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Examination of the effect of the annealing cation on higher order structures containing guanine or isoguanine repeats

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Abstract

Isoguanine (2-oxo-6-amino-guanine), a natural but non-standard base, exhibits unique self-association properties compared to its isomer, guanine, and results in formation of different higher order DNA structures. In this work, the higher order structures formed by oligonucleotides containing guanine repeats or isoguanine repeats after annealing in solutions containing various cations are evaluated by electrospray ionization mass spectrometry (ESI-MS) and circular dichroism (CD) spectroscopy. The guanine-containing strand (G9) consistently formed quadruplexes upon annealing, whereas the isoguanine strand (Ig9) formed both pentaplexes and quadruplexes depending on the annealing cation. Quadruplex formation with G9 showed some dependence on the identity of the cation present during annealing with high relative quadruplex formation detected with six of ten cations. Analogous annealing experiments with Ig9 resulted in complex formation with all ten cations, and the majority of the resulting complexes were pentaplexes. CD results indicated most of the original complexes survived the desalting process necessary for ESI-MS analysis. In addition, several complexes, especially the pentaplexes, were found to be capable of cation exchange with ammonium ions. *Ab initio* calculations were conducted for isoguanine tetrads and pentads coordinated with all ten cations to predict the most energetically stable structures of the complexes in the gas phase. The observed preference of forming quadruplexes versus pentaplexes as a function of the coordinated cation can be interpreted by the calculated reaction energies of both the tetrads and pentads in combination with the distortion energies of tetrads.

Introduction

The assembly of guanine base repeats in tetrads around central cations results in the formation of higher order DNA structures known as quadruplexes.(1) Quadruplexes have been studied extensively due to their role in halting both the immortalization of cells and tumor growth by preventing the binding of telomerase, a key enzyme responsible for the elongation of DNA. (2–5) Bases other than guanine are also capable of forming higher order DNA structures including triplexes, quadruplexes, and pentaplexes.(6) In addition to the growing body of evidence that supports the presence and function of higher order DNA structures formed by natural DNA bases, there is increasing interest in designing and exploiting the ability of both

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modified bases and non-standard bases to engage in intermolecular interactions, creating novel architectures for sensing applications and possible use as ion channels.(7–9)

Isoguanine, a non-standard but naturally occurring base, has been shown to form higher order complexes similar to guanine (Scheme 1). (8,10–15) While the difference between guanine and isoguanine only involves the transposition of the carbonyl and amino groups, the change in the structure of isoguanine has been shown to favor the formation of pentameric rather than tetrameric multimers in studies of the interactions of the free bases with large cations. (16, 17) This striking change in multimeric assembly is rationalized based on the decrease in the hydrogen bonding angle between bases which favors inclusion of a fifth base in the ring to maintain planarity. In addition, several computational studies have reported that isoguanine pentads formed around various monovalent cations have high interaction energies between bases and are planar complexes while the tetrads are far less planar. (18–21) Previous studies have noted a strong preference for the self-assembly of isoguanine around cesium over potassium ions. (22) In addition, individual isoguanosine units functioning as ionophores were found to be selective for singly charged cations in the order $\text{Cs}^+ \gg \text{Rb}^+ > \text{K}^+ > \text{Na}^+$ and for doubly charged cations in the order $\text{Ca}^{2+} > \text{Sr}^{2+} > \text{Ba}^{2+} > \text{Mg}^{2+}$. (15) The cation in the central cavity also has a significant template role in the formation of multimeric DNA structures from guanine- and isoguanine-containing strands. Both monovalent and divalent cations can support the formation of quadruplexes from guanine-rich strands.(23–27) In contrast, isoguanine-containing strands form pentaplexes around cesium cations but form quadruplexes around potassium, whereas the analogous guanine-containing strands consistently form quadruplexes. (28) The impact of the central cation on the formation and stability of pentaplexes by isoguanine-containing strands has not been systematically explored.

ESI-MS has been increasingly employed as a versatile and sensitive tool for the study of higher order DNA complexes.(29,30) While most of the studies have focused on quadruplexes(31–36) and their interactions with drugs or small molecules,(37–44) DNA triplexes have also been characterized.(45) ESI-MS allows ready assessment of the stoichiometries of complexes containing different numbers of cations and differentiation of single strand and multimeric species. Circular dichroism (CD) is also an established technique for studying biomolecular structures, including guanine quadruplexes in which the stacking of guanine bases affords strong optical activity.(42,46–50) While CD is rather unresponsive to changes in fine structure or the sequences of the strands involved in the quadruplexes, it is sensitive to the overall conformations of the quadruplexes.(51) In addition, most salts and buffers are compatible within the range of wavelengths of interest for studying DNA complexes. Polyisoguanine strands have also been studied previously by CD spectroscopy(52) as have complexes composed of individual bases around a central cation.(15)

Unlike guanine quadruplexes, isoguanine quadruplexes and pentaplexes have not been studied experimentally in detail. However, high level *ab initio* calculations provide an alternative approach to predict the structures of higher order DNA complexes. While the study of full quadruplex or pentaplex complexes is extremely resource-intensive due to the large number of atoms involved in the calculations, high level *ab initio* calculations have been carried out on smaller-scale isoguanine tetrads and pentads.^{18–22} Most investigations completed previously focused on alkali metal ions alone and did not explore a full range of metals cations.

In this work, the formation of quadruplexes and/or pentaplexes by strands containing either guanine repeats or isoguanine repeats is studied by ESI-MS and CD spectroscopy. The strands are annealed in solutions containing a variety of singly charged and doubly charged template cations including Li^+ , Na^+ , K^+ , Rb^+ , Cs^+ , NH_4^+ , Mg^{2+} , Ca^{2+} , Sr^{2+} , and Ba^{2+} . The wide range in ionic radii of these cations allows investigation of the effects of charge and ion size on the formation of higher order complexes. ESI-MS is used to determine the extent of complex

formation as well as the identity and number of the central cations incorporated in the complexes. CD spectroscopy provides information about the structures and conformations of the complexes and is used to reveal how sample preparation affected the structures of interest. *Ab initio* optimizations with density functional theory are undertaken for isoguanine tetrad and pentad structures incorporating all ten cations. For each complex, three different symmetries, specifically C_{4h} , D_4 and S_8 for isoguanine tetrads and C_{5h} , D_5 and S_{10} for isoguanine pentads, are investigated. The computational results are used to support the experimentally observed trends concerning the formation of quadruplexes and pentaplexes by isoguanine-containing strands.

Materials and Methods

Materials

The isoguanine-containing strand studied here, dTlIgIgIgTTTT (Ig9), where Ig refers to isoguanine, was synthesized as described previously.⁽⁷⁾ The corresponding guanine-containing strand, dTGGGGTTTT (G9), was obtained from IDT DNA, Inc (Coralville, IA). The Ig9 strand with a 5'-end modification consisting of phosphocholine bound to a linker (Ig9PC) was synthesized as described.⁽⁷⁾ The latter structure is shown in Scheme 2. Spectroanalyzed methanol, HPLC grade water, and ammonium acetate were purchased from Thermo Fisher Scientific (Waltham, MA). Lithium chloride, rubidium chloride, cesium chloride, magnesium chloride dihydrate, calcium chloride dehydrate, strontium chloride hexahydrate, and barium chloride were purchased from Sigma Aldrich (St. Louis, MO). Sodium chloride and potassium chloride were obtained from EM Science (Gibbstown, NJ). All salts were dissolved in water to make stock solutions. Ionic radii are based on Goldschmidt hard sphere ionic radii.⁽⁵³⁾

Methods

Both G9 and Ig9 were annealed in a similar manner. The strands and appropriate salts at concentrations described in the text were placed in a hot water bath and cooled to room temperature overnight (approximately 12 hours). The concentration of the single strands during annealing was approximately 100–125 μ M. The annealed solutions were placed in the freezer until analyzed. Immediately before ESI-MS analysis, all solutions except those containing ammonium acetate were desalted using Millipore (Billerica, MA) Biomax Ultrafree 5 kDa molecular weight centrifugal filters. After desalting, ammonium acetate and methanol were added to the solutions to assist desalting and enhance desolvation of the analytes. The ammonium acetate (50 mM final concentration) and methanol (20% by volume) were added no more than two minutes before analysis. Assuming complete formation of the quadruplexes, in the case of G9, or pentaplexes, in the case of Ig9, the final concentrations of the complexes were approximately 3 μ M. Ig9PC was annealed in 150 mM ammonium acetate under similar conditions to the G9 and Ig9. ESI-MS analysis was performed on a Thermo LTQ mass spectrometer (San Jose, CA). Analysis was performed in the negative mode with a heated capillary temperature of 90°C and a spray voltage of 3.5 kV. The tube lens voltage was approximately -250 V.

To estimate the relative complex formation of quadruplexes or pentaplexes from the ESI mass spectra, peak areas of the complexes were summed and divided by the peak areas of the complexes and the free single strands as shown in the following equation:

$$\text{Relative Complex Formation} = \frac{PA_Q \text{ or } PA_P}{PA_Q + PA_P + PA_{SS}}$$

where PA_Q and PA_P refer to the peak areas of the quadruplex and pentaplex species, respectively, and PA_{SS} refers to the peak area of the single strand. There were no pentaplexes detected for the G9 solutions, resulting in $PA_P = 0$ for all G9 solutions. Results discussed in this work are the average of three analyses.

Annealed solutions of G9 and Ig9 were also analyzed by CD spectroscopy via a Jasco (Easton, MD) J-815 circular dichroism instrument. The solutions were analyzed in a 0.1 cm cuvette over a wavelength range of 320 to 220 nm. Three sets of spectra were collected for each mixture: the first after simple dilution in water to 300 μ l, the second after filtration with the 5kDa molecular weight filters as described above, and the third after adding ammonium acetate to the filtered solution so that the final concentration of ammonium acetate in the solution was 50 mM. Five scans were averaged for each spectrum. For the solutions analyzed before desalting, individual blanks containing the salt used for annealing were subtracted through the software. A water blank and a 50 mM ammonium acetate blank were subtracted from the spectra taken after desalting and the spectra taken after addition of ammonium acetate, respectively. CD spectra were not normalized against one another.

For each complex, three conformations representing three molecular point groups were constructed for *ab initio* optimizations using B3LYP/lacv3p**.(54) This basis set, which utilizes effective core potentials for K^+ , Rb^+ , Cs^+ , Ca^{2+} , Sr^{2+} , and Ba^{2+} , and 6-311G** for the other cations, offers a reasonable balance between efficiency and accuracy in studying complexes containing metallic elements. The molecular symmetry was enforced during the optimization. All the calculations were performed using the Jaguar module (version 7.5) of Schrodinger, LLC. Considering that the formation of isoguanine quadruplexes requires the tetrad planes be parallel to each other, a constrained optimization that enforces the planarity of tetrad was employed for each of the tetrads. Technically, the cation was placed in the origin and the tetrad planes were perpendicular to the Z axis. The constraint was imposed by making the Z-coordinates z for the four isoguanines above the XY plane and $-z$ for the four isoguanines below the XY plane, where z is an optimized parameter. Similar conditions were used for the isoguanine pentad calculations. It should be noted that neither a frequency analysis nor BSSE (basis set superposition error) calculation was performed since the major purpose was to study the geometries of the complexes and to compare the relative template ability of the ten cations to promote the formation of isoguanine tetrads and pentads. According to recent literature, the zero point and BSSE corrections should not change the overall trends.(18,20)

Results and Discussion

ESI-MS analysis of G9

G9 was annealed in the presence of ten different cations, and the resulting solutions were analyzed by ESI-MS after removal of excess salts. Representative spectra from these solutions are shown in Figure 1. The spectra obtained for G9 with Li^+ , Mg^{2+} , Na^+ , and Cs^+ show little or no presence of quadruplexes, either due to lack of formation during annealing or due to dissociation of the quadruplexes during clean-up (as discussed more later). In contrast, the spectra obtained for G9 with Sr^{2+} , K^+ , NH_4^+ , Ba^{2+} , Rb^+ displayed large abundances of quadruplexes. G9 annealed in Ca^{2+} resulted in approximately equal abundances of single strands and quadruplexes. The results are summarized in bar graph form in Figure 2 in the order of increasing ionic radius of the annealing cation. It is clear that the cations within a certain range of radii afford far more efficient quadruplex formation as opposed to cations with very large (Cs^+) or small (Li^+) radii.

In addition to allowing the ready assessment of the efficiency of quadruplex formation, ESI-MS allows the determination of the identity and number of central cations present within the structures, as summarized for ions in the dominant 6- charge states in Table 1. For most of the

solutions, the ESI mass spectra revealed that the number of central cations in the dominant form of the quadruplex was three, along with far less abundant complexes containing varying numbers of cations. This is consistent with a pattern reported previously in which $n - 1$ central cations are associated with n number of tetrads in a tetramolecular quadruplex.(55) Based on the G9 sequences used in the present study, the formation of four tetrads is possible, supporting the inclusion of three cations. Overall, the number of cations included generally followed the $n - 1$ rule for singly charged cations. However, for the divalent cations Sr^{2+} and Ba^{2+} , only two cations were associated with the quadruplexes. While these cations are large, several monovalent cations that were larger or the same size promoted the incorporation of three cations. Thus, it appears that the greater charge density of Sr^{2+} and Ba^{2+} prevents the inclusion of a third cation in the quadruplex. In contrast to the results for the solutions containing Sr^{2+} and Ba^{2+} , four Mg^{2+} ions were incorporated in the quadruplexes. This ion, with a radius approximately half that of Ba^{2+} , is the smallest one retained within the complex. It appears that quadruplex formation and cation retention are related to both the size and charge of the annealing cations.

In several cases, the m/z values of the quadruplexes in the mass spectra indicate that the central cations are not the original templating cations used during annealing but instead are ammonium ions. Quadruplexes in which the cations were replaced by ammonium are denoted by a box in Figure 2. Ammonium acetate is added to each solution after the desalting procedure that is essential for successful ESI-MS analysis of solutions containing high concentrations of non-volatile salts. Cation exchange within G-quadruplexes has been observed previously by Ma et al. and attributed to the sequential replacement of cations of lower affinity with those of higher affinity.(31) The quadruplexes that undergo exchange of the annealing cations for NH_4^+ include those originally annealed in the presence of Li^+ , Ca^{2+} , Rb^+ , and Cs^+ . In fact, the Li^+ and Cs^+ solutions produced very low abundance quadruplexes, possibly indicative that these quadruplexes were only formed after the addition of ammonium acetate and never actually incorporated the very small Li^+ or very large Cs^+ ions (supported by CD results discussed below). However, both the Ca^{2+} and Rb^+ solutions exhibited high abundances of quadruplexes, indicating the ability to facilitate quadruplex formation despite the subsequent exchange with ammonium. Rb^+ is the only cation that was larger than ammonium in terms of ionic radius yet was still able to promote quadruplex formation. The larger size of Rb^+ and rather different electrostatic interactions may have contributed to its ready exchange for NH_4^+ prior to ESI-MS analysis. The formation of quadruplexes in the presence of Ca^{2+} and then subsequent exchange of Ca^{2+} for NH_4^+ is interesting as Ca^{2+} is the only divalent cation to exchange with NH_4^+ . The other two doubly charged cations, Sr^{2+} and Ba^{2+} , for which quadruplexes were very abundant, were larger than Ca^{2+} . It is possible that the size and charge density of Ca^{2+} was suitable for facilitating the initial quadruplex assembly but ultimately the greater NH_4^+ affinity caused cation exchange during the desalting and ammonium acetate dilution required for ESI-MS analysis. In general, the exchange of template cations for ammonium seen here is consistent with previous results reported by Ma et al.(31)

ESI-MS of Ig9

As with G9, the annealed Ig9 solutions were analyzed by ESI-MS after desalting. Representative spectra are shown in Figure 3. The Ig9 solutions showed large relative abundances of higher order complexes versus the single strands for all cations regardless of size or charge. In addition, the majority of the solutions resulted in almost entirely pentaplex formation with minimal quadruplex formation except for the Ig9 annealed in K^+ and NH_4^+ solutions. Even the smallest singly charged cation, Li^+ , promoted almost exclusive pentaplex formation. Ig9 annealed in the NH_4^+ solution resulted in approximately equal abundances of quadruplexes and pentaplexes. As summarized in Figure 4, the change in the relative abundances of quadruplexes and pentaplexes between solutions containing different annealing

cations is striking, even for those cations with similar ionic radii. For example, Sr^{2+} , with an ionic radius of 1.27 Å, stabilizes the formation of the pentaplex, whereas K^+ , with an ionic radius of 1.33 Å, preferentially stabilizes the quadruplex. In this case, the increased charge density of Sr^{2+} likely plays a role in pre-organizing the wider central cavity of the pentaplex as opposed to the quadruplex. A similar situation is seen for the NH_4^+ and Rb^+ solutions in which the ionic radii, 1.43 Å for NH_4^+ and 1.49 Å for Rb^+ , are very similar, whereas the distributions of quadruplexes and pentaplexes present are quite different. Rb^+ promoted only pentaplex formation yet annealing in NH_4^+ yielded both quadruplexes and pentaplexes. There appears to be a narrow range of acceptable ion sizes capable of supporting the formation of quadruplexes for Ig9 that is only met by K^+ and, to a lesser extent, NH_4^+ . In contrast, the pentaplexes are formed in the presence of cations of a broad range of sizes and charge densities.

The mass spectra of the Ig9 solutions were also examined to determine the number and identity of the included central cations. In most of the pentaplex complexes, the central cations appeared to have been replaced by ammonium cations. This was true for Li^+ , Mg^{2+} , Na^+ , Ca^{2+} , and Rb^+ . Also, the pentaplexes contained three central cations which is consistent with the $(n - 1)$ rule seen for guanine-based quadruplexes. Only the pentaplexes formed around the large doubly charged cations, Sr^{2+} and Ba^{2+} , retained these cations. The pentaplexes annealed in Cs^+ retained two cesium cations with one additional NH_4^+ cation. Given the apparent preference for three cations in the central cavity, it appears that one NH_4^+ cation replaced one Cs^+ cation during the desalting and ammonium acetate dilution procedure. The Ig9 annealed in a K^+ solution resulted in both quadruplexes and low abundance pentaplexes that incorporated three K^+ cations.

ESI-MS of G9 and Ig9 annealed in low salt concentration solutions

To determine the effects of salt concentration during annealing on complex formation, both G9 and Ig9 were annealed in solutions containing 10 mM or 150 mM salts. The resulting solutions were desalted in a similar fashion to solutions annealed in 440 mM salt, reconstituted in 50 mM ammonium acetate, and analyzed by ESI-MS. The results obtained for the G9 solutions are summarized in Figure 5a, and a summary chart comparing the results from all three salt concentrations can be seen in Supplemental Figure S-1. In several cases, there was virtually no change in the relative abundances of quadruplexes when compared to the ESI mass spectra obtained for the high salt concentration solutions discussed in Figure 2. G9 annealed in Sr^{2+} , K^+ , NH_4^+ , and Ba^{2+} retained high levels of quadruplexes, while Li^+ , Na^+ , and Cs^+ showed similar low abundances of quadruplexes. The two cases of greatest interest were the G9s annealed in Mg^{2+} and Ca^{2+} . For the Ca^{2+} solutions, the decrease in salt concentration of the annealing solution resulted in a corresponding decrease in quadruplex formation, indicating that the stability of these quadruplexes is dependent on the salt concentration of the solution and suggesting that the quadruplexes are less robust. In contrast, for the Mg^{2+} solutions a decrease in salt concentration of the annealing solution caused an increase in the abundances of quadruplexes observed in the ESI mass spectra. In addition, the peaks corresponding to the quadruplexes incorporating Mg^{2+} were much broader than usually observed (spectra not shown), a feature that indicates the quadruplexes possess different numbers and identities of cations, thus yielding a range of complexes rather than a single dominant complex as was observed with the other cations. CD results discussed below indicate that Mg^{2+} is capable of templating quadruplex formation with G9 although the structures do not survive desalting. It is possible that the formation of these quadruplexes under low salt conditions better enables the Mg^{2+} complexes to maintain their survival during the desalting procedure.

The impact of the salt concentration on the formation of higher order complexes of Ig9 was evaluated more selectively due to the limited quantity of Ig9. Ig9 was annealed in 10 mM Na^+ , K^+ , NH_4^+ , Ba^{2+} , or Cs^+ , a subset of cations that includes a range of ionic radii. The relative

abundances of pentaplexes and quadruplexes under these conditions are summarized in Figure 5b, and ESI mass spectra can be found in Supplemental Figure S-2. G9 annealed in the presence of 10 mM Na⁺, Ba²⁺, or Cs⁺ resulted in similar distributions of complexes to those obtained for the solutions annealed at higher salt concentrations (seen in Figure 4). However, there were notable differences for the 10 mM K⁺ and NH₄⁺ solutions. Unlike Ig9 annealed at high salt conditions, the solutions annealed in 10 mM K⁺ or NH₄⁺ resulted in formation of mostly pentaplexes rather than quadruplexes that dominated at higher salt concentrations. In addition, the isoguanine pentaplexes formed around potassium at low salt concentrations tended to exhibit loss of an isoguanine base. It is unclear why the loss of isoguanine would occur solely under these conditions since it did not occur for other solutions annealed in the presence of 10 mM cations (i.e. low salt conditions) nor did the loss of isoguanine base occur for the solutions annealed in the presence of high concentrations of potassium. The differences seen for the solutions containing potassium and ammonium suggest that the pentaplexes are more stable than the quadruplexes under low salt conditions, but that the quadruplex can be stabilized at higher salt concentrations. Because the solutions containing G9 or Ig9 retained different distributions of complexes depending on the nature of the salt, it is apparent that the desalting and ammonium acetate dilution procedure used for processing all the solutions did not cause systematic disassembly and re-organization of the complexes to one uniform species.

CD spectra of G9 and Ig9

To further evaluate the structures of the quadruplexes and pentaplexes formed by G9 and Ig9, respectively, and to determine the impact of the desalting and ammonium acetate dilution procedures used for the ESI-MS analysis, the solutions described above were analyzed by CD spectroscopy before desalting (after annealing in 440 mM salt), after desalting, and after addition of ammonium acetate (to 50 mM). Both the quadruplex structures and pentaplex structures formed by G9 and Ig9 are expected to provide good CD spectra as stacking between the bases, which is present in quadruplexes and pentaplexes, imparts optical activity to the structures. Since the salts are not significantly optically active under circularly polarized light from 220 to 320 nm, the presence of these salts does not hinder analysis of the solutions. Representative CD spectra are shown in Figures 6 and 7 for the G9 and Ig9 solutions, respectively, and all the results are summarized in Tables 2 and 3. CD spectra from all samples can be found in Supplemental Figures S-3 and S-4 for G9 and Ig9 solutions, respectively.

The quadruplexes formed by G9 are parallel tetramolecular quadruplexes which have a positive band around 264 nm and a smaller negative band around 243 nm. Table 2 provides a summary of peak locations and intensities for all solutions. Solutions of G9 annealed in the presence of Na⁺, Sr²⁺, K⁺, NH₄⁺, Ba²⁺, and Rb⁺ produced strong positive bands at 264 nm before desalting. The band intensities at 264 nm were moderate for the Mg²⁺ and Ca²⁺ solutions, and low for the Li⁺ and Cs⁺ solutions. Examples of strong, moderate, and weak positive bands produced by solutions annealed in K⁺, Mg²⁺, and Cs⁺, respectively, are shown in Figure 6a and Figure 6b before and after desalting. When compared to the CD spectrum of a DNA solution at 95 °C, which is a sufficiently high temperature to convert all higher order structures to single strands, the CD spectra acquired for the solutions annealed in Li⁺ and Cs⁺ are similar in both spectral shape and intensity, indicating that Li⁺ and Cs⁺ do not favor higher order structure formation, which is in agreement with the ESI-MS results. The loss of base stacking as the quadruplex converts to the single strand diminishes the optical activity and decreases the corresponding CD intensities, indicating a loss of secondary structure. The majority of the CD spectral findings are consistent with the ESI-MS results for quadruplex formation discussed above in terms of the relative abundances of quadruplexes, with the exception of the results for Mg²⁺ and Na⁺. Based on the mass spectral data, G9 annealed in the presence of Mg²⁺ and Na⁺ displayed low abundances of quadruplexes whereas the prevalence of the quadruplexes is high in the CD spectra.

To investigate the stability and survival of the quadruplexes, as well as to probe the reasons for the differences in the relative abundances of Mg^{2+} and Na^+ complexes obtained from the ESI-MS results versus the CD results, CD spectra were collected for the same solutions after desalting and after dilution in ammonium acetate, thus reproducing the conditions used for the ESI-MS analysis (see Figure 6c and d for example spectra). Table 2 contains data for all samples. The resulting spectra show that quadruplexes are maintained, albeit at lower abundances, for G9 annealed in Sr^{2+} , K^+ , Ba^{2+} , and Rb^+ , even after desalting, and the spectral features remain consistent with parallel quadruplexes. However, the CD spectra for G9 annealed in the presence of Mg^{2+} and Na^+ exhibit blue shifts for the bands from 264 nm to 257 nm and from 243 nm to 238 nm, as well as notable decreases in overall intensities which indicates a structural change, most likely denaturation to the single strands. Figure 6d highlights the changes seen for the quadruplexes annealed in the presence of Na^+ . This CD data suggests that the quadruplexes formed around the Mg^{2+} and Na^+ cations are less stable than those formed around other template cations as the salt concentration in solution decreases.

The final set of experiments entailed the examination of the CD spectra of the desalted G9 solution after the addition of ammonium acetate. Since quadruplexes can form around NH_4^+ cations, as has been seen in both the ESI-MS data and the CD spectra, it was necessary to see if the addition of ammonium acetate after annealing and desalting would affect the survival of the original quadruplexes formed in the initial annealing step. For all of the solutions, the CD results indicate no dramatic change in the prevalence of the quadruplexes after addition of ammonium acetate (refer to CD results summarized in Table 2). The solutions in which quadruplexes showed low prevalence after desalting (Li^+ , Mg^{2+} , Na^+ , Ca^{2+} , and Cs^+) displayed only slight increases in intensities after the addition of ammonium acetate. The solutions in which the initial quadruplexes were more prevalent (Sr^{2+} , K^+ , Ba^{2+} , and Rb^+ solutions) showed modest declines in intensities. This series of results suggests that the surviving quadruplexes are predominantly ones formed during the initial annealing procedure and that some of the less strongly bound template cations (Li^+ , Ca^{2+} , Rb^+ , Cs^+) can be exchanged for NH_4^+ without complete disruption or collapse of the quadruplex structures.

Five Ig9 solutions were chosen for further study by CD spectroscopy, and representative spectra are shown in Figure 7 for solutions annealed in Na^+ , K^+ , and Ba^{2+} . The CD spectra obtained for Ig9 annealed in the presence of Na^+ , NH_4^+ , Ba^{2+} , and Cs^+ are similar, whereas the spectra obtained for Ig9 in the presence of K^+ are quite different (Figure 7a). Overall, the intensities of the spectral features are lower than observed for the G9 solutions, possibly indicating a lower degree of planarity because the CD signals for these complexes largely arise from base stacking. The CD spectra of Ig9 annealed with Na^+ , NH_4^+ , Ba^{2+} , and Cs^+ exhibit strong positive bands at 307 nm and weaker negative bands around 289 nm. The wavelengths and intensities of spectral features are summarized in Table 3. These features are similar to the CD spectrum previously reported for poly(isoguanilic acid) with a positive band at 295 nm and a negative band around 275 nm. The prior measurements for poly(isoguanilic acid) were undertaken in 10 mM sodium chloride.⁽⁵²⁾ Similar results were also obtained for individual isoguanine bases bound to modified sugars when analyzed in dichloromethane with various cations present. (15)

The CD spectrum obtained for Ig9 annealed in K^+ was very different from the spectra obtained for the solutions containing other cations with a strong negative band at 310 nm and a weak positive band at 293 nm (Figure 7a). These bands contrast significantly from the CD features of G9 annealed in K^+ , which had a positive band at 264 nm and a negative band at 241 nm (Figure 6a), thus highlighting the impact of the change in bases from guanine to isoguanine. Despite the differences between the spectra, ESI-MS results indicate that both the G9 and Ig9 strands form quadruplexes under these conditions. The notable differences in CD spectra between the quadruplexes presumably formed by Ig9 and G9 suggest significant

conformational re-organization that accompanies the change from guanine to isoguanine in the strands. The isoguanine quartets are not anticipated to be as planar as the guanine quartets, a factor which would reduce the effects of base stacking interactions and lead to distorted stacked tetrads. Moreover, the change from guanine to isoguanine certainly alters the optical properties of the resulting quadruplexes due to the different structures of the constituent bases and their interactions.

Upon desalting of the Ig9 solutions, there are slight decreases in the peak intensities (Figure 7b) that are restored after dilution of the solutions in ammonium acetate (Figure 7c). The solution annealed in Ba^{2+} showed the greatest relative decrease in intensity after desalting and the addition of ammonium acetate, which may point to greater instability of the Ba^{2+} pentaplex in low salt environments.

Ab initio calculations of isoguanine complexes

To help rationalize some of the experimental findings regarding the formation of pentaplexes versus quadruplexes for the isoguanine strands in the presence of different cations, high-level *ab initio* optimizations were performed on three molecular symmetries for the smaller scale isoguanine complexes. As an example of the complexes formed, the optimized geometries of the Na^+ complexes are shown in Figures 8a and 8b for the tetrads and pentads, respectively, and the corresponding B3LYP/lacv3p** energies are listed in Table 4 for the tetrads and pentads. In each case S_8 is the most stable conformation for the tetrads containing most of the cations except for Rb^+ , Cs^+ and NH_4^+ , which have relatively larger ionic radii than the other cations. For the pentads, S_{10} is the most stable conformation for all five alkali metal complexes and the ones containing NH_4^+ and Ba^{2+} . In the optimized structures, the two (isoguanine)₅ pentad units are approximately parallel. Conversely, a significant degree of distortion was observed for the analogous tetrads, and double-bowl-like conformations were observed. The distortion parameter, d1, which measures the depth of the “bowl” is listed in Table 5 for each tetrad. The definition of d1 is demonstrated in Supplemental Figure S-5. Generally, the structures incorporating larger cations exhibited greater distortions. As the pentads are relatively planar, the d1 symmetry is not calculated for these complexes. However, the distances between the two planar (isoguanine)₅ units tended to increase with the radius of the cation (Table 5). The distortion in the tetrad planes compared to the planarity of the pentads supports the dominance of pentaplexes over quadruplexes for strands with isoguanine repeats.

To measure the relative template ability of each cation to promote isoguanine tetrads and pentads, the reaction energies, ΔE , were calculated. ΔE and the equations to obtain ΔE are listed in Table 6 (a full table, including the energies of the complexes and ions individually, can be found in Supplemental Table S-1). For the tetrads, the relative reaction energies, $\Delta\Delta E$, increased moving down the periodic table. In contrast, this trend was not observed for the pentads as those incorporating K^+ have larger $\Delta\Delta E$ values than those containing either Na^+ or Rb^+ . It should be noted that the trends changed after a hydration energy correction was performed. For example, after the hydration energy correction, the $\Delta\Delta E$ values determined for the tetrad complexes incorporating either Na^+ , K^+ or NH_4^+ were similar, and generally much more favorable than the values for the complexes incorporating the other monovalent cations. On the other hand, $\Delta\Delta E$ values for pentads were more favorable with Rb^+ , Cs^+ and NH_4^+ after the hydration energy correction. Overall, the energies of the pentads tended to be lower than the tetrads, supporting the tendency of the isoguanine strands to form pentaplexes.

The distortion energy, which is the difference between the energies of the fully optimized structures and the partially optimized geometries with the enforcement of the planarity of the isoguanine units, is listed in Table 7 for each tetrad. Interestingly, tetrads containing NH_4^+ or K^+ had the smallest distortion energies of all the complexes. This implies that a smaller energetic penalty was paid by incorporation of NH_4^+ or K^+ upon formation of planar isoguanine

tetrads compared to incorporation of other cations. This finding is consistent with the ESI-MS results showing that quadruplexes were the dominant species formed for strands annealed in high concentrations of K^+ and NH_4^+ . The greater $\Delta\Delta E$ of the K^+ -containing pentads in comparison to those incorporating neighboring cations Na^+ and Rb^+ may be another driving force for the formation of quadruplexes over pentaplexes in the presence of K^+ . The low distortion energies of the tetrads containing K^+ or NH_4^+ support the formation of isoguanine quadruplexes around these cations despite the lower energies of tetrads formed around smaller cations. The increased template abilities of K^+ and NH_4^+ with respect to formation of planar tetrad units appears to facilitate quadruplex formation over pentaplex formation under certain conditions despite the higher energy of the tetrads.

ESI-MS of Ig9PC

In addition to potential use as ion channels, multimeric complexes based on strands containing isoguanine could be used as scaffolds for multiplexed sensors. For example, C-reactive protein plays an important role in the inflammation response of the body and is a known marker for infection and cardiovascular diseases.(56) It is a homopentameric protein with five binding sites for phosphocholine, a molecule present in damaged cells.(57) An isoguanine pentaplex scaffold consisting of isoguanine-rich strands with a phosphocholine modification at the 5'-end could potentially be used to bind to C-reactive protein in diagnostic applications.

For the present study, strands of Ig9PC, Ig9 with a 5'-modification ending with a phosphocholine moiety (see Scheme 2), were annealed in a similar manner to the other solutions and subsequently analyzed by ESI-MS. One solution of Ig9PC was annealed in Cs^+ under high salt conditions and desalted as the other solutions (Figure 9a). The pentaplex is evident despite the presence of the elongated 5' modifier. The single strand is also detected in low abundance, but quadruplexes are not observed. It is interesting to note that the Cs^+ ions are not incorporated in these Ig9PC pentaplexes in contrast to the Ig9 solution, having been replaced by NH_4^+ cations upon dilution of the solutions with ammonium acetate prior to ESI-MS analysis. Ig9PC was also annealed directly in 150 mM ammonium acetate, and the resulting ESI-MS spectrum is shown in Figure 9b. Under these conditions, quadruplexes were favored, although modest abundances of pentaplexes were observed as well. The dominance of the quadruplexes in this spectrum, which were very low abundance for the Ig9PC solution annealed in Cs^+ , highlights the impact of the annealing cation in dictating the final higher order structures.

Conclusions

Quadruplexes and pentaplexes of G9 and Ig9, respectively, were annealed in solutions containing cations of varying charge and ionic radius. The formation of G9 quadruplexes was far more dependent on the identity of the central cation than the formation of Ig9 complexes. While Ig9 complexes were formed around all of the cations, annealing in only six of the ten cations produced high levels of G9 quadruplexes. In addition, five of various annealing cations, including Li^+ , Mg^{2+} , Na^+ , Ca^{2+} , and Rb^+ , were not retained but instead were replaced by NH_4^+ in the Ig9 pentaplexes when ammonium acetate was added to the solutions immediately before ESI-MS analysis. This was only true for G9 quadruplexes annealed in the presence of Li^+ , Ca^{2+} , Rb^+ , and Cs^+ . The ready exchange of central cations points to the utility of the Ig9 pentaplexes as efficient monovalent cation channels. Also, *ab initio* calculations indicate that the formation of planar isoguanine tetrads around the template cations K^+ and NH_4^+ require the least amount of energy, thus offering some rationalization for the formation of quadruplexes in the presence of high concentrations of these cations. CD results also confirm that many of the complexes retain their higher order structure throughout the desalting process, thus indicating the complexes are resistant to degradation in low salt environments. Finally, ESI-

MS analysis of an isoguanine strand modified with a sensing arm indicated the ability to form pentaplexes around cesium cations. These results indicate isoguanine complexes could serve as promising support architectures for sensing applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Spectra of G9 after annealing in a 440 mM solution of the salt noted in the spectra. Q refers to the quadruplex while ss denotes the single strand.

Figure 2.

Chart of relative quadruplex formation for G9 annealed in 440 mM salt as determined from the ESI-MS. The values represent the average of three samples. Boxes around the cations indicate that the ESI mass spectra showed replacement of the annealing cations with ammonium.

Figure 3.

Spectra of Ig9 after annealing in a 440 mM solution of the salt noted in the spectra. Q refers to the quadruplex, P refers to the pentaplex, and ss denotes the single strand.

Figure 4. Chart of relative complex formation for Ig9 annealed in 440 mM salt as determined from the ESI-mass spectra. The values represent the average of three samples. Boxes around the cations indicate that the ESI-mass spectra showed replacement of the annealing cations with ammonium. Only one of three Cs⁺ cations was exchanged.

Figure 5.

Charts of relative complex formation under low ionic strength conditions for G9 quadruplexes (a) and Ig9 quadruplexes and pentaplexes (b). G9 was annealed in 150 mM and 10 mM salts and Ig9 was annealed in 10 mM salts. The values represent the average of three samples. Boxes around the cations indicate that the ESI mass spectra showed replacement of the annealing cations with ammonium.

CD spectra of G9 samples annealed in MgCl_2 , KCl , and CsCl before desalting (a) and after desalting (b). CD spectra before desalting, after desalting, and after the addition of ammonium acetate for samples annealed in SrCl_2 (c) and NaCl (d).

Figure 6.

CD spectra of G9 samples annealed in the presence of Mg^{2+} , K^+ , and Cs^+ before desalting (a) and after desalting (b). Initial salt concentrations, after dilution to working volumes, was approximately 18 mM. Assuming complete quadruplex formation, the concentration of quadruplex after dilution was approximately 3 μM . CD spectra before desalting, after desalting, and after the addition of ammonium acetate for samples annealed in the presence of Sr^{2+} (c) and Na^+ (d).

CD spectra of G9 samples annealed in MgCl_2 , KCl , and CsCl before desalting (a) and after desalting (b). CD spectra before desalting, after desalting, and after the addition of ammonium acetate for samples annealed in SrCl_2 (c) and NaCl (d).

Figure 7.

CD spectra of Ig9 samples annealed in NaCl, KCl, and BaCl₂ before desalting (a), after desalting (b) and after the addition of ammonium acetate (c).

Figure 8. DFT optimized geometries of the isoguanine tetrads and pentads coordinated with Na⁺. For the tetrads, three molecular symmetries, i.e. C_{4h} , D_4 and S_8 , are shown from left to right (a). Top: the top views; bottom: the side views. For the pentads, C_{5h} , D_5 and S_{10} , are shown from left to right (b). Top: the top views; bottom: the side views.

Scheme 1.

a) Structures of deoxyguanosine and deoxyisoguanosine. B) Possible structures of guanine tetrad and isoguanine pentad around a central cation, M^+ . R refers to the sugar or sugar phosphate backbone.

Table 1

Table comparing the identity and number of cations found associated with the -6 charge state of the G9 intermolecular quadruplex. Asterisks denote number of ammonium cations present rather than the annealing cation.

Most abundant Q ⁶⁻ ion		
Cation	m/z	# cations
Li ⁺	1857	3*
Mg ²⁺	1864	4
Na ⁺	1861	3
Ca ²⁺	1857	3*
Sr ²⁺	1877	2
K ⁺	1867	3
NH ₄ ⁺	1857	3
Ba ²⁺	1894	2
Rb ⁺	1857	3*
Cs ⁺	1857	3*

Table 2

Wavelength maxima of positive peaks and intensities at 264 nm from CD spectra.

	Peaks (nm)		CD intensity at 264 nm (mdeg)			
	Before Desalting	After Desalting	After NH ₄ OAc	Before Desalting	After Desalting	After NH ₄ OAc
Li ⁺	257	257	258	Li ⁺	0.29	0.47
Mg ²⁺	260	262	259	Mg ²⁺	0.33	0.59
Ca ²⁺	261	264	260	Ca ²⁺	0.60	0.80
Na ⁺	265	259	259	Na ⁺	0.39	0.54
Sr ²⁺	263	263	263	Sr ²⁺	2.21	1.88
Ba ²⁺	264	264	263	Ba ²⁺	2.01	1.98
K ⁺	264	264	264	K ⁺	3.22	2.97
NH ₄ ⁺	263	-	264	NH ₄ ⁺	-	3.49
Rb ⁺	264	264	264	Rb ⁺	2.64	1.90
Cs ⁺	256	256	260	Cs ⁺	0.18	0.36

Table 3

Wavelength maxima and corresponding intensities of major CD spectral features.

	Peak/Trough (nm)			CD intensity at peak/trough (mdeg)	
	Before Desalting	After Desalting	After NH ₄ OAc	Before Desalting	After Desalting
Na ⁺	307	307	307	Na ⁺ 1.77	1.58 1.77
K ⁺ /[a]	310	306	312	K ⁺ /[a] -0.60	-0.19 -0.36
Cs ⁺	308	308	308	Cs ⁺ 0.93	0.75 1.00
Ba ²⁺	306	306	306	Ba ²⁺ 1.09	0.50 0.33
NH ₄ ⁺	307	306	306	NH ₄ ⁺ 0.60	0.71 0.47

[a]_{K⁺} results are reported for the trough since the peak for this sample was relatively less intense.

Table 4

The energies of the isoguanine tetrads and pentads in three symmetries at the B3LYP/lacv3p** level.

Tetrad	E(S ₈) Hartree	E(C _{4h})-E(S ₈) kcal/mol	E(D _{4h})-E(S ₈) kcal/mol	Pentad	E(S ₁₀) Hartree	E(C _{5h})-E(S ₁₀) kcal/mol	E(D _{5h})-E(S ₁₀) kcal/mol
Li ⁺	-4349.291856	8.83	0.28	Li ⁺	-5434.732948	15.09	8.46
Na ⁺	-4504.083846	6.80	0.93	Na ⁺	-5589.536896	16.07	9.51
K ⁺	-4369.934679	4.32	3.52	K ⁺	-5455.400848	8.95	3.29
Rb ⁺	-4365.651431	2.84	-0.98	Rb ⁺	-5451.139677	19.63	8.73
Cs ⁺	-4361.649461	-7.95	-0.59	Cs ⁺	-5447.157049	16.61	9.61
NH ₄ ⁺	-4398.873927	1.23	-5.65	NH ₄ ⁺	-5484.364241	11.58	10.23
Mg ²⁺	-4541.688798	17.90	1.04	Mg ²⁺	-5627.099895	61.98	-8.23
Ca ²⁺	-4378.232217	11.47	0.77	Ca ²⁺	-5463.642931	19.80	-1.15
Sr ²⁺	-4372.114214	8.26	0.45	Sr ²⁺	-5457.537268	6.36	-3.36
Ba ²⁺	-4366.895045	0.70	0.75	Ba ²⁺	-5452.363727	9.38	0.06

Table 5

The distortion parameters of isoguanine tetrads and the distances between isoguanine pentad units. For the tetrads, $d1$ is the distance between *Plane 1* and *Plane 2* as demonstrated in Figure S-5. $d2$ is the distance between the metal and the center of *Plane 2*. For the pentads, the metal is located in the geometric center of the pentad units.

Tetrad	Symmetry	d1 (Å)	d2 (Å)	Pentad	Symmetry	Distance (Å)
Li ⁺	S ₈	2.366	1.254	Li ⁺	S ₁₀	3.43
Na ⁺	S ₈	2.363	1.283	Na ⁺	S ₁₀	3.33
K ⁺	S ₈	2.845	1.612	K ⁺	S ₁₀	3.34
Rb ⁺	D ₄	2.707	1.729	Rb ⁺	S ₁₀	3.63
Cs ⁺	C _{4h}	3.32	2.138	Cs ⁺	S ₁₀	3.73
NH ₄ ⁺	D ₄	3.09	1.66	NH ₄ ⁺	S ₁₀	4.08
Mg ²⁺	S ₈	2.572	1.18	Mg ²⁺	D ₅	3.07
Ca ²⁺	S ₈	2.5	1.284	Ca ²⁺	D ₅	2.91
Sr ²⁺	S ₈	2.968	1.425	Sr ²⁺	D ₅	2.87
Ba ²⁺	S ₈	2.343	1.549	Ba ²⁺	S ₁₀	2.93

Energies of isoguanine tetrad and pentad formation using density functional theory at the B3LYP//lacv3p** level. ΔE is the relative reaction energy. For the alkali metal and ammonium complexes, the reference is the Li^+ complex, while the reference is the Mg^{2+} complex for earth alkali metal complexes.

Table 6

Tetrad	Symmetry	ΔE hartree ^[a]	ΔE kcal/mol	Pentad	Symmetry	ΔE hartree ^[b]	ΔE kcal/mol
Li^+	S_8	-0.484702	0.00	Li^+	S_{10}	-0.545232	0.00
Na^+	S_8	-0.474138	6.63	Na^+	S_{10}	-0.546626	-0.88
K^+	S_8	-0.441495	27.11	K^+	S_{10}	-0.527102	11.38
Rb^+	D_4	-0.424808	37.58	Rb^+	S_{10}	-0.530931	8.97
Cs^+	C_{4h}	-0.407843	48.23	Cs^+	S_{10}	-0.5222	14.45
NH_4^+	D_4	-0.440594	27.68	NH_4^+	S_{10}	-0.541339	2.44
Mg^{2+}	S_8	-0.925669	0.00	Mg^{2+}	D_5	-0.969313	0.00
Ca^{2+}	S_8	-0.826041	62.52	Ca^{2+}	D_5	-0.858028	69.83
Sr^{2+}	S_8	-0.778971	92.05	Sr^{2+}	D_5	-0.826813	89.42
Ba^{2+}	S_8	-0.718436	130.04	Ba^{2+}	S_{10}	-0.806556	102.13

^[a] For the tetrads, ΔE , the reaction energy, is calculated using the following equation: $\Delta E = E_{\text{complex}} - (E_{\text{ion}} + 8 \times E_{\text{isoguanine}})$, where the energy of isoguanine is -542.690281 hartrees.

^[b] For the pentads, ΔE , the reaction energy, is calculated using the following equation: $\Delta E = E_{\text{complex}} - (E_{\text{ion}} + 10 \times E_{\text{isoguanine}})$ where the energy of isoguanine is -542.690281 hartrees.

Table 7

The distortion energies of the most stable symmetry of isoguanine tetrads. The distortion energy, $E_{\text{distortion}} = E_{\text{constrain}} - E$, where $E_{\text{constrain}}$ is the energy of the partially optimized geometry forcing the two tetrad planes parallel to each other, and E is the energy of the fully optimized geometry. The distance between two isoguanine planes are also listed.

Tetrad	Symmetry	$E_{\text{distortion}}$ kcal/mol	Distance (Å)
Li ⁺	S ₈	20.65	3.24
Na ⁺	S ₈	16.48	3.33
K ⁺	S ₈	13.73	3.52
Rb ⁺	D ₄	17.43	3.79
Cs ⁺	C _{4h}	25.47	4.34
NH ₄ ⁺	D ₄	11.66	3.78
Mg ²⁺	S ₈	46.87	3.07
Ca ²⁺	S ₈	38.52	3.21
Sr ²⁺	S ₈	34.31	3.28
Ba ²⁺	S ₈	29.55	3.42