Journal Name

COMMUNICATION

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/7

G-Quartet Hydrogels for Effective Cell Growth Applications

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Functional G-quartet hydrogels formed from natural guanosine cross linked with benzene-1,4-diboronic acid and Mg^{2+} support cell growth with no visible signs of gel degradation.

Constitutional self-organization (CSO), combining dynamic covalent and supramolecular bonding, provides an evolutional approach to generate adaptive materials for biomimetic systems,[1] such as supramolecular gels.[2] A prime example of CSO is formation of constitutional materials and gels based on the G-quartet.[3] Various guanosine analogs form hydrogels.[4] The mechanism involves cation-templated generation of the G-quartet, a H-bonded macrocycle. The cation further stabilizes the assembly by coordinating to carbonyls of neighbouring G-quartets. The resulting G-quadruplex, a columnar structure of stacked G-quartets, may further self-assemble *via* supplementary reversible covalent and non-covalent interactions to provide the gel network. Although supramolecular hydrogels made from peptides have found increasing use in tissue engineering and cell growth applications,[5] the use of nucleoside gels as platforms for cell growth has, to our knowledge, not yet been demonstrated.[6]

We recently described hydrogels made by simply mixing 0.5 eq. of $KB(OH)_4$ with guanosine (G **1**).[7] The G_2-borate (GB) diesters, formed by condensation of 2 equivalents of G **1** and borate, then form G-quartets that assemble to give an anionic gel that selectively absorbs cationic dyes from water and can incorporate 1,2-diols and guanines into its network.[7] The Sadler group has subsequently shown that related GB hydrogels, modified with photoactivatable drugs, are non-toxic to a number of cultured cell lines.[8] We reasoned that such facile self-assembly of the readily available natural product G **1** in the presence of boron cross-linkers,[9] might well provide robust hydrogel platforms for investigating cell growth and fabricating tissue outside of the organism.

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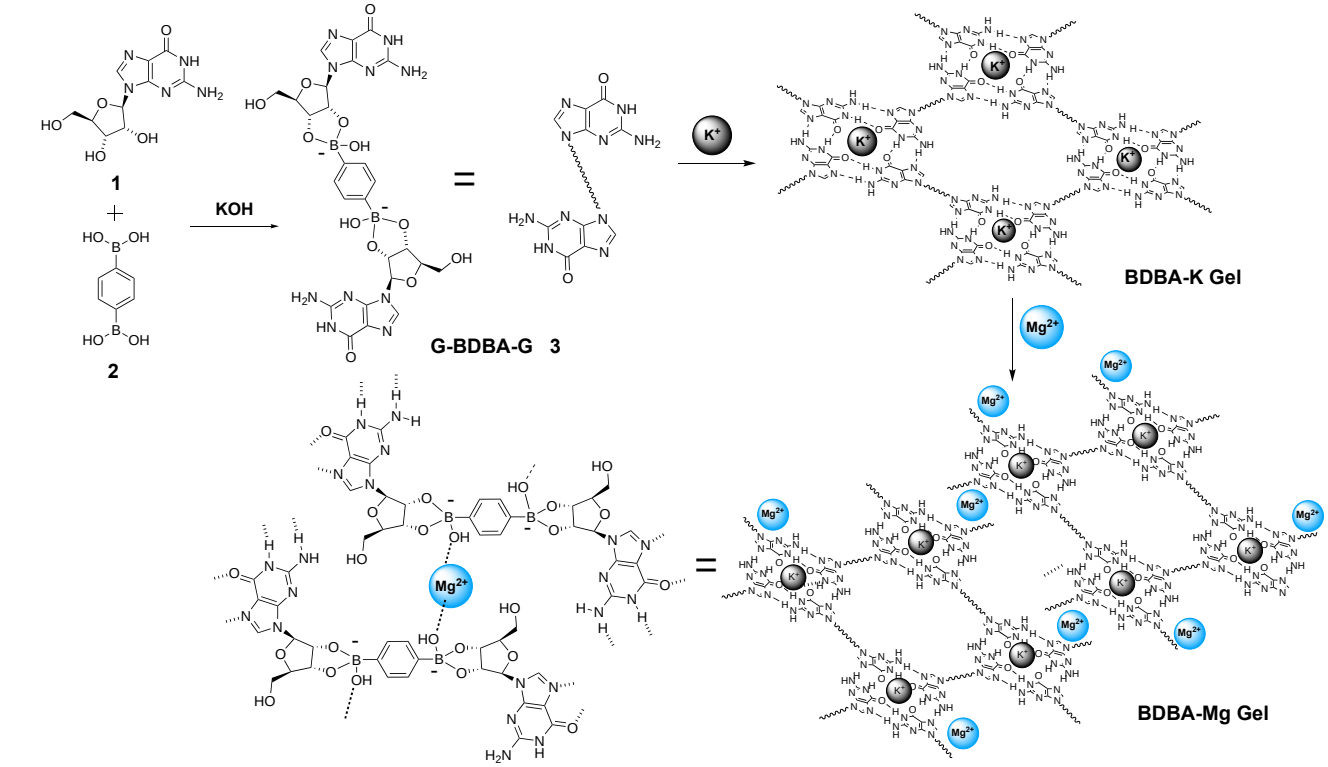
Electronic Supplementary Information (ESI) available: Experimental details, NMR, XPRD CD, Rheology, AFM, Cell growth experiments. See DOI: 10.1039/x0xx00000x

We now describe preparation and application of G-quartet hydrogels formed from guanosine (G **1**) and benzene-1,4-diboronic acid (BDBA **2**) using K^+ or Ba^{2+} as templating cations, wherein G-quartets formed *in situ* by bis-boronate G-BDBA-G **3** are further cross-linked with Mg^{2+} (Scheme 1). Compared to the parent GB hydrogel,[7] we sought to tune the gel's swelling properties by 1) changing the cross-linker from boric acid to ditopic BDBA **2** and 2) using Mg^{2+} cations as external stabilizers of the anionic bis-boronates G-BDBA-G **3**. These modifications lead to hydrogels with much increased water content, a necessary property for making biocompatible materials.[5] Importantly, we report that these new G-BDBA hydrogels, particularly a high-water content gel made with Mg^{2+}, support cell growth and show cell viability of up to 73 % after 24 hours.

Fig. 1 (a) Samples of self-standing hydrogels BDBA-K, 88 mM of G **1** in 3 mL of water (left), BDBA-Ba, 22 mM of G **1** in 10 mL of water (middle) and BDBA-Mg, 4 mM of G **1** in 44 mL of water (right), showing their different water retention capacities. Samples of varying G concentrations (mM indicated on vials) without (b) and with (c) Mg^{2+} made with G **1** : BDBA 2 : KOH ratio = 1 : 0.5 : 1. BDBA-Mg gels were made by preforming a BDBA-K gel and diluting with H_2O, then adding 5 mM Mg^{2+}.

We reasoned that, in the presence of a templating cation like K^+, hydrogelation would be driven by H-bonded G-quartets made from the boronate dimer G-BDBA-G **3**, itself formed by condensation of BDBA **2**[10] with the cis-1,2-diols of two G **1** monomers (Scheme 1). We first tested hydrogel formation by heating a solution of 0.05 g of G **1** (100 mM) with 0.015 g of BDBA **2** (0.5 eq.) and 1 eq. of KOH in 2.0 mL of distilled water. Upon cooling to ambient temperature we observed a transparent gel that held its own weight upon vial inversion (Fig. S1). This BDBA-K mixture showed a relatively narrow capacity for water retention, forming transparent gels in up to



Scheme 1. Reaction pathways for BDBA-K and BDBA-Mg hydrogels made from G **1** and BDBA **2**, stabilized with K^+ via G-quartet formation and with Mg^{2+} via external cross-linking.

3 mL of water (Fig. 1a, left), but collapsing to give a solution with 4 mL of water. Upon changing the cation that templates the G-quartets from K^+ to Ba^{2+}, the resulting BDBA-Ba hydrogel showed enhanced water retention, now requiring 10 mL of water to yield a transparent gel, when using the same amount of G **1** (Fig. 1a middle). We next investigated the potential stabilization of the BDBA-K hydrogel by divalent (Mg^{2+}, Ca^{2+}, Mn^{2+}) or trivalent (Fe^{3+}) cations, which are too small to bind within the G-quartet pore,[3] but could electrostatically crosslink anionic boronates in different G-quartet strands (Scheme 1). As a negative control, we first formed the building block G-BDBA-G **3** using LiOH instead of KOH, followed by addition of $MgCl_2$ or $CaCl_2$, which gave white, viscous suspensions (Fig. S2). This confirmed that K^+ is needed to template the G-quartet hydrogel. Indeed, addition of Mg^{2+} (5 mM) to a pre-made BDBA-K gel gave a new BDBA-Mg hydrogel (Fig. 1a right), one that was able to retain ~ fifteen times more water (44 mL) than the original BDBA-K gel. Surprisingly, addition of Ca^{2+} or Fe^{3+} did not yield hydrogels, but a successful experiment with Mn^{2+} gave a BDBA-Mn gel with similar water-retention ability as BDBA-Mg (Fig. S3). In another study, we compared the critical gelator concentration (cgc) for the BDBA-K and BDBA-

Mg gels. Using the vial inversion test we determined that cgc was ~ 36 mM G **1** for the BDBA-K gel (Fig. 1b). Adding Mg^{2+} to a preformed BDBA-K gel resulted in a significantly lower cgc of < 5 mM (Fig. 1c). The results in Fig. 1 indicate that Mg^{2+} is an effective cross-linker that significantly enhances the water-retention capabilities of hydrogels made from G **1** and BDBA **2**.

To obtain evidence for the structures depicted in Scheme 1 we used ^{1}H NMR to show that 3 species co-exist in the sol phase of BDBA-K, BDBA-Ba and BDBA-Mg gels: namely, free G **1**, a mono-substituted boronate G-BDBA and the key boronate diester G-BDBA-G **3** (Fig. S4a). For BDBA-Mg at 25 °C, the doublet at δ = 5.90 ppm corresponds to the H1' proton of G **1**, while broader peaks at δ = 5.80 ppm and δ = 6.10 ppm were due to H1' of the two G-boronate esters. Diffusion-ordered spectroscopy (DOSY)[11] of BDBA-Mg at 5 °C (Fig. S4b), confirmed these assignments: the peak at δ = 5.70 ppm had the largest diffusion coefficient (2.377 x 10^{-10} m^2/s) and belongs to the smallest species, G **1**. The next largest coefficient (1.766 x 10^{-10} m^2/s) belongs to the mono-ester G-BDBA at δ = 5.66 ppm. Lastly, the peak at δ = 5.91 ppm had the smallest diffusion coefficient (1.698 x 10^{-10} m^2/s), consistent with the largest species, di-substituted G-BDBA-G **3**, the building block for hydrogelation.

The key to hydrogel formation are the G-quartets that provide the basis for the gel's network. Powder X-ray diffraction (PXRD) and CD spectroscopy were consistent with stacked G-quartets in these hydrogels made from G **1** and BDBA **2**. PXRD data obtained from a freeze-dried sample of a BDBA-K gel showed a peak at 2θ ≈ 26.9° (d = 3.3 Å), in line with the π-π separation between stacked G-quartets (Fig. S5).[12] Stacked G-quartets show CD absorption bands in the 240-260 nm and/or 290-300 nm regions.[13] All three hydrogels had distinctive CD spectra, indicating different G_4-stacking interactions or different populations of syn/anti conformers. The CD spectrum of BDBA-K has an intense positive band at 300 nm with a broader negative band at 255 nm (Fig. S6a). In BDBA-Ba, the oppositely signed negative–positive bands lower than 290 nm can be related to a left-handed helical stacking of G-quartets (Fig. S6b),[13] indicating a different chiral structure than that in BDBA-K. Finally, the CD spectrum of BDBA-Mg has a small negative-positive band (240-260 nm), similar to BDBA-Ba, and a strong negative band at 290 nm, attributed to a unique G4-orientation induced by Mg^{2+} (Fig. S6c).

These hydrogels exhibited solid-like rheology, where the storage modulus (G') of the material is larger than its loss modulus (G'') (Fig. S7). Both BDBA-K and BDBA-Ba gels are stiffer than BDBA-Mg, which contains a higher water content and lower concentration of G **1**. The important point is that, even at 5 mM G **1**, BDBA-Mg has rheological characteristics of a hydrogel, with a constant G' value that is greater than G'' over the entire strain range of 0.1-100%.

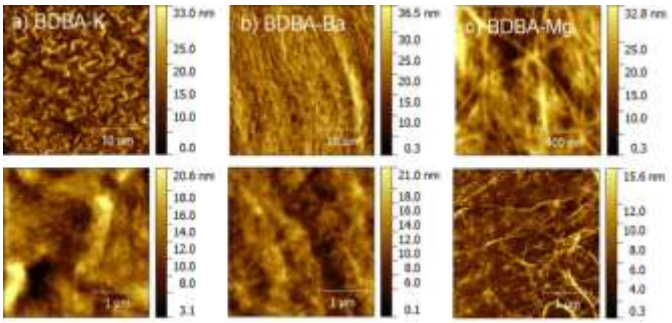


Fig. 2 AFM images of a) BDBA-K, b) BDBA-Ba and c) BDBA-Mg hydrogels.

Insights on gel morphology were obtained from atomic force microscopy (AFM) (Fig. 2) and scanning electron microscopy (Fig. S8). Each sample exhibited distinct morphologies at the microscale: BDBA-K has an organized "wave"-like structure with unit size of 1 µm (Fig. 2a), while BDBA-Ba shows uniform fibrillar layers (Fig. 2b). At the sub-µm scale, BDBA-K and BDBA-Ba hydrogels have similar structures (bottom Fig. 2a,b). Importantly, the BDBA-Mg gel has a distinct morphology, with interconnected fibers up to several µm in length (Fig. 2c). These AFM results, along with the tip-test, CD and rheology data, highlight that Mg^{2+} serves as a bridging element between G-quartets formed by bis-boronate G-BDBA-G **3**. The width of the fibers suggests that 15-23 G-quartets, each having a diameter of ~3 nm,[3,4] may self-assemble *via* Mg^{2+} external cross-linking, to form an aggregated fibrillar structure of 50-70 nm. The AFM studies show differences not only between mono and divalent cations, but also between the divalent cations, Ba^{2+} and Mg^{2+}. The absence of the fibres for BDBA-Ba in the AFM is consistent with Ba^{2+} not forming bridges, but instead stabilizing the H-bonded G-quartets. A related cross-linking motif was reported for adenosine monophosphate hydrogels stabilized by selective synergetic interactions of Zn^{2+} with phosphate and adenine groups.[14]

To check the potential applicability of these G-quartet hydrogels in tissue engineering, we performed cell growth and viability tests on the NHDF (Normal Human Dermal Fibroblasts) cell line using freshly-prepared hydrogels BDBA-K (2 mL), BDBA-Ba (9 mL) and BDBA-Mg (10 mL) (Fig. 3).[15] The high pH values of the initially prepared hydrogels were adjusted to near neutral by washing with TAE buffer: BDBA-K (pH=9.5 to pH=7.6), BDBA-Ba (pH=8.8 to 7.5) and BDBA-Mg (pH=9.5 to pH=7.4). The BDBA-K gel was stable under both buffered and unbuffered conditions. The BDBA-Ba gel became cloudy after buffer treatment and no cell growth was observed. Although the buffer-treated BDBA-Mg gel initially supported cell growth it was unstable under these experimental conditions, starting to degrade after 24 h and it was almost all destroyed after 48 h (Fig. S9). Based on these findings, the cell growth experiments were monitored on buffered BDBA-K and unbuffered BDBA-Ba and BDBA-Mg gels, at 0, 4 and 24 hours of cell cultivation, and pictures were taken at each time interval (Fig. 3).

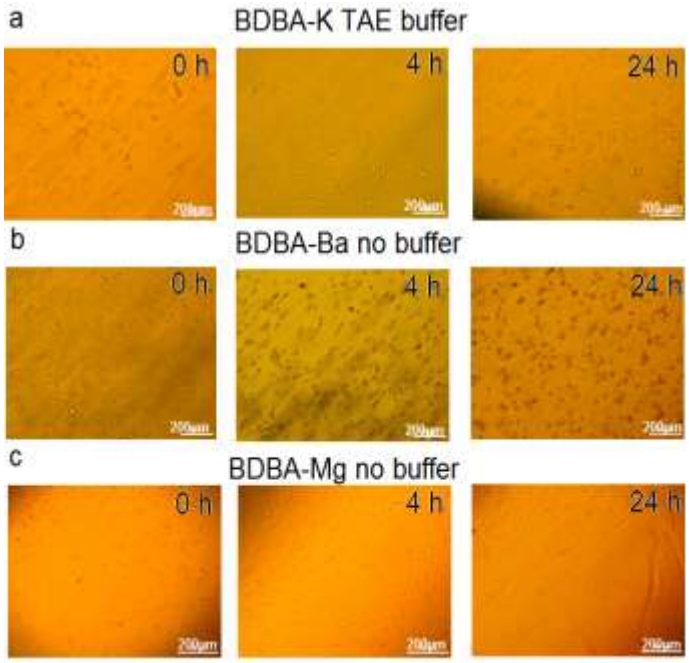


Fig. 3 NHDF cells on G-quartet hydrogels made from G **1** and BDBA **2**: a) BDBA-K washed with 3 x TAE/Mg^{2+} buffer; b) BDBA-Ba and c) BDBA-Mg without buffer treatment visualized at t= 0 h (a), after 4 h (b) and after 24 h (c).

As shown in Fig. 3a, after 4 h, the cells had begun to attach to the BDBA-K surface, changing from spherical to ellipsoidal, with a clear improvement in cell density after 24 hours. The most spectacular results were obtained with BDBA-Ba and BDBA-Mg hydrogels that were not treated with TAE buffer. As seen in Fig. 3b, the cells adhered to the surface of unbuffered BDBA-Ba hydrogel, forming a network of linked cells after 24 h. Similarly, untreated BDBA-Mg hydrogels showed excellent properties as cell growth supports, with a dense network of connected cells, and the gel platform didn't present any apparent signs of degradation over 24 h (Fig. 3c).

Cell viability on the BDBA hydrogels was evaluated using a colorimetric MTS cell proliferation assay and was calculated as a percentage relative to the viability of untreated cells supported by the culture medium.[16] Our experiments showed that cell viability after 24 hours was 42% for BDBA-K, 47% for BDBA-Ba, but was significantly greater (73%) for the highly swelled and less dense BDBA-Mg hydrogel (Fig. S10).

In conclusion, we have designed and prepared G-quartet hydrogels made readily from the natural product G **1**, diboronic acid **2** and various templating and bridging cations. These gels show interesting cation-dependent physical and functional properties. Tip tests, NMR, CD and AFM microscopy data showed that the structure and water content of the supramolecular hydrogel is governed by the identity of the cations used for internal stabilization of G-quartets (K^+ vs Ba^{2+}) as well as by external cross-linking of anionic borates by Mg^{2+}. The composition of each hydrogel determines the amount of water it can incorporate, being able to sustain 15-fold more water if a Mg^{2+} cross-linker is used. This gel swelling property makes these hydrogels perfect candidates for cell growth applications. Our initial findings using the NHDF cell line demonstrate that these G-quartet hydrogels can support significant cell growth on their surfaces. We are currently screening other formulations (pH, ionic strength, buffers) to find optimal conditions for growing cells on these easily prepared and biocompatible G-quartet hydrogels.

Acknowledgements

This work was supported by ANR DYNAFUN, ANR-15-CE29-0009-02, by Romanian National Authority for Scientific Research and Innovation, CNCS – UEFISCDI, project number PN-II-RU-TE-2014-4-1444, by the European Commission, Horizon 2020 WIDESPREAD 2-2014: ERA CHAIR SUPRACHEMLAB No. 667387. Research carried out by TNP and JTD was funded by the Office of Basic Energy Sciences of the U.S. Department of Energy by grant DE-FG02-98ER14888. TNP thanks the U.S. Dept. of Education for a GAANN fellowship.

Conflicts of interest

There are no conflicts of interest to declare.

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