Published in final edited form as:

Phys Chem Chem Phys. 2016 June 1; 18(22): 15447–15455. doi:10.1039/c6cp01121g.

Distance Measurements between Paramagnetic Ligands Bound to Parallel Stranded Guanine Quadruplexes

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INTRODUCTION

Under physiological conditions, nucleic acid sequences which are guanine (G) rich have a propensity to self-assemble into four-stranded helical structures known as quadruplexes. G-quadruplexes display a rich diversity in structural topology, and may fold either intramolecularly through the association of a single nucleic acid strand containing several guanine rich repeats, or through the intermolecular assembly of multiple strands¹.

Regardless of the nature of the folding pathway, the central units of all G-quadruplex structures are comprised of Hoogsteen hydrogen-bonded arrays of guanines (termed G-quartets) (Figure 1a). Monovalent cations, such as Na⁺ or K⁺, located between or within G-quartets coordinate oxygen atoms from guanine bases in an antiprismatic bipyramidal geometry, and form a central ion channel through the G-quadruplex².

Highly conserved repeats of G-rich sequences on the 3' overhang strand of telomeres have been shown to form G-quadruplexes *in vitro* through an extensive variety of biophysical methods, including crystallography, nuclear magnetic resonance (NMR) and electrospray ionization mass spectrometry^{3–8}. The tertiary structures of G-quadruplexes within the telomeric overhang have been implicated as an impediment to cellular propagation by inhibiting telomerase activity⁹. As such, investigations of ligands that target and stabilize G-quadruplexes have garnered intensive attention as avenues towards prospective anticancer agents^{2, 10–15}.

For a wide range of ligands, including acridines, naphthalene diimides, quindolines and porphines, the most commonly reported binding site is onto the planar surface of a G-quartet; this mode of binding is referred to as end-stacking^{16–23}. These ligands feature large aromatic surfaces to maximize π - π stacking interactions and are also typically polycationic, which induces stabilizing electrostatic interactions with the quadruplex backbone and increases the hydrophilicity of the molecule. To our knowledge, there are currently (as of December 2015) 36 three-dimensional structures of ligand-quadruplex complexes deposited in the Protein Data Bank, comprising roughly 15 % of all known quadruplex nucleic acid structures^{16, 17, 24–45}. Of the deposited structures of ligand G-quadruplex complexes, none contain sequences with more than four contiguous guanines. Ligand-free sequences with single thymines flanking a long poly-G stretch (i.e. d(TG_nT), where $n > 6$), however, have been suggested to fold intramolecularly to create structures in which some guanines are not

part of the quartet and instead are found in loop regions⁴⁶. Moreover, a recent high resolution NMR structure of d(TTG₁₅T) indicates this intramolecular quadruplex adopts a parallel-stranded fold⁴⁷. In contrast to the ligand-free studies, evidence has been presented that supports a model in which ligands end-stack onto tetramolecular quadruplexes formed by d(T₄G₄₋₁₀T₄) sequences⁴⁸. Ligand binding to quadruplexes formed by long G tracts has potential biological implications, and therefore a clearer understanding of the structures of these motifs is warranted.

In this work, we report on double electron electron resonance (DEER) measurements applied to the copper derivative of the porphyrin 5,10,15,20-tetrakis(1-methyl-4-pyridyl)-21*H*,23*H*-porphine (H₂TMpyP4, Figure 1b) bound to quadruplexes containing *n* G-quartets, where *n* = 4, 6, 8 or 10 (see Table 1 for quadruplex sequences). The DEER technique has rapidly emerged as a powerful tool for probing structures of biomacromolecules on the nanometer scale (for recent reviews, see⁴⁹⁻⁵¹). It is a two frequency pulsed electron paramagnetic resonance (EPR) spectroscopy method that selectively isolates the dipolar interaction between unpaired electron spins, and is sensitive to distances in the range of 2 nm to 8 nm. The distance distributions extracted between pairs of paramagnetic reporter molecules provide constraints to the range of possible conformations the macromolecule can adopt. DEER previously has been used to map conformations of human telomeric quadruplexes⁵²⁻⁵⁴ and a G-rich sequence found in *Treponema pallidum*⁵⁵. These studies utilized site directed spin labeling, in which nitroxide spin labels are attached covalently to a deoxyribose group, to probe quadruplex conformation and folding motifs. For the samples reported herein, the coordinated Cu²⁺ ion serves as the paramagnetic spin probe with which to investigate the local structure of the G-quadruplex. Distance measurements using pulsed EPR techniques between copper centers are far less common than those between nitroxide radicals, though an increasing number of measurements on copper ions bound to biomacromolecules have been made in recent years⁵⁶⁻⁶². The distance measurements reported in this study are unique in that the copper centers are not covalently bound to the macromolecule and no nucleic acid modifications are required to obtain distance information. Nitroxide spin labeling has the potential to sterically perturb the conformation of the ligand-quadruplex assembly, whereas a label free approach, through the addition of a single transition metal ion to the center of the porphyrin, decreases the probability of perturbing the complex. We find a linear increase in the Cu²⁺-Cu²⁺ distances as the number of guanines in the sequence increases, indicative of tetramolecular complex formation with the ligands end-stacked onto the quadruplex. Moreover, based on the mean distances measured between the paramagnetic centers, we discover a monotonic decrease in the ligand end-stacking distance as the quadruplex length is extended.

MATERIALS AND METHODS

Materials

Water with a resistivity of 18.2 MΩ cm was used for all experiments. CuTMpyP4 was purchased as the chloride salt. Solutions of CuTMpyP4 in H₂O were stored in the dark to prevent unwanted photochemical reactions. All chemicals were purchased from commercial suppliers and used without further purification.

All oligonucleotides were purchased from a commercial supplier as lyophilized pellets, resuspended in water and stored at 4 °C. The concentration of each oligonucleotide was determined by measuring its absorbance at 260 nm using extinction coefficients calculated using the nearest-neighbor method as reported previously⁴⁸. Concentrations of oligonucleotides are per strand unless otherwise stated. Solutions containing 2.0 mmol L⁻¹ G4, G6, G8 and G10 were each combined with 1.0 mmol L⁻¹ CuTMPyP4 in 50 mmol L⁻¹ tris(hydroxymethyl)aminomethane, 50 mmol L⁻¹ boric acid at pH 8.3 and heated to 95 °C. After 5 min, KCl was added to achieve a final potassium concentration of 10 mmol L⁻¹ in each sample, and the sample mixtures were heated at 95 °C for an additional 5 min. Samples were then slowly cooled to room temperature over a 2 h to 3 h period and subsequently stored at 4 °C overnight.

Methods

Polyacrylamide gel electrophoresis—Sample purification was performed using non-denaturing polyacrylamide gel electrophoresis (PAGE) on 20 % polyacrylamide gels containing 10 mmol L⁻¹ KCl. Gel electrophoresis was carried out at 4 ± 1 °C for 4 h to 6 h at 250 V. The running buffer was 44.5 mmol L⁻¹ tris(hydroxymethyl)aminomethane, 44.5 mmol L⁻¹ boric acid, 1 mmol L⁻¹ ethylenediaminetetraacetic acid (0.5× TBE) with 10 mmol L⁻¹ KCl. All gels were wrapped in plastic and placed on a fluorescent thin layer chromatography (TLC) plate for UV shadowing and for photographing the visible image (see Figure S1). Bands containing purified quadruplexes with bound CuTMPyP4 were excised from the gel using a clean razor blade and then isolated using the crush and soak protocol⁶³.

Circular dichroism spectroscopy—Circular dichroism (CD) measurements were conducted on a spectropolarimeter at room temperature. Solutions for CD experiments in 10 mmol L⁻¹ potassium phosphate with 50 mmol L⁻¹ KCl contained gel-purified G4, G6, G8 and G10, with bound CuTMPyP4 at concentrations of 37.6 μmol L⁻¹, 40.0 μmol L⁻¹ and 38.6 μmol L⁻¹, respectively, at a matched oligonucleotide optical density of 4.0. Each sample was loaded into a 0.1 mm path length quartz cylindrical cuvette and a total of five scans were acquired and subsequently averaged to produce each spectrum. A background spectrum, consisting only of buffer solution, was collected and subtracted from each of the averaged sample spectra. All spectra displayed characteristic lineshapes for parallel stranded quadruplexes, with a maximum near 263 nm and a local minimum near 240 nm^{64, 65}. CD spectra are displayed in Figure S2, with ellipticity expressed in millidegrees.

EPR spectroscopy—H₂O and mono/di-basic potassium phosphate were exchanged for deuterium oxide and deuterated mono/di-basic potassium phosphate by three rounds of sequential concentration and dilution using centrifugal concentrators. For G4 samples, concentrators with a nominal molecular weight limit (NMWL) of 3 000 were used, while for G6, G8 and G10 samples concentrators with a NMWL of 10 000 were used. A final buffer exchange using 10 mmol L⁻¹ deuterated potassium phosphate, 50 mmol L⁻¹ KCl with 100.14 g d8-glycerol in 20.0276 g deuterium oxide at pD = 7.0 ± 0.05 pH units was performed, and samples were stored at 4 °C until EPR measurements were made. Deuterated solutions containing 600 μmol L⁻¹ G4 plus 920 μmol L⁻¹ CuTMPyP4, 204 μmol L⁻¹ G6

plus 660 $\mu\text{mol L}^{-1}$ CuTMpyP4, 430 $\mu\text{mol L}^{-1}$ G8 plus 630 $\mu\text{mol L}^{-1}$ CuTMpyP4 and 310 $\mu\text{mol L}^{-1}$ G10 plus 500 $\mu\text{mol L}^{-1}$ CuTMpyP4 were used for the pulsed EPR experiments.

Pulsed EPR measurements at X-band (approximately 9.5 GHz) were performed on a commercial pulsed EPR spectrometer using a split ring resonator with a maximum sample access of 5 mm. The resonator was over-coupled in all experiments to a quality factor of approximately 100. All experiments were conducted at 30 K. At this temperature, the phase memory time of the samples was found to be approximately 11 μs by monitoring Hahn echo decay, $\pi/2$ -T- π -echo. The longitudinal relaxation time was measured to be approximately 430 μs using a picket-fence saturation sequence of 20 successive π pulses, where the time between saturation pulses was 400 ns, and a four step phase cycle was used⁶⁶. Temperature stabilization within a continuous flow cryostat was controlled by a commercial temperature controller. The dead-time free four pulse DEER sequence, $\pi/2_{\text{observe}}-\tau_1-\pi_{\text{observe}}-T-\pi_{\text{pump}}-\tau_2-\pi_{\text{observe}}-\tau_2$ -refocused echo, was employed for all measurements⁶⁷ (see Figure S3 for the pulse sequence). The refocused echo was integrated over the full width at about 1/3 height and taken as the signal. The video bandwidth was set to 20 MHz. All samples for the DEER experiments were loaded into quartz tubes of 4.0 mm outer diameter, flash frozen in liquid nitrogen, and inserted into the precooled cryostat.

Within conventional bridge designs, microwave pulses for resonant excitation are shaped by fast diode switches. The rise and fall times of these switches are on the order of the observer pulse lengths for DEER at X-band, consequently leading to deviations from ideal rectangular shapes. Technical limitations of the spectrometer's electronics, in addition to the value of the loaded quality factor of the resonator, further restrict the excitation bandwidth of the pulses⁶⁸. Moreover, in the frequency domain, the sidebands of $\sin x/x$ excitation profiles can generate non-selective excitation and lead to unwanted artifacts in the detected signal. Perhaps of greater consequence than the non-ideality of pulse shapes within the field of EPR spectroscopy, the spectral width of transition metal species often falls in the gigahertz range, far exceeding the excitation bandwidth of ideal rectangular pulses by at least one order of magnitude. These aforementioned limitations must be considered during pulsed EPR experiments using rectangular shaped pulses to manipulate polarization transfer or coherence order in multilevel spin systems.

Alternative excitation schemes using shaped pulses to provide considerably wider bandwidths than rectangular pulses have shown superior performance in Fourier transform-EPR experiments^{69, 70} pulsed EPR distance measurements⁷¹ and also in two-dimensional correlation spectroscopy⁷². Such experiments rely on an arbitrary waveform generator (AWG) with (sub)ns time resolution to generate the shaped pulses. In this work, a commercially available AWG with a 625 ps time resolution was used with an existing commercial EPR spectrometer to modulate the microwave local oscillator. The output of the AWG is supplied to a commercial 1 kW traveling-wave tube amplifier and subsequently directed to the EPR resonator. In the particular case of DEER spectroscopy conducted on transition metal complexes, where selective orientational excitation is not desired, pulses based on adiabatic full passage are uniquely suited to uniformly invert transverse magnetization of the spin system over a broad bandwidth, thereby increasing the sensitivity of the measurement⁷³. As a member of the adiabatic pulse family, a sech amplitude

modulation and tanh frequency modulation (sech/tanh) pulse was selected for broadband inversion at the pump frequency⁷⁴. This pulse shape was previously used and optimized by the group of Prisner to perform DEER on a cobalt-nitroxide biradical⁷⁵. The apposite variables describing the microwave pulses originating from the AWG are described in detail below.

The pump frequency was set to coincide with the resonant frequency of the resonator. At this frequency, a sech/tanh pulse, 200 ns in length with a bandwidth of 150 MHz, was applied to achieve broadband inversion⁷⁵. The pulse length is expected to be short relative to the inverse of the dipolar coupling frequency, which is necessary in order to avoid a time-dependent offset of the dipolar evolution⁷⁶. Pulse power was optimized at the pump frequency using the inverted-echo sequence, sech/tanh-T- $\pi/2$ - τ - π - τ -echo, so that the echo exhibited maximum inversion. All observer pulses used to generate the refocused echo were conventional rectangular shaped pulses. The frequency of the observer pulses was set to be 135 MHz higher than that of the pump pulse. This arrangement permitted approximately 50 MHz separation between pump and observer stopbands, thereby minimizing excitation overlap. The increased steepness of the transition from passband to stopband of the sech/tanh pulse relative to that of a rectangular pulse further decreases the probability of observer/pump overlap. The lengths of the observer pulses were optimized for maximum amplitude of a Hahn echo formation. Usually this configuration resulted in 25 ns and 50 ns $\pi/2$ and π pulses, respectively. The shot repetition time was set to 600 μ s, and spectra were typically averaged over a one-to-three day time frame.

An eight step phase cycle was used to eliminate all unwanted coherence-transfer pathways, as the pump and observer pulses produced from the AWG are phase locked. In order to suppress unwanted deuterium electron spin echo envelope modulations (ESEEM) to the DEER signal, τ_1 , the time between the first and second observer pulse, was set to equal one oscillation period of the Larmor precession of ^2H . The pump pulse was stepped in 8 ns increments, starting 220 ns before primary echo formation and ending at slightly more than one pump pulse length away from the starting edge of the third observer pulse.

RESULTS AND DISCUSSION

DEER Results

The objective of this study is to ascertain topological details of G-quadruplexes possessing varying lengths of G-tracts with bound ligands. Pulsed EPR measurements on samples with paramagnetic ligands afford nanometric structural resolution without the use of extrinsic labels, thereby ensuring preservation of the native-like structure of the ligand-quadruplex complexes. DEER spectroscopy at X-band employing an AWG for broadband inversion at the pump frequency is used towards this end.

A two-pulse echo detected field swept spectrum of G10 + CuTMpyP4 at both the pump and observer frequencies is displayed in Figure 2. The presence of orientational selectivity was probed by acquiring DEER spectra at multiple magnetic fields; two positions in the $g_{\perp}$ region and one in the $g_{\parallel}$ region, as suggested by Yang et al⁷⁷. For each quadruplex-ligand sample, the DEER experiments were performed at each field position numbered in Figure 2,

with a constant offset of 135 MHz between the observer and pump frequencies. In an effort to simultaneously reduce potential orientational selectivity effects and also to increase the modulation depth, an amplitude and frequency swept sech/tanh pulse with a bandwidth of 150 MHz was used at the pump frequency.

Each data set was analyzed using two open source programs for comparison and validation of the extracted distance distributions; DeerAnalysis2015⁷⁸ was used to conduct Tikhonov regularization on the background subtracted time domain trace, and DD⁷⁹ to perform model based fitting with multiple Gaussian peaks (up to three in this work). Moreover, DD was used to fit the DEER data without *a priori* background correction to obtain unbiased results from the analysis. For each field position, the DEER time traces are shown within the **a** panel of Figures 3–6. Following background removal (**b** panels, Figures 3–6), distance distributions modeled using a single Gaussian are shown in the **c** panels of Figures 3–6.

Determination of the Distance Distributions

The background subtracted DEER time domain traces display no appreciable differences in their modulation periods for each sample at the various field positions. In addition, the perpendicular singularities of the frequency domain spectra (see Figure S4), in which the static magnetic field and interspin vectors are orthogonal, show nearly comparable splittings at each field position. The similarities of the spectra denote the paucity of orientational selection. Broadband inversion from the sech/tanh pump pulse reduces the effects of orientational selectivity compared to that of a rectangular pulse with the same B_1 maximum, yet even this bandwidth is small relative to the spectral range between field positions 1 and 3. Experiments conducted using all rectangular pulses yield nearly identical results for time and frequency domain spectra as well as for the distance distributions (Figure S9). In such a situation where orientational selectivity effects are negligible, it is advantageous to use broadband shaped pulses to increase the modulation depth of the DEER traces, and ultimately the SNR. At field position 1, the modulation depth is increased by a factor of 1.5 when pumping with the sech/tanh pulse compared to that of a 26 ns rectangular pulse.

A single Gaussian peak was found to suitably fit the data, with insignificant improvement upon fitting the data to two or three Gaussians. For all DEER measurements except G4 + CuTMpyP4 at field position 2 and G6 + CuTMpyP4 at field position 2, the model in which the error to the fit was best matched to the noise in the data was that of a single Gaussian as determined from the reduced chi squared values (see Figure S11, panels **a–d**). Moreover, the change in the Akaike information criterion corrected⁸⁰ is insignificant when the number of Gaussian peaks is increased from one to three (Figure S11, panels **e–h**), implying a single Gaussian sufficiently describes the dataset, and multiple Gaussians could lead to overfitting. Thus, a single Gaussian was chosen as the model with which to describe the distance distribution. These statistical results indicate that the data are well characterized by an isolated two electron spin system, reflecting a 2:1 ratio of CuTMpyP4 to G-quadruplex, consistent with previous work on this system.⁴⁸

Distance distributions extracted using Tikhonov regularization and Gaussian models are superimposed in panels **g–i** of Figures S5–S8 and display similar probabilistic values for both the maximum and full width at half maximum. Using the combined results obtained

from the goodness of fit comparison of multimodal Gaussian distributions, the lack of observable orientational selectivity effects and the clear similarities between distributions derived from both Tikhonov regularization and model based approaches, a single Gaussian peak at field position 1 most adequately describes the Cu^{2+} - Cu^{2+} distance constraints. This selection enables analytical error analysis of the fitting parameters, an unbiased background subtraction to the raw DEER data, and also ensures optimal resolution of the dipolar modulations by pumping at the maximum amplitude of the absorption spectrum.

Analysis of Single Gaussian Distributions

Broad distance distributions are observed for each of the quadruplex samples. Standard deviations of the distributions using a single Gaussian peak range from 0.48 nm to 0.65 nm. These results contradict a model in which two rigid coplanar Cu^{2+} ligands are bound to the termini of a G-quadruplex, where orientational selectivity effects would be maximized and narrower distance distributions would be expected (Figure 7a). Rather, they suggest that some motional degrees of freedom exist when CuTMPyP4 binds the G-quadruplex (Figure 7b). This situation likely arises from the non-covalent nature of the binding, where the complex is stabilized by electrostatic and π - π interactions. The previously reported binding constants of CuTMPyP4 to quadruplexes G4 and G8 are approximately $5.6 \times 10^6 \text{ mol}^{-1} \text{ L}$ and $5.2 \times 10^7 \text{ mol}^{-1} \text{ L}$, respectively, determined using the Scatchard model¹⁸. Under such binding conditions, it is feasible that a distribution of the copper porphyrin around the central potassium channel of the quadruplex exists, which would result in a broadening of the observable Cu^{2+} - Cu^{2+} distances. In addition, deviations to the planarity of the external G-quartets in relation to those located in the interior of the quadruplex could result in a distribution of the relative orientations of the Cu^{2+} g -tensors, thereby decreasing orientational selectivity effects (Figure 7c). G-quartets close to the termini of a quadruplex have been shown to experience out-of-plane deformation^{17, 81}.

The mean values of the distance distributions display a linear increase as the G-quadruplex is extended by additional quartets. For G4, the mean Cu^{2+} - Cu^{2+} distance is 2.91 nm; for G6, G8 and G10 it is 3.35 nm, 3.96 nm and 4.30 nm, respectively (Table 2). Ligand binding through intercalation would not be expected to generate such a strong linear relationship. Moreover, if the longer sequences G8 or G10 were to adopt a monomeric fold-back G-quadruplex structure, significantly shorter Cu^{2+} - Cu^{2+} distances, approaching a factor of two relative to the observed values, would be expected. Therefore, a 2:1 ratio of CuTMPyP4 to quadruplex implies the ligands are end-stacked and that the quadruplex is an in-register tetramolecular structure. Using the known high-resolution crystal structure of a parallel stranded tetramolecular G-quadruplex⁸², we assumed a distance of 0.36 nm between all interior G-quartets in the ligand-quadruplex complex. Moreover, from NMR studies of TMPyP4 end-stacked onto the *Pu241* quadruplex in the human *MYC* promoter region³⁵, we infer a distance of 0.42 nm between the terminal G-quartets and bound CuTMPyP4. These stacking distances allow for some prediction of expected Cu^{2+} - Cu^{2+} distances based on the oligonucleotide sequence length. It is important to keep in mind, however, that both the X-ray diffraction and NMR experiments were performed at much higher concentrations than used in our experiments. These predictions are plotted against the measured distances in Figure 8a.

Structural Implications for Tetramolecular G Quadruplexes with End-Stacked Ligands

The mean values of the measured Cu^{2+} - Cu^{2+} distances for all G-quadruplexes display superb correlation with oligonucleotide sequence length, yielding a coefficient of determination equal to 0.987. However, the slope of linear regression line, equal to 0.66, is less than unity. Consequently, as seen in Figure 8a, the measured distance of G10 is in excellent agreement with the predicted value, yet as the oligonucleotide sequence shortens, measured distances increasingly deviate from those of prediction, so that the shortest sequence, G4, is nearly 1 nm greater than its predicted value. As DEER is an ensemble measurement, a combination of two possible scenarios may be responsible for this observation. Either the ligand stacking energy is dependent on the overall length of the quadruplex structure, thereby altering only the ligand stacking distance, or the overall morphology of the ligand bound quadruplex structure is variable for quadruplexes of varying sequence lengths. These two extreme scenarios are depicted in Figure 8b. Here, the open circles represent the distance between an end-stacked ligand and a terminal G-quartet, where the quartet-to-quartet distance within the quadruplex is fixed at 0.36 nm. Conversely, the filled circles assume a model where the entire quadruplex-ligand structure is permitted to stretch or compress along the axis normal to the plane of a quartet. Of these two extreme models, the one in which the entire quadruplex-ligand structure is allowed to oscillate is the most physically realistic, as there is an enthalpic binding penalty associated with an increased ligand distance from the G-quartet. We hypothesize that this oscillation would manifest as a change in the helical pitch of the quadruplex; an increase in the number of consecutive G-quartets is complimented possibly by a decrease in the pitch of the quadruplex backbone. A model depicting a conformational change in helical pitch from G8 to G4 is shown as a cartoon in Figure 9.

As mentioned above, with an ensemble measurement, only extreme values regarding the inter-quartet distances may be deduced; either all inter-quartet distances are fixed (open circles, Figure 8b) and the ligand-quartet distance is solved for, or all are distances are equally varied (filled circles, Figure 8b). The more likely scenario is that the rise and planarity between G-quartets varies over the length of the quadruplex. For example, in parallel quadruplex structures, it has been shown that G-quartets closer to the quadruplex termini experience buckling and deformation^{40, 84}. Though the DEER technique is not capable of generating atomistically resolved structures of G-quadruplexes, i.e. it cannot be used to precisely assign individual quartet-quartet distances, the obtained distance constraints between the paramagnetic end-stacked ligands clearly indicate deviations in inter-quartet distances as the number of quartets in the G-quadruplex changes. The conformational changes associated with the poly-G sequence length put forth here could in fact have biological relevancy, such as the potential for progressively longer G-quadruplexes to exhibit altered drug binding affinities.

In summary, we have reported on the first DEER experiment conducted between transition metal pairs that are non-covalently bound to a biomacromolecule, the nature of which is postulated to reflect the breadth of the distance distributions. The experiments employed pulse shaping at the pump frequency for broadband inversion, and were performed on copper porphyrins bound to model, parallel stranded G-quadruplexes with varying numbers

of guanine quartets. The results indicate a degree of conformational flexibility of the ligand greater than expected. Average Cu^{2+} - Cu^{2+} distances linearly increase as the poly-G repeat lengthens, though the stacking distance between the ligand and an external quartet monotonically decreases as the number of quartets increases. This suggests that the average inter-quartet distance is dependent on the length of the entire quadruplex. Our results show that a single Gaussian distribution was found to suitably represent the data, indicative that relatively long poly-G stretches, up to ten, form tetramolecular quadruplexes with two end-stacked ligands.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Prof. Liliya A. Yatsunyk (Swarthmore College) is thanked for her critical reading of the manuscript and for stimulating discussions on the topic of G-quadruplexes. Drs. Curt W. Meuse and Jason K. Streit (National Institute of Standards and Technology, Gaithersburg) are acknowledged for their help in conducting circular dichroism measurements. Dr. Sergey Milikisyants (North Carolina State University) is acknowledged for helpful discussions regarding pulsed EPR experiments.

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Figure 1.
a Hoogsteen hydrogen-bonded G-quartet displaying the arrangement of the bases. **b** Structure of the Cu²⁺ derivative of 5,10,15,20-tetrakis(1-methyl-4-pyridyl)-21*H*,23*H*-porphine (CuTMpyP4).

Figure 2.

Field swept echo detected ($\pi/2$ - τ - π , where $\pi/2 = 15$ ns $\pi = 30$ ns $\tau = 200$ ns) EPR spectra of G10 + CuTMpyP4 recorded at the pump frequency (red) and the observer frequency (black). The offset frequency ($\nu_{\text{observer}} - \nu_{\text{pump}}$) is 135 MHz. The numbered arrows indicate the magnetic field positions at which DEER experiments were performed.

Figure 3.

Analysis and extraction of distance distributions from G4 + CuTMpyP4 at field positions indicated on Figure 2. **a** DEER traces (solid lines) before subtraction of the background factor (dashed lines). **b** DEER traces after background subtraction (dashed lines) with the simulated form factor (solid lines) within DEERAnalysis2015. The vertical dashed line is a guide to the eye for the first modulation period. **c** Distance distributions extracted without *a priori* background correction using a single Gaussian peak, scaled to the respective modulation depths.

Figure 4. Analysis and extraction of distance distributions from G6 + CuTMpyP4 at field positions indicated on Figure 2. Panels **a–c** carry the same descriptors as for Figure 3.

Figure 5. Analysis and extraction of distance distributions from G8 + CuTMpyP4 at field positions indicated on Figure 2. Panels **a–c** carry the same descriptors as for Figure 3.

Figure 6. Analysis and extraction of distance distributions from G10 + CuTMpyP4 at field positions indicated on Figure 2. Panels **a–c** carry the same descriptors as for Figure 3.

Figure 7.

Possible models for CuTMpyP4 end-stacking onto G-quadruplexes based on DEER distance distributions. G-quartets (blue rectangles) forming a rigid, parallel stranded tetramolecular G-quadruplex with end-stacked CuTMpyP4 ligands (green rectangles). **a** The lack of orientational selectivity and the breadth of the distance distributions suggest flexibility in ligand binding, where the two CuTMpyP4 molecules are neither coplanar nor rigidly bound to the quadruplex. **b** Translational flexibility of CuTMpyP4 around the central potassium channel would result in an increase in the width of the distance distribution. **c** An inclination of the exterior quartets would increase the distribution of the relative orientations of the two Cu^{2+} g -tensors.

Figure 8.

a A comparison of the measured Cu^{2+} - Cu^{2+} distances obtained from fitting to a single Gaussian distribution as a function of predicted Cu^{2+} - Cu^{2+} distances. Filled squares indicate the mean extracted distances, the error bars indicate one standard deviation and the red diamonds represent predicted values for each quadruplex. Blue curly brackets denote the residual distance between the mean extracted distances and predicted distances for each quadruplex. The dashed line is a linear regression of the mean extracted distances, where the coefficient of determination is equal to 0.987. **b** Estimations of the average stacking distance between a CuTMpyP4 molecule and a terminal G-quartet. Open circles represent the situation in which the quartet-quartet stacking distance is fixed. Filled circles represent the case in which the entire complex is permitted to stretch along the axis normal to the quartet plane, with the center of mass fixed.

Figure 9.

Model of parallel stranded tetramolecular G-quadruplexes of different continuous G-quartet lengths upon ligand binding, where G8 is represented by the cartoon on the left and G4 by the cartoon on the right. Helical pitch compression is postulated to occur in G-quadruplexes with progressively longer G-tracts. Green polygons represent the bases, and white polygons and ribbons represent the sugar phosphate backbone. For clarity, only a single oligonucleotide strand within the quadruplex is depicted. Oligonucleotide coordinates for both images were obtained from PDB code 352D⁸² and the cartoons rendered using Discovery Studio Visualizer 4.1⁸³. Images have been manipulated to convey the structural differences between G4 and G8 as put forth in the model within the main text.

Table 1

Sequences of single stranded oligonucleotides

Abbreviation	Sequence
G4	TTTTGGGGTTTT
G6	TTTTGGGGGGTTTT
G8	TTTTGGGGGGGGTTTT
G10	TTTTGGGGGGGGGGTTTT

Table 2

Summary of mean distances and standard deviations from single Gaussian fitting to background subtracted DEER traces at field position 1

Quadruplex name	Cu ²⁺ -Cu ²⁺ <r> (nm)	Cu ²⁺ -Cu ²⁺ σ (nm)
G4	2.91	0.59
G6	3.35	0.54
G8	3.96	0.48
G10	4.30	0.65