Non-covalent interactions enable construction of large structural motifs from small molecules. Molecular self-assembly, an important process typically driven by non-covalent interactions, is often dynamic and generally under thermodynamic control. With interest in the application of the self-assembly process to biomedical research, there is a growing demand with respect to preparing stable non-covalent assemblies in a biocompatible environment. However, supramolecular structures built through H-bonds are often studied in less polar aprotic solvents (such as CH$_2$Cl$_2$) to avoid the competition of H-bond interactions between substrates and solvents. Thus, the development of novel supramolecular systems which are stable in a polar protic solvent is highly desirable, though very challenging.

A G-quartet is an interesting supramolecular scaffold formed by H-bonds. As shown in Scheme 1, with an approximately 90-degree angle between the H-bond donor and acceptor, four guanine units are held together to form a G-quartet. Through ion-dipole interactions, alkali and alkaline earth metal cations can enhance the process by serving as the template to coordinate with central oxygen atoms. Stacking of G-quartets gives G-quadruplexes as bioactive building blocks found in DNA and RNA folding. In this case, the extent of G-quartet stacking in a G-quadruplex will be determined by the phosphate backbone, which is often associated with the formation of a counter folding subunit, such as the i-motif.

Inspired by this unique H-bond assembly, researchers have been devoted to developing guanine derivatives to achieve controllable G-quadruplex formation from small molecules. Some interesting applications have been identified with various G-quartet assemblies, including lipophilic ion channels, supramolecular hydrogels, nanomaterials, potential targets for cancer therapy, and more. Although many examples of G-quartet formation through various modified

![Scheme 1](Image)
guanine derivatives have been reported, studies on controlling G-quartet stacking molecularity are relatively rare.48,49 Factors to be taken into consideration include the cation concentration,50 the solvent,51 the anion,52 and so on.53 In many cases, mixtures of various “stacking isomers” (G₄₋, G₁₂ or G₁₆) were observed, which highlights the significant challenges associated with controlling the vertical stacking.44–46 Moreover, the assembly of a specific and stable G-quadruplex in H-bond competitive solvents remains a challenging task.46 Herein, we report the construction of the first G-octamer with structures characterized by single crystal X-ray diffraction through monomer conformational design. Moreover, with this new system, stable G-quadruplexes were formed with significantly improved stability. Through the design of cross-layer H-bonds and covalent linkage, G-octamers were prepared with excellent stability in MeOH (no dissociation) and even in 50% DMSO, which offers a potential opportunity to extend the H-bond assembly system into biosystems for future applications.

Results and discussion
Design, synthesis and characterization of G-octamers
Ideally, a G₄-tetramer would be the most concise target towards the construction of a simple and stable assembled structure. However, with a metal template in solution, further stacking of G₄-tetramers leads to the mixture of G-quadruplex species.59 Thus, G₄-tetramers are unfavorable for the formation of well-defined supermolecules.

The simplest plausible G-quadruplex would be a G-octamer which is likely to adopt either top-to-top (T–T) or bottom-to-bottom (B–B) stacking patterns (Scheme 2A).50 In previous studies, Meijer et al. employed different concentrations of a guanosine derivative resulting in the formation of tunable G-octamers.41 Spada has reported G-octamer formation upon exposure to UV light through alkene isomerization.51 Wu presented several excellent examples of G-octamer formation through π–π interactions using NMR and MS studies.52 Hirao and coworkers applied Au(i)–Au(i) interaction between two G-quartet layers to achieve a G-octamer confirmed by NMR and CD spectra.55 However, to the best of our knowledge, no single crystal structure of a G-octamer has ever been reported, implying the challenging nature of preparing a stable and discrete G-octamer in a dynamic equilibrium.

To tackle this problem, we set out to design a G-derivative where the structure is rigid and predisposed for the formation of a potential octamer structure so as to minimize the entropy cost involved in the self-assembly process. As shown in Scheme 2B, modification of guanosine often occurs on two positions: C-8 of purine and the hydroxyl of ribose. The Sessler group first reported on 8-aryl substituted guanosine in the formation of a G-quartet without templating cation in both solution and solid state.54

This seminal work initiated the concept of conformational control for G-quartet formation: the steric effect between the aryl substituent and protected ribose helped guanosine to adopt a syn conformation, preventing the ribbon formation and giving a tetramer as the dominant conformation. On the other hand, Davis’s group developed lipophilic guanosine with bulky ribose to form a G-hexadecamer (Scheme 2B) both in solution and solid state (Me₄Sn = K⁺, Ba²⁺, Sr²⁺, and Pb²⁺).55 Notably, a picrate anion bridge played a crucial role: as revealed by single crystal X-ray diffraction (Scheme 2C), four picrate anions linked two G-octamers through H-bonds between anion and two inner G-quartets. The two G-octamers (from adjacent inner and outer G₄) gave top-to-bottom stacking with ribose interdigitated between the adjacent layers. Interestingly, the two inner layers adopted bottom-to-bottom (B–B) stacking, which is more favorable than the T–T mode with the cation binding on the more “naked” convex face between the two layers. This result aroused our interest in developing a G-octamer through similar B–B stacking.

Considering the steric interaction between the C-8 substituent and ribose, we postulated that incorporation of C-8 aryl and the rigid ribose ring might provide a new system with steric hindrance between the G-quartet to force the G₄ bowls to stack in a bottom-to-bottom manner, while obstructing ribose interdigitation at the top-face (Scheme 2C). To confirm this idea, compound 1b was designed, prepared and applied to assemble with various alkali and alkaline earth metal cations.1H NMR spectra were obtained and selected regions of the 1H NMR spectra of these G-quadruplexes were compared with the G-hexadecamer from 1a as shown in Fig. 1.

As previously reported, treating 1a with alkali and alkaline earth metal salts (K⁺, Ba²⁺, Sr²⁺ and so on) gave two sets of signals in the 1H NMR spectra, corresponding to the inner and outer G-quartet.56 Conducting similar cation binding experiments with 1b in CDCl₃ gave a single set of 1H NMR signals in all cases (M = K⁺, Ba²⁺, and Sr²⁺; A⁻ = Picrate⁻ or PF₆⁻). Furthermore, ESI-MS demonstrated a clear doubly charged
peak at m/z = 2123.01, corresponding to a mol. wt of 4246.68 for ([1b]Ba)$^{2+}$. The experimental and calculated isotope patterns further suggested an octameric composition. In addition, traveling wave ion mobility mass spectrometry (TWIM-MS) confirmed no formation of stacking isomers, which excluded the formation of random aggregates in gas phase (see ESI† for details).

Finally, single crystal structures were obtained and unambiguously verified the G₈-octamer formation with the proposed bottom-to-bottom stacking (Fig. 2A). The top view of the crystal structure (Fig. 2B) shows the G-quartet self-assembly in the tail to tail orientation. The five crystal structures obtained with ligand 1b include monovalent cations (K⁺ and Rb⁺) and divalent cations (Ba²⁺ and Sr²⁺). Picarete anion showed no clear binding with the G-quartet, consistent with what was observed in the ¹H NMR spectra (see Fig. S2†). A complex with non-coordinated PF₆⁻ anion was also successfully obtained, [([1b]K)⁺·(PF₆⁻)], confirming the “anion-free” binding mode of this new type of G₈-quadruplex. The distances of the H-bond within the G₄-quartet and between the G₁ layers are compared in Table 1.

According to these crystal structures, all G-octamers from 1b gave G-quartets with an [N1···O6] and [N2···N7] H-bond distance of around 2.9 Å, similar to that of the inner and outer layer in the G₁₆-hexadecamer formed from 1a.⁴⁶ These results indicated that both cations and the C-8 phenyl substituent had little influence on the H-bond in the G₁₄-quartet.

However, the size of the G₈ was influenced by the average O-M distances of all these complexes from 1b, which might follow the trend where higher ionic potential (Z/r) resulted in shorter O-M distances (2.75–2.81 Å). Interestingly, in comparison with the G₁₆-hexadecamer [([1a]₁₆Ba)⁴⁺·(Pic)⁴⁻], the G₈-octamers [([1b]₁₆Ba)⁴⁺·(Pic)⁴⁻] gave a slightly shorter distance between the two G₁ layers (2.89 Å vs. 3.06 Å and 3.58 Å). This result implied the stronger cation interaction of the (1b)₈-quartet than the (1a)₈-quartet. This improved cation interaction has been supported by G₄-binding studies with Rb⁺ using the 1b ligand and led to the first crystal structures of Rb⁺ coordinated G-quadruplexes. Among all the G₈ crystals, [([1b]₁₆Rb)⁺·(Pic)⁻] gave the longest O-M and G₄-G₄ distance due to its large radii³⁸ and low valency. Very few examples of Rb coordinated G-quartets have been reported so far, indicating how challenging it is for guanosine to bind with Rb to form a discrete G-quadruplex.⁷⁹ To the best of our knowledge, this is the first single crystal structure of a G-quadruplex containing Rb⁺, clearly suggesting the promising cation binding ability of guanosine derivative 1b.

Having successfully confirmed a new concise G₈-quadruplex structure in solution (NMR), solid state (XRD) and gas phase (ESI-MS and TWIM-MS), we evaluated its stability in MeOH. Dissolving octamer [([1b]K)⁺·(Pic)⁻] in CD₃OD gave a mixture of two sets of signals in NMR spectra, suggesting partial decomposition of this G₈-octamer (vide infra). To obtain MeOH stable G-quadruplexes, further modification is still needed.

Cross-layer H-bonded G-octamers

To further improve the stability of the G-quadruplex, we sought to establish the interactions between the G-quartet layers. As highlighted in Fig. 2A, the 8-phenyl group in 1b adopted a tilted conformation and reached out from the G-quartet. This geometry provided an opportunity to further enhance the supramolecular structure by introducing new interactions between the two G-quartets.

Notably, the Rivera group have reported the formation of an intralayer H-bond between carbonyl oxygen with N(2)H within the same G-quartet by using 2'-deoxy guanosine derivatives without rigid ribose functionalization.³⁴,³⁵,³⁶,³⁸ This work suggested the possibility of forming extra H-bonds by using both hydrogens of the N(2)-NH₂ group. Inspired by this work, a carbonyl group was introduced at the meta-position of the 8-aryl position of 1b as illustrated in Fig. 3A. Based on this design, we hypothesized that N(2)-Hₐ would form a H-bond with neighboring guanosine within the G-quartet, while the N(2)-Hₐ could interact with the carbonyl group by forming a cross-layer H-bond.

To confirm this idea, compound 1c was synthesized and applied to G-quadruplex construction upon interacting with metal cations. According to the ¹H NMR spectra, treating 1c with Ba(Pic)₂ led to the formation of a new G-quadruplex with one set of signals, similar to the G₈-octamer obtained from 1b. Analysis of the NMR sample (in CDCl₃) by ESI-MS gave a dominant, doubly charged peak with m/z at 2291.34, corresponding to [([1c]₁₆Ba)⁴⁺ (mw = 4582.68).
Having confirmed the G₈-octamer [([1b]₀Ba)²⁺·(Pic⁻)₂] formation, our next goal was to determine if there was a cross-layer H-bond as designed above. The ¹H NMR spectra of [([1b]₀Ba)²⁺·(Pic⁻)₂] and [([1c]₀Ba)²⁺·(Pic⁻)₂] did not show peaks corresponding to N(2)H at room temperature. This is likely due to the rapid exchange between the two NH₂ protons, even with the formation of a H-bond. Thus, the exchange rate of the two protons provides a direct indication of the H-bond strength in the G₄-quartet. To explore the dynamic structure, variable temperature (VT) NMR experiments with [([1b]₀Ba)²⁺·(Pic⁻)₂] and [([1c]₀Ba)²⁺·(Pic⁻)₂] were performed and are summarized in Fig. 4.

For complex [([1c]₀Ba)²⁺·(Pic⁻)₂], the NH₂ protons started appearing as broad peaks at 0 °C with the chemical shift at 10.28 ppm (H₄) and 7.25 ppm (H₆). In contrast, the VT NMR spectra of [([1b]₀Ba)²⁺·(Pic⁻)₂] did not show apparent peaks of the N(2)-H signals until further cooling the sample to −40 °C. The results indicated that there might be an extra H-bond in the 1c complex to lock the N(2)-NH₂ from rapid exchange. Furthermore, a significantly downfield shifted chemical shift (7.25 ppm) was ascribed to the N(2)-H₆ proton in the (1c)₈-octamer compared with the (1b)₈-octamer (5.98 ppm). These observations provide clear evidence of the formation of a cross-layer H-bond in the (1c)₈-octamer as designed.

Finally, the G₈-octamer was verified by X-ray crystallography as shown in Fig. 3B. The crystal structure also confirmed the presence of the cross-layer interactions with a mean H-bond distance of 3.09 Å and a bond angle of 161.5°, suggesting a weak cross-layer H-bond present in solid state (Fig. 3C). This makes the structure a “self-assembled molecular-cuboid” purely generated by H-bond linkage with all eight guanosine units. On the other hand, the distance of the two G-quartet layers (2.90 Å) in [([1c]₀Ba)²⁺·(Pic⁻)₂] remained similar to the 1b complex. Considering the specific “cage” size in the G₈-octamer shown in Table 1, the cross-layer H-bond was not strong enough to generate extra enthalpy gain to balance the entropy cost caused by holding the two layers tighter.

With the confirmed cross-layer H-bonds, we evaluated the complex stability of [([1c]₀Ba)²⁺·(Pic⁻)₂] in methanol. The results showed a similar stability to the complex formed with 1b (vide infra). Although the cross-layer H-bond approach could not boost G₈-quadruplex stability in MeOH as anticipated, it provided an effective and novel approach to enhance G-quadruplex stability from monomer conformational design. Structural amendment was required to further improve the stability of the G₈-octamer.

Cross-layer linkage through a covalent bond

To increase the stability to a new level, we turned to establishing a potential covalent linkage between the two G-quartets. By scrutinizing the crystal structure of [([1b]₀Ba)²⁺·(Pic⁻)₂], we found that the distance between the two meta position of the phenyl ring from each tetramer was 4.0 Å, a distance similar to three single bond lengths.⁵⁴ According to the observation, the meta position of the two phenyl groups could serve as a reference site for constructing cross-layer covalent linkers. The guanosine dimer 1d and 1d' were then prepared using the synthetic route summarized in Fig. 5A.
Self-assembly of 1d with K⁺ and Ba²⁺ cations in CDCl₃ gave a similar one-set-of-signal in the ¹H NMR spectra, consistent with the formation of a G₈-octamer. ESI-MS of complexes from 1d and Ba²⁺ gave a doubly charged peak at m/z = 2175.55 as the dominant signal, indicating a mol. wt. of 4351.1 for the super-molecule as [(Pic)K]⁺([Pic]–). An attempt to obtain a single crystal of the 1d complex failed initially, resulting in a rather thin, film-like solid formation. Fortunately, the single crystal structure was successfully obtained by switching the monomer to 1d’ using DMSO as a co-solvent, confirming the cross-layer covalent linked structure as proposed (Fig. 5B). Notably, for complex [(Pic)K]⁺([Pic]–), the dihedral angle between 8-aryl and guanine is 40.7 degrees, similar to the dihedral angles in complex [(Pic)K]⁺([Pic]–) (42.5 degrees). Overall, through G-monomer conformational analysis, a series of G₈-octamers was successfully prepared with functionalization at 8-phenyl (1b), a cross-layer H-bond linker (1c) and a covalent linker (1d).

G-quadruplex H-bond stability in MeOH

As discussed above, our intrinsic motivation in exploring these different G-quadruplexes was to develop H-bonded guanosine self-assembly that could survive in protic solvents (H-bond competitive). With all these diﬀerent G₈ and G₆ quadruplexes prepared, we dissolved them in CD₂OD to compare the ¹H NMR spectra. As shown in Fig. 6A, N(1)–H and N(2)–H protons did not appear in ¹H NMR spectra with CD₂OD as the solvent due to the H/D exchange. Thus, evaluation of the ¹H NMR spectra will mainly be focused on the non-exchangeable aromatic protons and ribose protons. Dissolving the G₆-hexadecamer [(Pic)K]⁺([Pic]–) in CD₂OD gave only one set of signals, identical to the 1a monomer in CD₂OD. The result suggested complete dissociation of the G₆-hexadecamer to 1a monomer in MeOH. Interestingly, the G₈-octamer [(Pic)K]⁺([Pic]–) and [(Pic)K]⁺([Pic]–) in CD₂OD gave two sets of signals, indicating the existence of dissociated monomer and possible oligomers or a G-quadruplex in the solution phase. Although the exact structures of the guanosine species in these two cases are not determined at this moment, the fact that H-bonded guanosine complexes were formed with 1b and 1c clearly suggests the improved H-binding ability of these two monomers over 1a. Surprisingly, when dissolving the G₈-octamer [(Pic)K]⁺([Pic]–) in CD₂OD, only one set of signals was observed. Notably, in this case, N(1)–H gave a broad signal at 12.95 ppm, clearly suggesting the formation of a G-quadruplex through a H-bond. NMR solvent signal suppression was applied for the G-quadruplex to improve H-binding ability of these two monomers over 1a. Overall, the G₈-octamer failed initially, resulting in a rather thin, film-like solid formation. Fortunately, the single crystal structure was successfully obtained by switching the monomer to 1d’ using DMSO as a co-solvent, confirming the cross-layer covalent linked structure as proposed (Fig. 5B). Notably, for complex [(Pic)K]⁺([Pic]–), the dihedral angle between 8-aryl and guanine is 40.7 degrees, similar to the dihedral angles in complex [(Pic)K]⁺([Pic]–) (42.5 degrees). Overall, through G-monomer conformational analysis, a series of G₈-octamers was successfully prepared with functionalization at 8-phenyl (1b), a cross-layer H-bond linker (1c) and a covalent linker (1d).

Evaluating G-quadruplex stability

With the success in maintaining G-quadruplex stability in protic solvent MeOH, we sought to evaluate whether a similar
stability trend exists with polar aprotic solvents. DMSO is a strong polar solvent, which can disrupt the H-bond in G-quartets and cause the decomposition of G-quadruplexes. To evaluate how the incorporation of the phenyl group and cross-layer interaction impact on thermodynamic stability, G-quadruplexes were treated with CDCl3/DMSO-d6 solvent mixture. A summary of 1H NMR spectra from these experiments is shown in Fig. 7.

As shown in the 1H NMR spectra, G-quadruplexes from 1a, 1b and 1c started to dissociate in mixed solvents containing 20% DMSO-d6. Compared with the reported G16-hexadecamer from 1a,46 the G8-octamer formed by 1b and 1c showed comparable stability in 20% DMSO-d6. Eventually, all three G-quadruplexes gave complete dissociation in 50% DMSO-d6 solution with only one set of signals corresponding to the monomer. In contrast, [(1d)K]+·(Pic−)] shows significantly improved stability, with only 2% complex dissociation in 50% DMSO-d6. This result demonstrates the significantly enhanced stability of G-quadruplexes constructed by 1d.

To quantify the thermodynamic stability of G-quadruplexes,17 VT NMR experiments were carried out for pure complexes of [(1a)K]+·(Pic−), [(1b)K]+·(Pic−), [(1c)K]+·(Pic−), and [(1d)K]+·(Pic−) in CDCl3/DMSO-d6 with a fraction of 20% DMSO-d6. The values of complex dissociation enthalpy and entropy for each complex [(Pic−)] can be calculated from van’t Hoﬀ plots and are significantly enhanced compared with the monomer. In contrast, [(1b)K]+·(Pic−) was treated with CDCl3/DMSO-d6 with a fraction of 20% DMSO-d6. The expected G8-octamer was conﬁrmed by mass spectrometry with significant fragmentation of G-quadruplex cation fragments of [(Pic−)] only showed a doubly charged peak at m/z = 2123.31 corresponding to [(1a)Ba]2+, indicating that the picate bridge dissociated under the MS conditions. Further comparison of all the cation fragments of the G-quadruplexes revealed a clear stability trend as [(1d)Ba]2+ > [(1c)Ba]2+ > [(1b)Ba]2+ > [(1a)Ba]2+. Overall, the covalent linking strategy signiﬁcantly helped to stabilize the G8-octamer, both in H-bond competitive solvents and gas phase.

Conclusions

In summary, with modiﬁcation on both guanine (8-aryl) and ribose (sterically hindered 2,3’ position), a stable G8-octamer was formed with its structure characterized by single crystal X-ray diffraction for the ﬁrst time. Through the analysis of cross-layer interactions, a covalently tethered 8-aryl guanosine dimer was designed and prepared for supramolecular assembly. The expected G8-octamer was conﬁrmed by X-ray, MS and NMR spectroscopy with signiﬁcantly improved stability in MeOH and 1 : 1 DMSO/CDCl3 mixture. To the best of our knowledge, this is the ﬁrst example of discrete G-quadruplexes formed from small molecules with enhanced stability in a protic solvent (MeOH) and a polar aprotic solvent (DMSO). Meanwhile, formation of the stable G8-octamer with a concise and well-deﬁned bottom-to-bottom stacking mode provides a novel supramolecular platform. Incorporation of this new system into material and biological applications is expected and currently undergoing in our group.

Conflicts of interest

There are no conﬂicts to declare.

Acknowledgements

We are grateful to the NSF (CHE-1665122), the NIH (1R01GM120240-01), and NSF (21629201) for ﬁnancial support.

Notes and references

§ All the structures reported in this article have been deposited with the Cambridge Crystallographic Data Centre. The accession numbers for [(1b)K]+·(Pic−): [(1b)K]+·(Pic−): [(1b)K]+·(Pic−)]; [(1b)Rb]+·(Pic−); [(1c)Ba]2+·(Pic−); [(1d)K]+·(Pic−) reported in this paper are:
27 T. Bhattacharyya, P. Saha and J. Dash, ACS Omega, 2018, 3, 2230–2241.