

## A $G_4 \cdot K^+$ Hydrogel Stabilized by an Anion

Gretchen Marie Peters,<sup>†</sup> Luke P. Skala,<sup>†</sup> Taylor N. Plank,<sup>†</sup> Brooke J. Hyman,<sup>†</sup> G. N. Manjunatha Reddy,<sup>‡</sup> Andrew Marsh,<sup>§</sup> Steven P. Brown,<sup>‡</sup> and Jeffery T. Davis<sup>\*,†</sup>

<sup>†</sup>Department of Chemistry and Biochemistry, University of Maryland, College Park, Maryland 20742, United States

<sup>‡</sup>Department of Physics, University of Warwick, Coventry CV4 7AL, U.K.

<sup>§</sup>Department of Chemistry, University of Warwick, Coventry CV4 7AL, U.K.

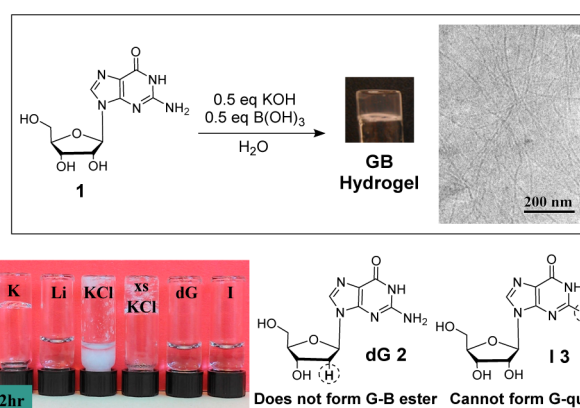
### S Supporting Information

**ABSTRACT:** Supramolecular hydrogels derived from natural products have promising applications in diagnostics, drug delivery, and tissue engineering. We studied the formation of a long-lived hydrogel made by mixing guanosine (G, **1**) with 0.5 equiv of  $\text{KB}(\text{OH})_4$ . This ratio of borate anion to ligand is crucial for gelation as it links two molecules of **1**, which facilitates cation-templated assembly of  $G_4 \cdot K^+$  quartets. The guanosine–borate (GB) hydrogel, which was characterized by cryogenic transmission electron microscopy and circular dichroism and  $^{11}\text{B}$  magic-angle-spinning NMR spectroscopy, is stable in water that contains physiologically relevant concentrations of  $K^+$ . Furthermore, non-covalent interactions, such as electrostatics,  $\pi$ -stacking, and hydrogen bonding, enable the incorporation of a cationic dye and nucleosides into the GB hydrogel.

Self-assembly is an efficient way to make new materials, such as supramolecular hydrogels.<sup>1</sup> Hydrogels have potential in drug delivery, cell culture, and tissue engineering. Analogues of guanosine (G, **1**), especially 5'-GMP, have long been known to form hydrogels, typically involving  $G_4 \cdot M^+$  quartets.<sup>2,3</sup> Various  $G_4$  hydrogels are known, with recent emphasis on improving their stability, enhancing their physical properties, and using them in biological contexts.<sup>4,5</sup> Two shortcomings of many  $G_4$  hydrogels, especially those made from poorly soluble **1**, are their propensity to crystallize and the requirement for excess  $K^+$ . The feasibility of using  $G_4$  gels for biological function would improve if such issues could be overcome. It would also be useful if drugs could be incorporated into  $G_4$  gels.<sup>6</sup> We describe a functional hydrogel made from **1** where an anion drives assembly of the  $G_4$  structure.

We and others have previously shown that anions, when non-covalently associated, can influence the structure, stability, and dynamics of lipophilic  $G_4 \cdot M^+$  quadruplexes.<sup>7</sup> This paper describes a situation where the anion is again crucial but now is covalently incorporated into the  $G_4 \cdot M^+$  assembly. We were intrigued by a 1970 report that **1** gels water in the presence of 0.5 equiv of boric acid and NaOH.<sup>8,9</sup> On the basis of viscosity data, the authors proposed that gelation is due to the formation of anionic borate diesters and consequent self-assembly of G dimers via hydrogen bonds. That report did not suggest the involvement of  $G_4 \cdot \text{Na}^+$  quartets or any synergy between the cation and anion in the gelation of water. We suspected that if  $G_4$  quartets were involved in this unique guanosine–borate (GB) gel, then there is

likely cooperativity between the cation that templates the  $G_4 \cdot M^+$  quartet and the anion that enables dimerization of G.<sup>10</sup> To test this hypothesis, we used inversion tests to examine the influence of the cation, anion, and nucleoside on gelation (Figure 1). The



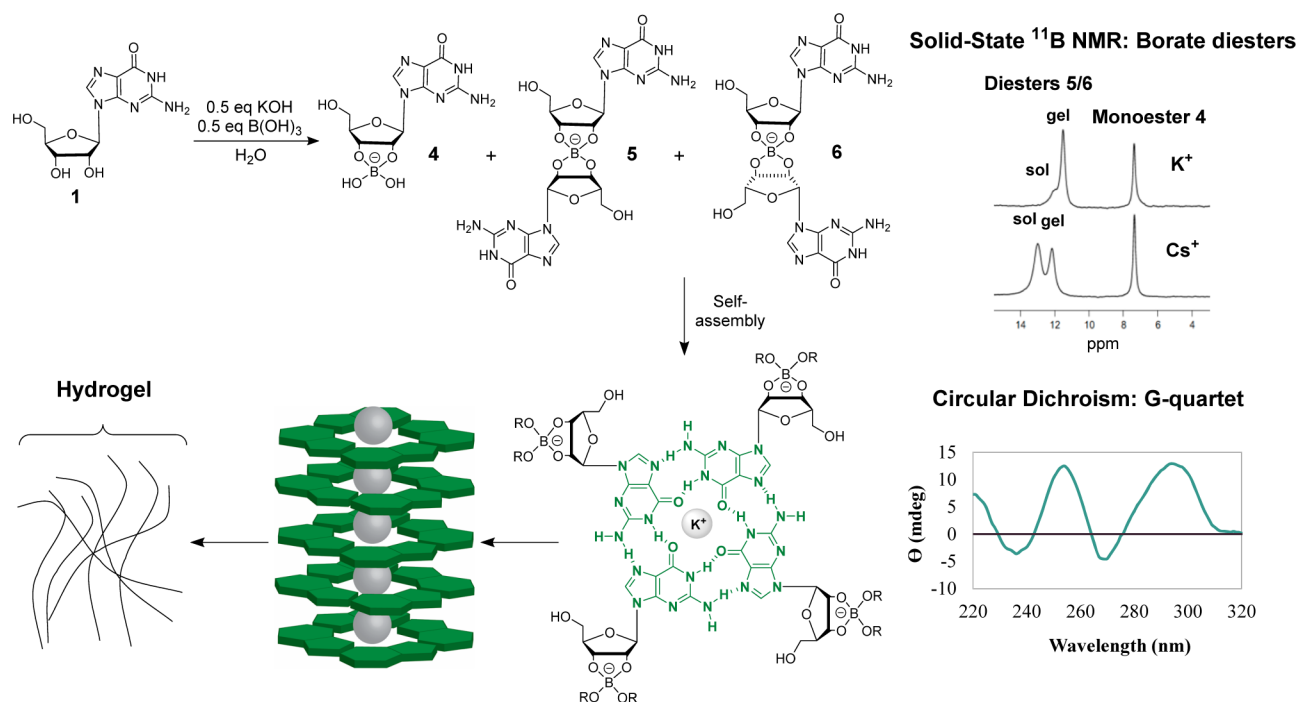
**Figure 1.** (top) A transparent hydrogel is formed from G (**1**) and 0.5 equiv of  $\text{KB}(\text{OH})_4$ . Cryo-TEM shows a network of nanofibers (also see Figure S2). (bottom) Gel (1 wt %) formed in water from 36 mM **1** and 18 mM  $\text{KB}(\text{OH})_4$  (vial K). **1** and  $\text{LiB}(\text{OH})_4$  (18 mM) gave a solution (vial Li). Samples prepared with 18 mM KCl or excess KCl (180 mM) gave precipitate or crystals (vials KCl and xs KCl). Exchanging **1** for 2'-dG (**2**) (vial dG) or inosine (**3**) (vial I) gave nonviscous solutions.

cation's impact was striking and indicated that  $G_4 \cdot M^+$  quartets are integral for gelation. Addition of 0.5 equiv of  $\text{KB}(\text{OH})_4$  to **1** (1 wt %; 36 mM) in water gave a transparent gel (vial K). Cryogenic transmission electron microscopy (cryo-TEM) showed dense, entangled fibrils consistent with  $G_4$  nanowires (Figure 1; for more detail, see Figure S2 in the Supporting Information).<sup>11</sup> In contrast, addition of 0.5 equiv of  $\text{LiB}(\text{OH})_4$  to **1** in water gave a free-flowing solution (vial Li). The  $\text{LiB}(\text{OH})_4$  helped dissolve G by forming borate monoester **4** and diastereomeric borate diesters **5** and **6** (Figures 2 and S3), but the sample did not gel with  $\text{Li}^+$  as the cation. Of course,  $K^+$  is far better than  $\text{Li}^+$  at stabilizing  $G_4$  quartets.<sup>3c,12</sup>

Borate anion, together with  $K^+$ , is crucial for gelation. When 0.5 equiv of KCl was added instead of  $\text{KB}(\text{OH})_4$ , we observed that **1** (36 mM) precipitated from solution upon cooling (vial KCl). Addition of excess KCl (180 mM, 5 equiv) gave a gel

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**Figure 2.** In the presence of  $\text{KB}(\text{OH})_4$ , guanosine (**1**) forms borate monoester **4** and diastereomeric borate diesters **5** and **6**. The solid-state  $^1\text{H}$  (850 MHz)-decoupled  $^{11}\text{B}$  MAS (5 kHz) NMR spectra of 2 wt %  $\text{K}^+$  and  $\text{Cs}^+$  gels indicate that borate diesters **5** and **6** are key for gelation. Self-assembly of borate diesters **5** and **6** into a structure containing stacked  $\text{G}_4$  quartets was confirmed by CD spectroscopy.

initially, but crystal growth occurred within hours (vial vs KCl). In contrast, GB hydrogels made with 0.5 equiv of  $\text{KB}(\text{OH})_4$  have remained transparent for over a year. The correct stoichiometry of borate is critical for self-assembly and increased hydrogel lifetime, consistent with borate diesters **5** and **6** (Figure 2) being central to the gel structure.<sup>8,13</sup> When too little  $\text{KB}(\text{OH})_4$  (<0.5 equiv) was added, all of the G did not dissolve; with excess  $\text{KB}(\text{OH})_4$ , we observed solutions of varying viscosity, since excess borate favors the formation of monoester **4** at the expense of diesters **5** and **6** (Figure S1).

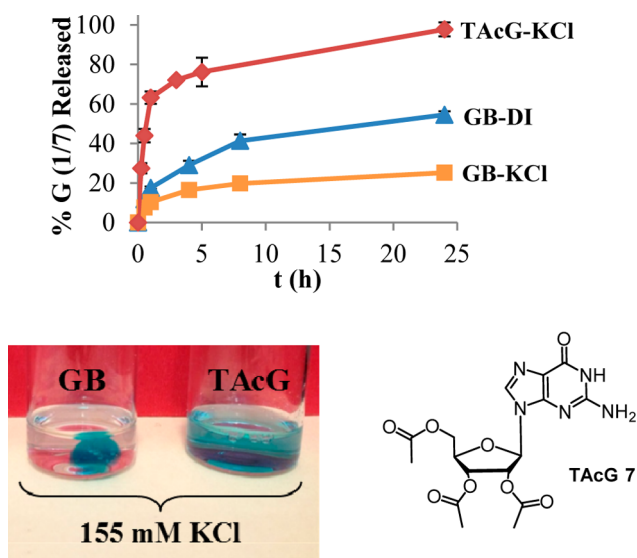
We next studied the impact of the nucleoside on gelation to confirm that both the borate diester and the hydrogen-bonded  $\text{G}_4\text{-K}^+$  quartet are critical for hydrogel formation. No gelation was observed for 2'-deoxyguanosine (dG, **2**) or inosine (**3**) when either G analogue was combined with 0.5 equiv of  $\text{KB}(\text{OH})_4$  in water (vials dG and I, respectively). These results highlight the importance of both the nucleoside's sugar and base in the self-assembly process. Since **2** lacks a vicinal diol, it cannot form borate diesters **5** and **6**; thus, **2** does not gel even though it should be able to form a  $\text{G}_4\text{-K}^+$  quartet.<sup>14</sup> On the other hand, **3** does form borate diesters **5** and **6**, but without the  $\text{NH}_2$  group, **3** does not favor a hydrogen-bonded quartet.

While the inversion tests provided macroscopic evidence for the structural model in Figure 2, we sought molecular-level evidence that the borate diester and  $\text{G}_4$  quartet motifs are integral to the GB hydrogel. Circular dichroism (CD) spectroscopy was used to assign the polarity of stacked  $\text{G}_4$  quartets, as a  $\text{C}_4$ -symmetric  $\text{G}_8$  octamer (head-to-tail stacking of  $\text{G}_4$  quartets) displays bands of opposite sign at 240 and 260 nm, whereas the CD bands for a  $\text{D}_4$ -symmetric  $\text{G}_8$  octamer (head-to-head stacking) are shifted to 260 and 290 nm.<sup>15</sup> The CD spectrum of a 2 wt % GB gel [72 mM **1**; 36 mM  $\text{KB}(\text{OH})_4$ ] showed positive peaks at 254 and 295 nm and troughs at 236 and 270 nm (Figure 2). This CD spectrum of the GB hydrogel is diagnostic of

$\text{G}_4$  quartets that are stacked in both head-to-tail and head-to-head orientations.

Although  $^{11}\text{B}$  NMR spectroscopy has been applied to characterize borate esters in solution,<sup>13,16</sup> it has found limited use in the solid-state characterization of hydrogels.<sup>17</sup> We used solid-state magic-angle-spinning (MAS)  $^{11}\text{B}$  NMR spectroscopy to confirm that borate diesters are crucial to the GB hydrogel structure. Figure 2 shows  $^1\text{H}$ -decoupled  $^{11}\text{B}$  MAS NMR spectra recorded at an  $^1\text{H}$  Larmor frequency of 850 MHz for a GB gel made from 0.5 equiv of  $\text{KB}(\text{OH})_4$  and a gel made using 0.5 equiv of  $\text{CsB}(\text{OH})_4$ . These spectra show resolved signals between 11 and 13 ppm, where NMR peaks are observed for nucleoside borate diesters in solution.<sup>13,16</sup> The  $\text{K}^+$  GB gel shows a sharp signal at 11.54 ppm and a smaller, broader peak at 12.10 ppm. In contrast, the weaker  $\text{Cs}^+$  GB gel gave a  $^{11}\text{B}$  NMR spectrum whose downfield peak at 13.00 ppm is larger than the upfield signal at 12.20 ppm. We interpret these data to mean that (1)  $^{11}\text{B}$  NMR signals for borate diesters in the gel and sol states can be resolved by MAS NMR and that (2) the  $\text{K}^+$  GB sample has more borate diester in the gel state than the weaker  $\text{Cs}^+$  sample.<sup>18</sup> Overall, the evidence from the inversion tests and spectroscopy indicates that anionic borate diesters and hydrogen-bonded  $\text{G}_4\text{-K}^+$  quartets are critical for gelation of G.

As a first step toward evaluating its biocompatibility, we discovered that the  $\text{K}^+$  GB gel dissolves in water but not in a solution of 155 mM KCl, a typical intracellular concentration for  $\text{K}^+$ . We prepared a "blue" gel (2 wt %) from 72 mM **1**, 36 mM  $\text{KB}(\text{OH})_4$ , and 11  $\mu\text{M}$  methylene blue (MB, **8**) and soaked it in different solutions. Gel dissolution was monitored (a) by UV spectroscopy, which quantified release of G from the gel, and (b) by visual observation of MB going into solution. We observed that when the GB gel was placed in water, it swelled and eventually dissolved completely. As shown in Figure 3, ~55% of **1** used to make the GB sample had dissolved in water after 24 h (GB-DI). The same GB hydrogel was much more stable in 155

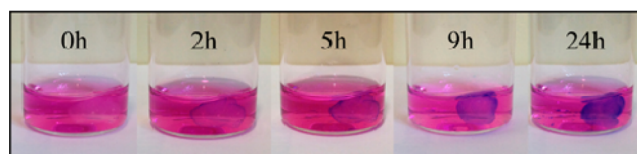
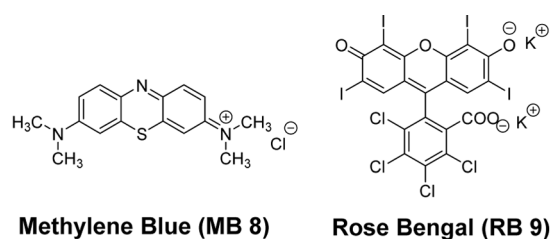


**Figure 3.** (top) G (1) or TAcG (7) released from gels into deionized water (DI) or 155 mM KCl solution as determined by UV spectroscopy. (bottom) Photographs 24 h after addition of 2 wt % GB gel or 2 wt % gel from 60:40 G/TAcG to 155 mM KCl. Methylene blue was added to visualize the gel.

mM KCl, as only ~25% of 1 had leached out of the gel after 24 h and beyond (GB-KCl). Any 1 released from the hydrogel in the first 24 h was likely material trapped in the pores and not part of the gel (Figure S4). This “blue” GB gel has remained intact in 155 mM KCl for over a year without any leaching of MB. The  $K^+$  in solution must stabilize the  $G_4$  quartets and allow the GB hydrogel to stay intact.

The borate anion's importance in making such a stable hydrogel was highlighted by comparing the hydrolytic stability of the GB gel with another known  $G_4 \cdot K^+$  hydrogel, one made from a 60:40 mixture of G and triacetylguanosine (TAcG, 7) and excess KCl (5 equiv, 354 mM).<sup>5</sup> Since the properties of the G/TAcG/KCl hydrogel are known, we felt that this “binary”  $G_4$  gel would be ideal for comparison with the GB hydrogel. Figure 3 shows that the GB hydrogel is far more stable than the G/TAcG gel. In 155 mM KCl, where the GB hydrogel remained intact for months, >75% of the G/TAcG hydrogel had dissociated after just 5 h (TAcG-KCl). As shown by the blue solution in Figure 3b, the G/TAcG hydrogel had completely dissolved after 24 h. The data in Figure 3 show that the  $B(OH)_4^-$  anion cooperates with the  $K^+$  cation to template self-assembly of G to give a robust, non-covalent hydrogel that remains intact indefinitely in salt water.

With an understanding of the GB gel's structure and stability, we aimed to incorporate compounds into the network using both non-covalent interactions and covalent bonds. We first investigated absorption of a cationic aromatic dye from solution into the GB gel, since we anticipated that the anionic borate esters and  $\pi$  faces of the  $G_4$  quartets would enable non-covalent binding. We compared the uptakes of MB and rose bengal (RB, 9) from solution by the GB hydrogel (Figure 4). Cationic MB is a  $G$ -quadruplex ligand,<sup>19</sup> and it has also been used as a dye for uptake studies by other hydrogels.<sup>3c,20</sup> RB is a nonplanar, anionic dye not known to interact with  $G_4$  quartets. We added a cube of 2 wt % GB gel to a KCl solution (155 mM) that contained MB and RB (12.5  $\mu$ M each). As shown in Figure 4, after 2 h the colorless gel showed a blue hue around its edges due to absorption of MB. Over time, the gel turned bluer around the edges and the dye



**Figure 4.** Suspension of a 2 wt % GB gel in KCl solution (155 mM) containing 12.5  $\mu$ M MB and RB. The gel turned increasingly blue, indicating that MB was selectively absorbed.

diffused into the interior. After 24 h, the gel was all blue, whereas the solution remained pink.

We also quantified the absorption of MB and RB into the GB hydrogel by monitoring the UV–vis absorbance of the dye that remained in solution (Figure S5). Whereas the GB gel absorbed almost all of the MB after 24 h (~90%), little change was seen in the concentration of RB. Presumably, the GB gel's selectivity for absorbing MB is due to electrostatic interactions of the cationic dye with the anionic borates and stacking interactions with the  $G_4$  quartets. This GB hydrogel may have potential to bind  $G$ -quadruplex ligands, which are potential anticancer drugs.<sup>21</sup>

Next, we envisioned incorporating nucleosides other than G into the GB gel by using (1) exchange reactions of 1,2-diols with the borate ester bonds<sup>22</sup> and (2) hydrogen bonding. As a proof of concept, we first explored the selectivity of incorporating dG, adenosine (A, 10) or 2'-deoxyadenosine (dA, 11) into the GB gel by carrying out competition experiments during the gelation process. Thus, we added equimolar A and dA (3 mM each) to a GB hydrogel [50 mM G/25 mM  $KB(OH)_4$ ], heated the mixture to 90  $^{\circ}C$ , and then let the mixture cool to reform a transparent hydrogel. We then used variable-temperature  $^1H$  NMR spectroscopy to measure the amounts of A and dA in the sol phase. As shown in Table 1, the GB gel was selective for incorporating A over its 2'-deoxy analogue dA. At 20  $^{\circ}C$  the GB gel showed a 4.5:1 selectivity for uptake of A (25.2%) over dA (5.7%), and that selectivity further increased to 8.5 at 37  $^{\circ}C$ . We attribute this marked selectivity for incorporation of diol A into the GB gel to either covalent bond formation via B–O exchange with the gel's

**Table 1. Incorporation of Nucleosides into the GB Hydrogel Network<sup>a</sup>**

Chemical structures of dG 2, A 10, and dA 11 are shown. dG 2 is a deoxyribose sugar with a guanine base. A 10 is a ribose sugar with an adenine base. dA 11 is a deoxyribose sugar with an adenine base.

T ( $^{\circ}C$ )	% dG	% A	% dA
20	83.4 $\pm$ 0.3	25.2 $\pm$ 1.7	5.7 $\pm$ 1.3
37	73.2 $\pm$ 1.6	18.9 $\pm$ 1.4	1.9 $\pm$ 1.1

<sup>a</sup>The values are the percentages of a 3 mM solution of nucleosides dG, A, and dA that were incorporated into a 50 mM GB gel, as determined by  $^1H$  NMR spectroscopy.

borate diesters or effective hydrogen bonding of the 1,2-diol with the anionic borates. Lastly, even though dG does not form a hydrogel in the presence of 0.5 equiv of  $\text{KB}(\text{OH})_4$  (Figure 1), it is readily incorporated into the GB gel (83.4%). Since dG cannot form a borate diester, it likely forms mixed G quartets with G units in the hydrogel.<sup>6</sup>

We have described a transparent  $\text{G}_4$  hydrogel formed from G and  $\text{KB}(\text{OH})_4$  that is indefinitely stable in 155 mM KCl solution. The borate anions function to give this  $\text{G}_4$  hydrogel by (a) solubilizing G and (b) reacting with G to form covalent dimers **5** and **6**, which work in concert with  $\text{G}_4 \cdot \text{K}^+$  self-assembly to give remarkably stable hydrogels. Moreover, the GB gel, with its anionic borate esters, binds cationic MB and also selectively incorporates nucleosides ( $\text{A} > \text{dA}$  and  $\text{dG} > \text{dA}$ ). In the future, we hope to better understand the supramolecular structure of this unique GB hydrogel and plan to explore its use for a variety of potential applications.

## ■ ASSOCIATED CONTENT

### Supporting Information

Experimental procedures and data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

## ■ AUTHOR INFORMATION

### Corresponding Author

[jdavis@umd.edu](mailto:jdavis@umd.edu)

### Notes

The authors declare no competing financial interest.

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