give access to these angles which would allow comparing the different values. An example for this is given in Fig. S51, where the data of $Cu_2[Ld(G_5)LdT]_4$ (*R*)-L (Cs⁺) already shown in Fig. S45 are reconsidered. In Fig. S51, the geometrical parameters are the same as those used in Fig. S45, except for the angle ζ , which was restrained to high values.



Fig. S51: Orientation selective PELDOR time traces of $Cu_2[Ld(G_5)LdT]_4$ (*R*)-L (Cs⁺) (full, black lines) and PeldorFit simulations thereof (dotted). The experimental data and the geometrical parameters are identical to those used in Fig. S45, except for the angle ζ , which amounts to 80° in this Figure.

As can be seen in Fig. S51, the dipolar frequencies at low effective g values are now far too high, in agreement with the geometrical model outlined in Figure 5 in the main text. The second angle β does not influence the dipolar coupling frequency in the orientationally selective PeldorFit simulations. However, it has a marked effect on the predicted modulation depth. This makes sense, as a large angle β would mean that the planes of the two copper complexes are strongly tilted with respect to each other. In such a situation, exciting along the equatorial plane of one copper complex would lead to an alignment along the axial direction of the other copper complex, which has a far higher effective g value. Thus, a high angle β would prevent effective excitation of both copper centres simultaneously. This effect is also observed in the simulations, if the value of β is fixed to be close to 90° (Fig. S52). Thus, the simulations suggest that high values for ζ and for β are not sensible, in agreement with the model outlined in Figure 5 in the main text and with results obtained by MD simulations.



Fig. S52: Orientation selective PELDOR time traces of $Cu_2[Ld(G_5)LdT]_4$ (*R*)-L (Cs⁺) (full, black lines) and PeldorFit simulations thereof (dotted). The experimental data and the geometrical parameters are identical to those used in Fig. S45, except for the angle β , which amounts to 80° in this Figure.

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