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# A Molecular Chaperone for G4-Quartet Hydrogels

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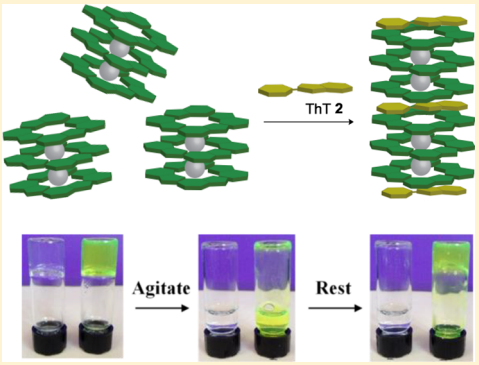
Supporting Information

**ABSTRACT:** Thioflavin T (ThT) functions as a molecular chaperone for gelation of water by guanosine and lithium borate. Substoichiometric ThT (1 mol % relative to hydrogelator) results in faster hydrogelation as monitored by $^1$H NMR and visual comparison. Vial-inversion tests and rheology show that ThT increases the stiffness of the $Li^+$ guanosine-borate (GB) hydrogel. In addition, the dye promotes relatively rapid and complete repair of a $Li^+$ GB hydrogel destroyed by shearing. We used rheology to show that other planar aromatics, some cationic and one neutral dye (methylene violet), also stiffened the $Li^+$ GB hydrogel. Data from powder X-ray diffraction, UV, and circular dichroism spectroscopy and ThT fluorescence indicate that G4 quartets are formed by the $Li^+$ GB system. We observed a species in solution by $^1$H NMR that was intermediate in size between monomeric gelator and NMR-invisible hydrogel. The concentration of this intermediate decreased much faster when ThT was present in solution, again showing that the dye can accelerate hydrogel formation. We propose that ThT functions as a molecular chaperone by end stacking on terminal G4-quartets and promoting the assembly of these smaller fragments into longer G4-based structures that can then provide more cross-linking sites needed for hydrogelation.




## BACKGROUND AND RATIONALE

Chaperone proteins promote assembly and folding of biomolecules by stabilizing key intermediates and destabilizing undesirable aggregates.[1] In addition to proteins, small molecules can also regulate the formation of biomolecular assemblies, as in the case of polymerization of tubulin into microtubules, which depends on the ligands 5′-GTP and 5′-GDP.[2] Small molecule chaperones have also been used to build supramolecular assemblies that can carry out various functions.[3−6] For example, in the area of controlling DNA structures, Hud and colleagues demonstrated that intercalators of the correct size and shape, which they coined "molecular mid-wives", enabled polymerization of short oligonucleotides into longer DNA strands.[4] Sleiman's group demonstrated that intercalators could also program self-assembly of oligonucleotides into well-defined DNA nanostructures.[5] Both of these examples underscore the power of using chaperones to control form and function in supramolecular assemblies, particularly ones made from DNA nucleobases.

Our interest in the potential of molecular chaperones to modulate the properties of supramolecular assemblies arose from our studies into interactions of dyes with supramolecular hydrogels made from guanosine **1** and borate salts.[7,8] Guanosine hydrogels certainly have a venerable history, and water-soluble derivatives of guanosine, such as 5′-guanosine monophosphate (5′-GMP) have been known to form gels in water for over a century.[9] Gelation of water by other guanine derivatives has led to a number of interesting systems.[10] However, hydrogelation using the parent nucleoside G **1** itself is hampered by the compound's terrible solubility and by the fact that it tends to crystallize from solution relatively quickly (within hours to days). Recent progress in making hydrogels using G **1** has come about through the design and discovery of binary mixtures that employ other guanosine derivatives as co-additives.[11] Recently, we have reported that the simple combination of G **1** and 0.5 equiv of $KB(OH)_4$ gives transparent, strong, and long-lived hydrogels.[7] These robust hydrogels form by self-assembly involving two orthogonal processes. One factor that drives self-assembly and subsequent hydrogelation is formation of a guanosine-borate (GB) diester that uses the borate anion to link two ribose nucleosides.[7,12,13] The second critical feature for hydrogelation using G **1** and $KB(OH)_4$ is the cation-templated formation of the hydrogen-bonded G4-quartet motif. Stacking of the G4-quartets into columnar structures and lateral interaction and bundling of these G4-wires gives rise to the physically entangled fibers that make up the $K^+$ GB hydrogel.[7]

In addition to their interesting structural properties, these anionic GB hydrogels are functional assemblies, as they bind cationic dyes, including thioflavin T (ThT **2**). We found that ThT **2** fluoresces quite strongly when incorporated into the GB gels.[7b] Based on precedent for ThT **2** binding to G4-DNA,[14] we concluded that this response was due to ThT **2** docking in a rigid, planar conformation to G4-quartets within the hydrogel (Figure 1). Our recent efforts have focused on testing this proposed mechanism and exploring the functional implications of adding ligands to GB hydrogels. The latter goal is timely due to the growing interest in using additives to modify the structure and properties of supramolecular gels.[15]

We report that 1 mol % of ThT **2** (relative to G **1**) acts as a molecular chaperone to (1) speed up hydrogelation by G **1** and

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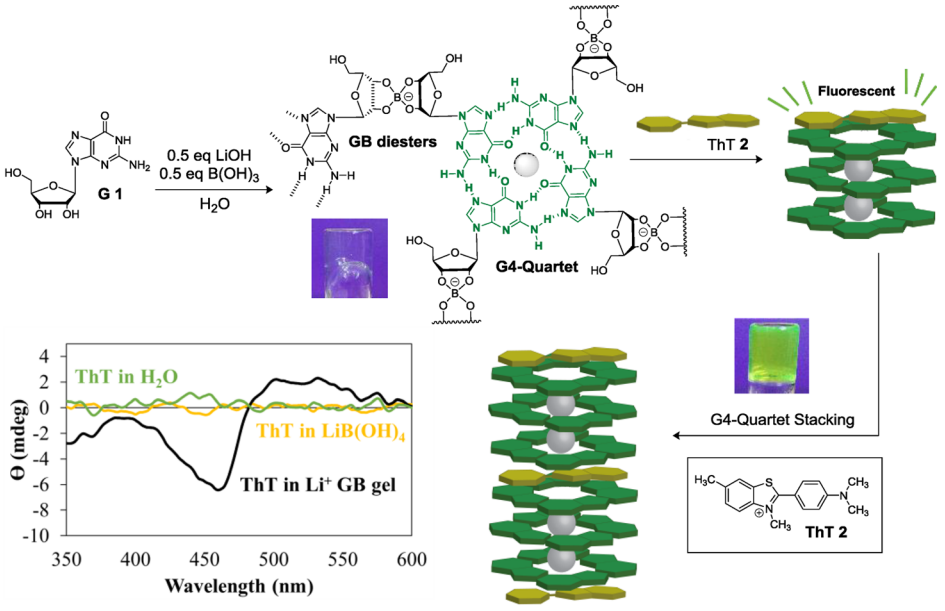


**Figure 1.** Addition of ThT **2** to a $Li^+$ GB hydrogel (72 mM G **1**, 36 mM $LiB(OH)_4$, 0.5 mM ThT **2**) results in a fluorescence response and a stiffer gel. We propose that ThT **2** binds to smaller G4-assemblies and promotes their stacking to ultimately form a hydrogel network with more cross-linked fibers. The gray spheres inside the G4-quartets represent cations, either $Li^+$ cations or adventitious $Na^+$ (see text below). The induced CD signal for ThT **2** supports the idea that the dye is stacking on a G4-quartet structure.

lithium borate $LiB(OH)_4$, (2) stiffen the $Li^+$ GB gel, and (3) help repair a GB-$Li^+$ gel destroyed by stress. We hypothesize that ThT **2** functions as a chaperone by stacking on G4-intermediates in solution and sandwiching these fragments together to stabilize larger G4-assemblies that can then give hydrogel fibers (Figure 1).[16] In doing so the small molecule ThT **2** not only stabilizes the G4-quartet structures but also likely shifts the equilibrium to disfavor other $G_n$ aggregates (such as the well-known hydrogen-bonded $G_n$ ribbons)[9c,d] that are not part of the self-assembly program for GB hydrogelation. As depicted in Figure 2, we

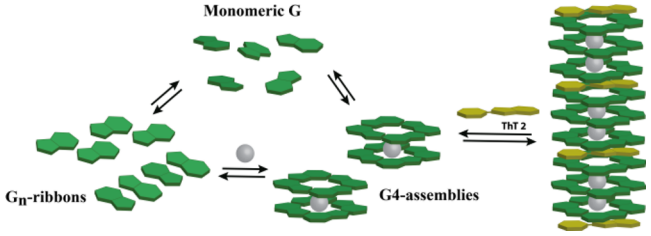


**Figure 2.** ThT **2** (yellow) functions as a molecular chaperone by assisting in the formation of larger G4-assemblies by G **1** (green) and $LiB(OH)_4$, while simultaneously shifting the equilibrium away from undesirable structures such as the hydrogen-bonded $G_n$ ribbons. The gray spheres inside the G4-quartets represent cations, either $Li^+$ cations or adventitious $Na^+$ (see text below).

propose that ThT **2** fits the definition of a molecular chaperone because it assists in the productive formation of G4-quartet-based assemblies and also selects against the formation of competing (and presumably undesirable) self-assembled structures such as the $G_n$ ribbons.

## RESULTS AND DISCUSSION

**Structural Evidence for Formation of G4-Quartets by G 1 and Lithium Borate and for ThT 2 Binding by the $Li^+$ GB Hydrogel.** To study the impact of substoichiometric ThT **2** on hydrogelation, we decided to work with the weakest of the GB gels: the $Li^+$ version.[7b] We reasoned that a relatively weak system teetering on the sol–gel boundary, like $Li^+$ GB, might respond best to the influence of a molecular chaperone. In the presence of 0.5 equiv of $LiB(OH)_4$, the typically insoluble G **1** (2 wt %, 72 mM) was fully soluble in water and initially gave a free-flowing solution. After a few hours, however, a weak gel formed (Figure 1 inset). Vial inversion tests showed that this gel was stiffened by addition of substochiometric ThT **2**. Thus, a $Li^+$ GB (100 mM in G **1**) melted between 45 and 46 °C. Addition of 2 mM of ThT **2** to this gel increased the gel–sol melting point to 55–56 °C, the first clear indication that a small molecule could stiffen the gel.

Compared to $Na^+$ and $K^+$, the smaller $Li^+$ cation is typically not as effective at stabilizing G4-quartets in solution.[17] There are, however, a number of known guanine-based systems where mass spectrometry and/or $^1H$, $^7Li$ NMR has shown that G4-quartets can bind $Li^+$.[18] Various measurements indicated that the $Li^+$ GB sample contained G4-quartets. First, powder X-ray diffraction of a dried gel made from G **1** and $LiB(OH)_4$ showed a major peak at $2\theta \approx 27.0°$ ($d = 3.3$ Å), characteristic of the $\pi$–$\pi$ distance for stacked G4-quartets (Figure S5).[7b] Second, circular dichroism (CD) spectra of a 2 wt % $Li^+$ GB gel (72 mM G **1**; 36 mM $LiB(OH)_4$) showed positive peaks at 247 and 275 nm, a spectrum that is diagnostic of some sort of stacked G4-quartet assembly (Figure S6).[19] Notably, these positive peaks in the CD were enhanced considerably when just 1 mol % of ThT **2** was added to the $Li^+$ GB hydrogel, consistent with the dye inducing and stabilizing G4-based structures. Lastly, ThT **2** was strongly fluorescent in the $Li^+$ GB hydrogel (Figure S4), diagnostic of its binding to G4-quartets.[7b,14] We also observed a 44 nm red shift in the UV–vis spectrum for ThT **2** (Figure S7) and an induced CD signal for ThT **2** in the 450 nm area (Figure 1) when the dye was in a $Li^+$ GB hydrogel. Both of these last spectroscopic results are again consistent with ThT **2** stacking on exposed G4-quartets within the framework of a $Li^+$ GB hydrogel.[14a]

The above evidence, while indirect, is consistent with formation of $G_4$-quartets when G **1** is mixed together with $LiB(OH)_4$.


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We do not, however, have direct evidence that $Li^+$ is bound within the center of these $G_4$-quartet structures. Attempts to identify $Li^+$ within the gel phase using solid-state $^7Li$ NMR spectroscopy have so far been unsuccessful, as these weak gels do not survive the rotor speeds used to record the $^7Li$ spectra.[20] A reviewer suggested that $Na^+$ contaminants might influence the properties of this $Li^+$ GB system, given the $Li^+$ cation's high hydration energy and its relatively weak affinity for the G4-quartet cavity.[17b] To address this issue, we recorded $^{23}Na$ NMR spectra of the $Li^+$ GB gel both below and above its melting temperature and with an internal standard (Figure S8). Based on integration of this $^{23}Na$ NMR data, we determined that there was 0.33 mM of $Na^+$ in the gel phase of a GB hydrogel made from 50 mM of G **1** and 25 mM $LiB(OH)_4$. Since the LiOH used to prepare the hydrogel contained no detectable $Na^+$ (by elemental analysis), this $Na^+$ contaminant must be introduced during sample preparation and manipulation. So, another possibility for the enhanced stiffness of this hydrogel is that the combination of a small amount of $Na^+$ cation and aromatic dye serves as the nucleation site for the growth of interconnected fibers needed for hydrogelation. We will continue to look for MS and NMR methods to confirm whether or not $Li^+$ or $Na^+$ is bound to the G4-quartets of this $Li^+$ GB hydrogel. But, our major finding in this present study, that ThT **2** promotes hydrogelation and stabilizes an intrinsically weak G4 hydrogel, is not changed by a 1−1.5% $Na^+$ contaminant in the $Li^+$ GB gel.

**NMR Spectroscopy Reveals a "Large" Intermediate in Solution Whose Lifetime Is Influenced by ThT 2.** Solution $^1H$ NMR spectra of the $Li^+$ GB sample, before it had formed a gel, showed a separate set of broad signals in slow exchange with the much sharper signals for the "monomeric" G **1** and its borate esters (Figures S9−S11). These relatively broad $^1H$ NMR signals, observed here for the $Li^+$ GB sample, are never observed for either the $Na^+$ or $K^+$ GB hydrogels. We postulated that the broadened signals were due to some type of self-associated $G_n$-intermediate, either a G4-based structure or a $G_n$ ribbon structure as depicted in Figure 2. Diffusion-ordered spectroscopy[21] indicated that this species with broad signals was indeed significantly larger than the monomeric G **1** and its borate esters **3**−**5** (see Figure S10). Using the well-resolved H1′ signals for the various species, we measured the diffusion coefficient for G **1** to be $4.04 \times 10^{-10}$ $m^2$ $s^{-1}$, whereas the putative $G_n$ species with the broad signals was obviously much larger, as seen by its reduced diffusion coefficient of $D = 1.95 \times 10^{-10}$ $m^2$ $s^{-1}$. Importantly, the H1′ signal for this $G_n$-intermediate decreased in intensity over time (Figures 3 and S11). Thus, after 96 h, about 50% of the original $G_n$-intermediate remained in solution. This loss in NMR signal with time is presumably due to a process wherein G **1** subunits within this larger $G_n$-assembly slowly enter the gel phase and become part of a structure that is too large to detect by solution $^1H$ NMR. Such a dynamic process, where the equilibrium concentration and possibly the structure of this self-assembled $G_n$ intermediate is changing, is also consistent with the observation that the chemical shift for the H1′ signal of the $G_n$ assembly moves downfield as the concentration of the intermediate changes over time (Figure 3).

Importantly, addition of ThT **2** (0.5 mM) to the $Li^+$ GB gel accelerated disappearance of signals for this $G_n$-intermediate (Figure 3B), as now 50% of that broadened H1′ signal remained after 24 h (instead of the 96 h it takes in the absence of the dye). This difference in the intermediate's lifetime in solution, as measured by $^1H$ NMR, correlated nicely with macroscopic changes in the hydrogel. Whereas the $Li^+$ GB sample remained

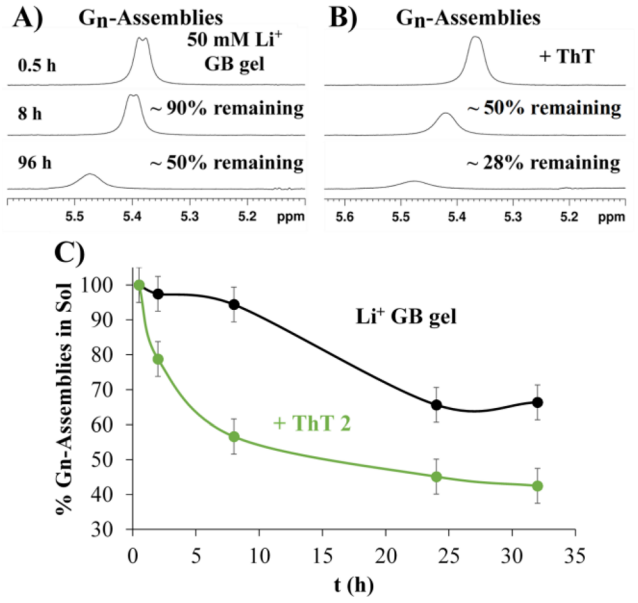


**Figure 3.** (A) The H1′ signal for the $G_n$-intermediate in a 50 mM $Li^+$ GB hydrogel (100 mM G **1**) decreased with time. (B) With 0.5 mM of ThT **2** present. (C) The % of $G_n$-intermediate present in the sol as a function of time.

free-flowing after 8 h, a sample containing ThT **2** (0.5 mM) formed a gel that held its weight when inverted. These comparative NMR data in Figure 3 indicate that ThT **2** promotes faster formation of the $Li^+$ GB gel, a hallmark of a molecular chaperone.[22]

To confirm the dye's impact on increasing the size of $G_n$-assemblies in solution, as suggested by the $^1H$ NMR data, we carried out dynamic light scattering (DLS) measurements. Table S1 shows that particle size increased with increasing concentration of ThT **2**. Whereas particles in the 50 mM $Li^+$ GB solution (2 h after preparation) had hydrodynamic radii of ∼200 nm, addition of 100 $\mu$M ThT **2** nearly doubled the radii to $R$ = 397 nm. Furthermore, as more ThT **2** was added to the solution, the particle radius increased to as high as $R$ = 700 nm in solutions containing 1 mM of ThT **2**.

**Rheology Confirms that Substoichiometric Amounts of ThT 2 Make the $Li^+$ GB Gel Stiffer.** The physical implications of adding ThT **2** to the $Li^+$ GB hydrogel are also striking, as rheology indicated that the gel is stiffened by low relative concentrations of ThT **2**. Dynamic frequency sweeps showed that the $Li^+$ GB hydrogel (100 mM) with ThT **2** (2 mM) has a much higher storage modulus ($G'$) than the system without dye (Figure S12). Also, as seen in Figure 4, strain sweeps indicated that the $G'$ value of a $Li^+$ GB hydrogel (100 mM G **1**, 50 mM $LiB(OH)_4$) increased 10-fold as the concentration of ThT **2** was raised from 0 to 1 mM. At concentrations >1 mM, the $G'$ value leveled off, indicating that the system was essentially saturated at this 100:1 molar ratio of hydrogelator G **1** to chaperone ThT **2** (Figure S13). This leveling effect at such a low molar ratio of dye to hydrogelator suggests that it is not the electrostatic interactions of the cationic ThT **2** with the anionic borate G4 polymers that is responsible for the strengthening of the hydrogel. If that were the case, one might expect that the $G'$ value would continue to increase as more cationic dye beyond 1 mM was added to the $Li^+$ GB solution.

In addition to increasing the mechanical strength of the $Li^+$ GB hydrogel, substoichiometric amounts of ThT **2** promoted repair


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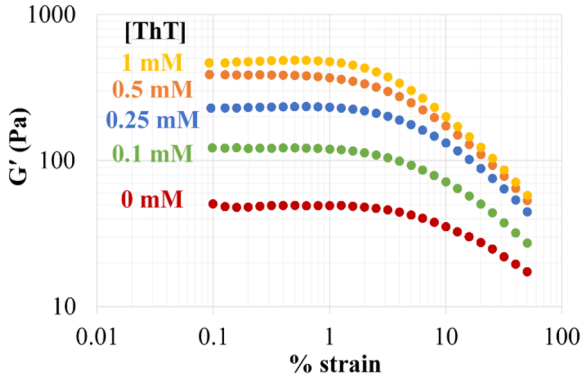


**Figure 4.** Strain sweeps on a $Li^+$ GB hydrogel (100 mM G **1**, 50 mM $LiB(OH)_4$) show that the storage modulus ($G'$) increases as a function of ThT **2** concentration (see also Figure S13).

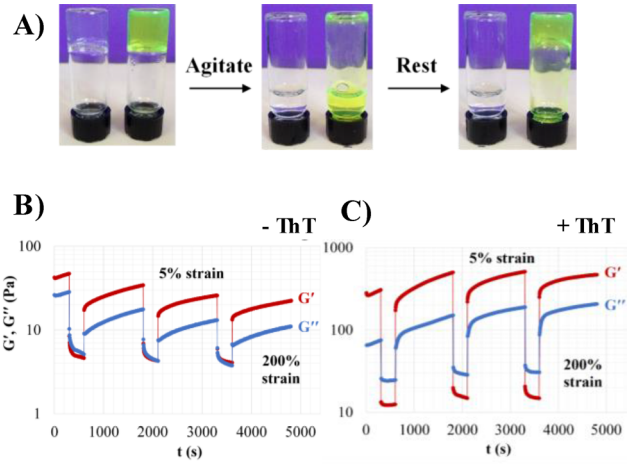


**Figure 5.** (A) In the presence of ThT **2** (0.5 mM), the $Li^+$ GB hydrogel (72 mM G **1**, 36 mM $LiB(OH)_4$) reformed faster after agitation. The samples were sonicated until liquid and allowed to rest. After 10 min, the system with ThT **2** (yellow) had reformed into a gel. (B) Hysteresis tests performed at constant angular frequency of 10 rad $s^{-1}$ show the gel undergoes shear-induced weakening. After each interval of high strain (200%), the GB gel weakens and does not rebound to its initial $G'$ value. (C) With added ThT **2** (0.25 mM), strain cycles do not impact the stiffness of the gel, and the gel fully recovers.

of the gel after it had been stressed. The $Li^+$ GB hydrogels undergo a shear-induced weakening that is apparent visually and can be quantified by rheology (Figures 5 and S14–15).[23] Figure 5A illustrates that the $Li^+$ GB hydrogel reformed faster after agitation if substoichiometric amounts of the chaperone were present. Hydrogels (72 mM G **1**, 36 mM $LiB(OH)_4$), either with ThT **2** (0.50 mM) or without dye, were sonicated until they turned into free-flowing solutions (~5 min). After 10 min of resting at room temperature, the sample containing ThT **2** had reformed a hydrogel that could support its weight. Conversely, the sample without ThT **2** remained a solution.

Rheology also demonstrated the influence of ThT **2** on reversible healing of a $Li^+$ GB hydrogel (100 mM G **1**, 50 mM $LiB(OH)_4$). As shown in Figure 5B, when the hydrogel ($G' < G''$) was subjected to high oscillatory strain ($\gamma$ = 200%; $\omega$ = 10.0 rads $s^{-1}$), it underwent a gel to sol transition ($G'' > G'$). When the strain was decreased ($\gamma$ = 5%; $\omega$ = 10.0 rads $s^{-1}$), a gel would reform but it was always weaker, as its final $G'$ value was less than the storage modulus value $G'$ at the beginning of each strain cycle (Figure 5B). In contrast, when ThT **2** (0.25 mM) was present the $Li^+$ GB hydrogel withstood 3 cycles of high strain and always fully rebounded to its initial $G'$ value (Figure 5C). This significant difference in the rheology data in Figure 5B and C indicates that substoichiometric amounts of the cationic ThT **2** are able to guide the reformation (or repair) of a stable hydrogel from G **1** and $LiB(OH)_4$.[24] One explanation for this property, again consistent with all the data, is that ThT **2** adopts a planar conformation and stacks on exposed G4-quartets in order to stabilize G4-intermediates and promote stacking of these fragments to give longer fibers that can form more connections with other fibers, thus stabilizing the hydrogel network.

**Other Aromatic Dyes Stiffen $Li^+$ GB Hydrogel.** If ThT **2** stiffens the $Li^+$ GB hydrogel by stabilizing G4-quartets in the fibrous network, we reasoned that other ligands that bind G4-quartets should also function as molecular chaperones for hydrogelation by G **1**. We used rheology to measure the difference in storage modulus ($\Delta G'$) for $Li^+$ GB gels that did and did not contain dyes (**6**–**12**) with different charges, sizes, and shapes (Figure 6). Cationic dyes, crystal violet (CV **6**), thiazole

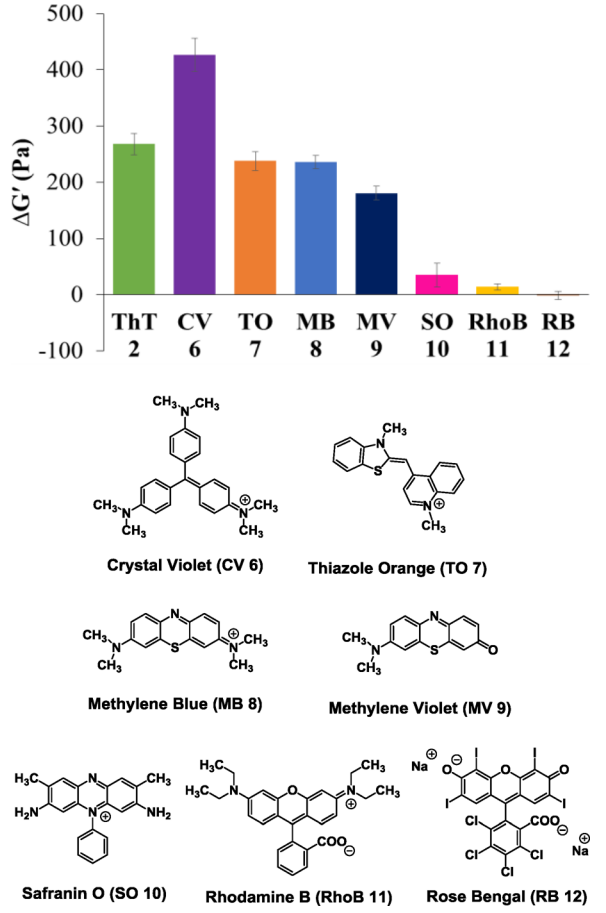


**Figure 6.** Change in storage modulus ($\Delta G'$) for $Li^+$ GB gels (100 mM G **1**, 50 mM $LiB(OH)_4$) that did and did not contain dyes **2** and **6**–**12** (250 $\mu$M). The $G'$ values were measured at 1% strain. For strain sweeps for these dyes, see Figure S18.

orange (TO **7**), and methylene blue (MB **8**), are known to bind to G4-DNA and other G4-structures.[25−27] As shown in Figure 6, each of these planar cationic compounds increased the storage modulus ($G'$) of the $Li^+$ GB hydrogel. Triarylmethyl cation CV **6** was the most effective ligand, as its $\Delta G'$ value was 1.5 times greater than that for ThT **2**.



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Methylene violet (MV **9**), a neutral analog of MB **8**, was also relatively effective at increasing the $G'$ value of the $Li^+$ GB gel. This result is important because it suggests that $\pi-\pi$ binding between the planar aromatic dye and G4-quartet, and not simply electrostatics, can drive molecular recognition in this case. To investigate the possibility that the basicity and ease of protonation of MV **9** might be shifted within the negative environment of the GB hydrogel, we determined the protonation state of MV **9** within the $Li^+$ GB hydrogel. The $pK_a$ of protonated $MVH^+$ has been determined to be $4.00 \pm 0.05$.[28,29] So, at pH 8.5 within the borate hydrogel, MV **9** should be neutral. We carried out a pH titration experiment and confirmed that MV **9** only begins to be protonated below pH 5. Furthermore, when we added MV **9** to the GB hydrogel (Figure S17) we saw UV absorbance bands only for the neutral form of MV and no absorbance bands at $\lambda$ = 470 nm for $MVH^+$. We conclude, therefore, that it is the neutral MV **9** that binds and strengthens the $Li^+$ GB gel.

Certainly, electrostatic interactions between cationic dyes like **2** and **6**−**8** and the hydrogel's anionic borate esters may be quite important. But, apparently not all cationic dyes interact with the $Li^+$ GB hydrogel. Safranin O (SO **10**),[30] which has a similar core as MB **8** and MV **9**, had little impact on hydrogel stiffness (Figure 6). Perhaps the phenyl substituent on the central ring of SO **10**, which is likely to be orthogonal to the tricyclic core,[30] inhibits binding to the G4-quartet. Another important point here is that SO **10** might be expected to stiffen the gel if only electrostatic interactions between the cationic dye and the gel's borate diester frame were necessary. The importance of a planar aromatic surface for binding the $Li^+$ GB gel was reiterated with nonplanar ligands rhodamine B (RhoB **11**) and rose bengal (RB **12**). Addition of zwitterionic RhoB **11** or anionic RB **12** had little effect on the gel's storage modulus ($G'$).[31] These findings with dyes **6**−**12**, combined with the fluorescence, UV, and CD spectroscopy results with ThT **2**, indicate that increased gel stiffness is largely due to $\pi-\pi$ stacking of planar aromatics with G4-quartets that make up the hydrogel.

## CONCLUSIONS

We found that substoichiometric amounts of ThT **2**, and some other planar aromatics, promote faster hydrogelation by G **1** and $LiB(OH)_4$, stiffen the resulting hydrogel, and enable the relatively fast and complete repair of gels that had sheared. By stabilizing G4-assemblies, and inhibiting other H-bonding motifs that are available to G **1**, ThT **2** functions as a molecular chaperone for hydrogelation (Figure 2). We are pursuing the idea that this unique hydrogel, made simply by mixing G **1** and $LiB(OH)_4$ in water, may be useful for identifying ligands that can bind to G4-quadruplex DNA.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b08769.

Experimental procedures and associated data (PDF)

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### Notes

The authors declare no competing financial interest.

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