Supporting Information for

G-Quartet Hydrogels for Effective Cell Growth Applications

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Abstract: Supramolecular hydrogels composed of natural components are of high interest in drug delivery and tissue engineering. In addition, formation of hydrogels with increased water content is crucial for cell growth and viability. Our strategy for making such functional hydrogels involves the use of G4·K^+ and G4·Ba^{2+} quartets, made from the natural product guanosine and 0.5 equiv. of benzene-1,4-diboronic acid. The resulting guanosine-borate hydrogel can be further modified by selectively cross-linking gel fibers with Mg^{2+} cations. The addition of Mg^{2+}, as an external cross-linker, provided an impressive swelling of the resulting hydrogels, a desirable property for biocompatible materials that are optimal for cell growth and tissue engineering. The guanosine-borate hydrogels were characterized by NMR, CD, AFM, and rheology. These guanosine-borate hydrogels support cell growth and do not show visible signs of degradation.

Experimental section

1. Hydrogel synthesis

Synthesis protocol BDBA-K: In a glass vial 0.05g, 0.2 mmol (1 eq) of guanosine (G **1**) is mixed with 0.0146 g, 0.1 mmol (0.5 eq) of benzene-1,4-diboronic acid (BDBA **2**). Distilled water (1.9 mL) is added, and the mixture is sonicated for a few minutes, until all the components are dispersed. The suspension is then heated in an oil bath preheated to 120°C until the solution becomes transparent. To avoid pressure in the vial upon heating a syringe needle in the plastic cap of the vial is used (**Fig. S1**). Then, 100 µL, 0.01 g, 0.2 mmol KOH (1 eq, stock solution of KOH: 0.66 g in 6.6 mL distilled water) is added and the mixture is heated and stirred for a few more minutes, and then left to cool down at room temperature. After cooling, a transparent and self-standing hydrogel is formed.

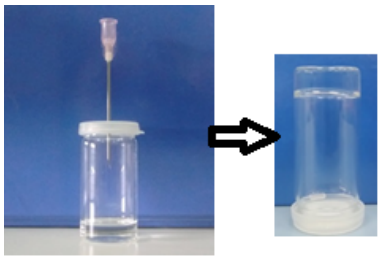

Figure S1: Vial with syringe needle in the plastic cap.

Synthesis protocol BDBA-Ba: In a vial, an amount of 0.05 g, 0.2 mmol (1 eq) of G **1** is mixed with 0.0146 g, 0.1 mmol (0.5 eq) of benzene-1,4-diboronic acid (BDBA **2**). Distilled water (6.58 mL) is added, and the mixture is sonicated for a few minutes, until all the components are dispersed. The suspension is then heated on an oil bath preheated to 120°C until the solution becomes transparent. To avoid pressure in the vial upon heating a needle in the cap of the vial is used. Then, 1.42 mL containing 0.015 g, 0.1 mmol $Ba(OH)_2{*}H_2O$ (0.5 eq, stock solution of $Ba(OH)_2{*}H_2O$: 0.31 g in 30 mL distilled water) is added and the mixture is heated and stirred for a few more minutes, and then left to cool down at room temperature. After cooling a transparent and self-standing gel is formed. Note that, with smaller amounts of water used, the gel is cloudy.

Synthesis protocol BDBA-Mg: In a vial, an amount of 0.05g, 0.2 mmol (1 eq) of G **1** is mixed with 0.0146 g, 0.1 mmol (0.5 eq) of benzene-1,4-diboronic acid (BDBA **2**). Distilled water (1.9 mL) is added, and the mixture is sonicated for a few minutes, until all the components are dispersed. The suspension is then heated on an oil bath preheated to 120°C until the solution becomes transparent. To avoid pressure in the vial upon heating a needle in the cap of the vial is used. Then, 100 µL containing 0.01 g, 0.2 mmol, KOH (1 eq) is added and the mixture is heated and stirred for a few more minutes, and then left to cool down at room temperature. After cooling, the gel is diluted with 38 ml of distilled water in a NUNC tube (50 mL). The mixture is then

sonicated for a few seconds to remove the air bubbles and then 4 mL of Mg^{2+} solution containing 0.041 g of $MgCl_2*6H_2O$ or 0.059 g 0.2 mmol (1 eq) of $Mg(NO_3)_2*6H_2O$ is added (stock solution: 0.59 g in 40 mL). We used initially chloride and then nitrate in the presence of TAE buffer to avoid $MgCl_2$/TAE pH change effects.

Synthesis Protocol for BDBA-LiOH: In a vial, an amount of 0.05g, 0.2 mmol, (1 eq) of G **1** is mixed with 0.0146 g, 0.1 mmol, (0.5 eq) of 1,4-benzene diboronic acid (BDBA **2**). Distilled water (1.85 ml) and 100 µl containing 0.0074g LiOH, 0.2 mmol, (1 eq, stock solution: 0.148 g in 2 mL distilled water) are added and the mixture is sonicated for a few minutes, until all the components are dispersed. The suspension is then heated on an oil bath preheated to 120°C until the solution becomes transparent. To avoid pressure in the vial upon heating, a needle in the cap of the vial is used. After cooling, the mixture does not form a gel. At this point 50 µl containing 0.0128 g $MgCl_2*6H_2O$ (0.5 eq, stock solution: 0.256 g in 1 mL distilled water) or 50 µl containing 0.0097 g $CaCl_2$ (0.5 eq, stock solution: 0.194 g in 1 mL distilled water) were added. After 30 minutes approximately, turbid and cloudy, non-viscous, free-flowing solutions are formed in both cases (**Figure S2**).

The concentration of K^+ in the BDBA-K hydrogel was 100 mM (2 mL gel volume volume).
The concentration Ba^{2+} in the BDBA-Ba hydrogel was 12.5 mM (8 mL gel volume).
The final concentration of K^+ and Mg^{2+} in the BDBA-Mg hydrogel was 0.5 mM (40 mL gel volume).

Figure S2: Pictures of the BDBA-containing hydrogels (normal – left, upside-down – right) prepared with LiOH with the subsequent addition of $CaCl_2$ (left vial) and $MgCl_2$ (right vial).

2. Comparison of water retention between BDBA-K, BDBA-Ba and BDBA-Mg hydrogels

We have observed the differences in the behavior of hydrogels containing K^+, Ba^{2+} and Mg^{2+} ions as jellifying triggers. In order to get a primary insight into the differences of hydrogel properties, we have performed water retention experiments. Two similar hydrogels were prepared using the standard protocol described above: hydrogel BDBA-K stabilized with K^+ and hydrogel BDBA-Ba stabilized with Ba^{2+}. Next, 2 mL of water to each sample was added and the reactions heated until melted. Then, the samples were cooled down and turned upside-down to check the self-sustainability (Fig. 1a). The gel BDBA-K is self-sustainable at maximum 3 mL of overall water content (Fig. 1a left), collapsing at the volume of 4 mL. Hydrogel BDBA-Ba is self-sustainable at volumes up to 14 mL (Fig. 1a middle). To check the influence of Mg^{2+} to the formation of hydrogel, we used the collapsed BDBA-K gel and step wise added water solution containing Mg^{2+} (12 mM). We have observed an astonishing effect of Mg^{2+} ions over the self-sustainability of the gel, the same amount of organic matrix was sustaining up to 44 mL of water (Fig. 1a right).

Below we present our attempts to stabilize the BDBA hydrogel with Li^+, guanidinium (G), Fe^{3+}, Mn^{2+} and Mg^{2+} cations (**Fig. S3**). Only the last two Mn^{2+} and Mg^{2+} cations formed a gel structure. We chose to move forward for the cell growth experiments only with Mg^{2+} because the Mn^{2+} gel had a brown colour, most likely due to the oxidation process, which might disturb monitoring of the subsequent cell growth.

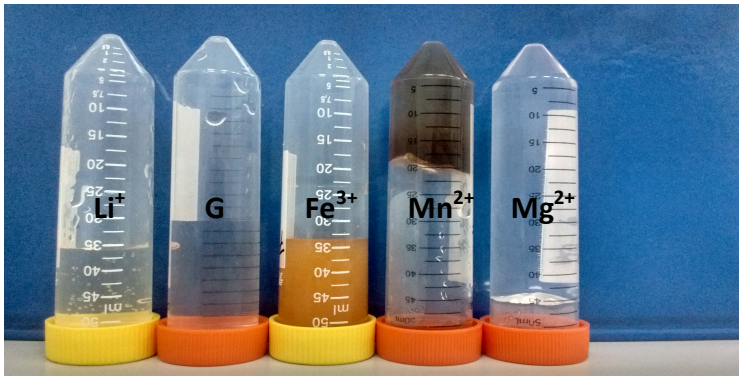

Figure S3: Pictures of the BDBA-containing solutions in the presence of Li^{+}, guanidinium (G), Fe^{3+}, Mn^{2+} and Mg^{2+} cations.

3. NMR Procedure: ^{1}H NMR Procedure

Gels were formed in D_2O according to the aforementioned procedures. In the case of the BDBA-K and BDBA-Ba gels, 0.5 mL of the hot solution was pipetted into an NMR tube immediately following the final heating. For the BDBA-Mg gel, 0.5mL of the mixture was pipetted into an NMR tube after addition of the $Mg(NO_3)_2$. Samples were allowed to sit overnight prior to running NMR experiments. Experiments were performed on a Bruker AVIII-600 at 25 °C and the solvent peak was used to calibrate the shifts.

Diffusion-Ordered Spectroscopy (DOSY) NMR Procedure: A Mg-BDBA gel was formed in D_2O according to the previously established procedure. After adding the $Mg(NO_3)_2$ 0.5mL of the mixture was pipetted into an NMR tube and the gel was allowed to sit overnight. Diffusion experiments were performed on a Bruker AVIII-600 at 5 °C to maximize the separation of the H1′ signals (**Fig. S4**) with 18 points of 256 scans, a delay of 5 s, a gradient pulse length of 2300 µs and a delta value of 0.10 s. The spectral width and offset were set at 8.0 ppm and 5.5 ppm respectively. The diffusion coefficients for each peak were calculated using their integration and the fitting function in the Bruker software.

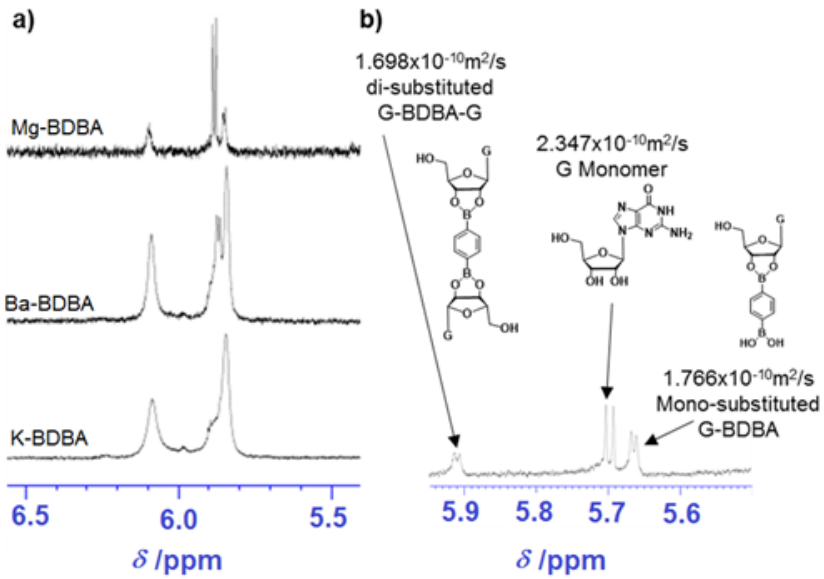

Figure S4: a) The H1′ region of the ^{1}H NMR spectra at 25°C in D_2O of the BDBA-K, BDBA-Ba and BDBA-Mg hydrogels; b) The diffusion coefficients at 5 °C for the H1′ peaks of different species of free, mono- and di-substituted G in the BDBA-Mg hydrogels.

4. Procedure for Powder X-ray diffraction: PXRD. A G-BDBA-K^+ gel was prepared according to the established procedure. The sample was allowed to sit at 20 °C overnight and was then frozen and dried on a lyophilizer to form a white powder. Diffraction measurements were performed using a Cu radiation source at 20 °C on a Bruker D8 Advance Bragg-Brentano Diffractometer with a LynxEye detector. The data is shown below in **Fig. S5**.

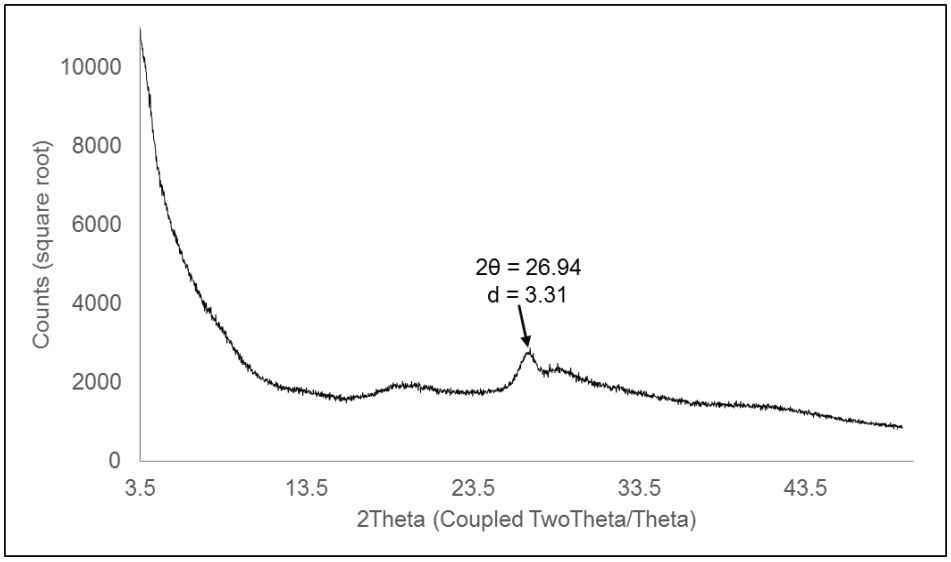

Figure S5: Data from powder x-ray diffraction shows a peak at 2θ=26.94 from a lyophilized sample of G-BDBA-K^+ gel. Using Bragg's law this was determined to correspond to a distance of 3.31 Å, matching the π-π distance between G-quartet layers.

5. CD-spectroscopy

Circular Dichroism (CD) spectra in **Fig. S6** were measured on a Chirascan plus (AppliedPhotophysics) spectrometer, using 0.1 mm path lamellar cells by adding 100 µL of a sample without dilution. Scanning range used for all samples was 220 – 320 nm.

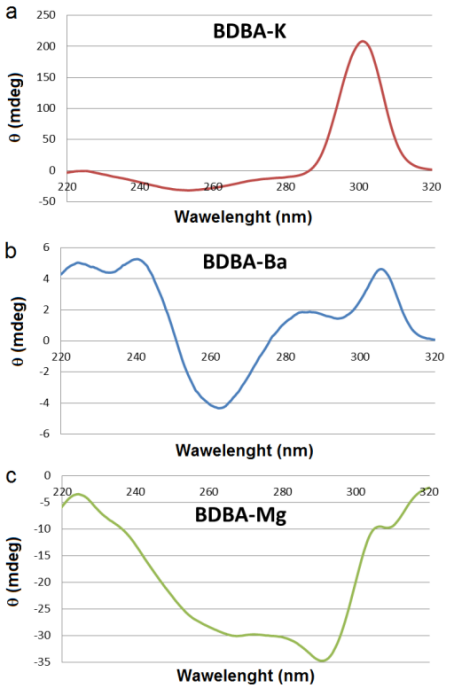

Figure S6: Circular dichroism spectra for BDBA-K, BDBA-Ba and BDBA-Mg hydrogels.

6. Rheology

Gels were made as described in the procedures above and allowed to sit at room temperature overnight before experiments were performed. Experiments were run on an AR2000 stress-controlled rheometer from TA Instruments in New Castle, DE at 20 °C with a 20 mm diameter parallel plate geometry. The gels were loaded onto the rheometer and allowed to equilibrate on the plate for 10 min before running the experiment. Frequency sweeps were performed at 0.2% strain (**Fig. S7**).

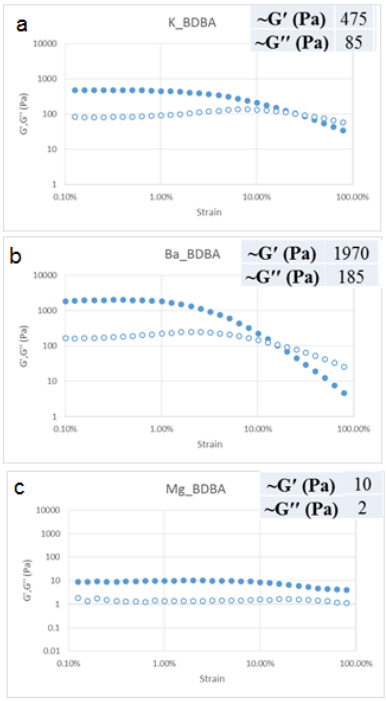

Figure S7: Rheological strain sweeps of the 3 gels show the storage modulus G′ (●) and the loss modulus G″(○).

7. Atomic Force Microscopy (AFM)

The Ntegra Spectra Atomic Force Microscope (NT-MDT, Russia) operated in tapping mode under ambient conditions was used to image gels structures. Silicon cantilever tips (NSG 10, NT-MDT) with gold reflecting coating, a resonance frequency of 140–390 kHz, a force constant of 3.1–37.6 N m^{-1} and a tip curvature radius of 10 nm were used. Sample preparation: a 10 mL aliquot of the gel solution was deposited on freshly cleaved mica substrates and dried in air at room temperature prior to imaging.

8. Scanning Electron Microscopy (SEM)

The freeze-dried BDBA-K hydrogel showed a sponge-like microstructure with pore diameters varying from 5 to 8 µm (**Fig. S8**).

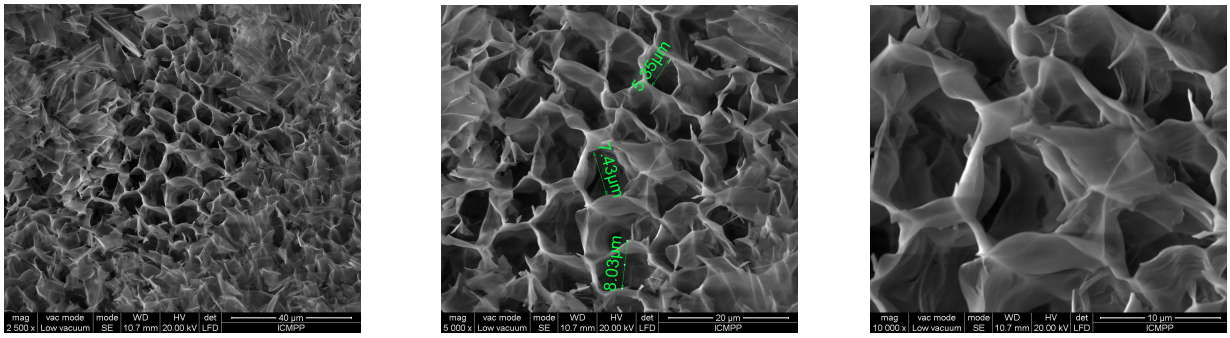

Figure S8: Examples of SEM images for freeze-dried BDBA-K hydrogel.

9. Cell growth experiments

100 µl of freshly prepared hot solutions of BDBA-K, BDBA-Ba and BDBA-Mg, prepared as previously describe in Section 1, were dispensed in each well with a pipette, and left to cool down and form hydrogels. Subsequently, 100 µl of 3 x Tris-acetate-EDTA – TAE buffer (119.86 mM Tris-acetate, 3 mM EDTA) was added to adjust the hydrogels to the physiological pH. In parallel, several wells containing all three hydrogels were left without TAE buffer treatment. After 2 hours of incubation, the buffer was removed, and DMEM culture medium supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin-Amphotericin B mixture (10K/10K/25 µg) was added (100 µl/well). One hour later, the cell suspension was added to each well ($1x10^4$ NHDF cells/well in a total volume of 100 µl/well).

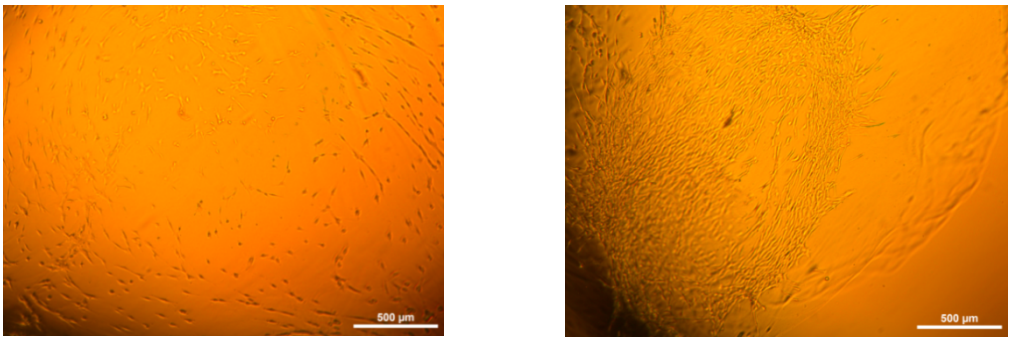

Figure S9: NHDF cells on BDBA-Mg hydrogel treated with 3 x TAE + KCl (5ml, 155 mM) visualized after 4 hours (A) and after 24 hours (B).

In the MEM culture medium the concentration of possible competitive ions is rather small (0.2 g/L Ca^{2+} and 0.1 g/L Mg^{2+}) but with a very high concentration of Na^+ (6.8 g/L) and, under these conditions we do not expect major ion exchange processes as previously observed with Na^+, 300-500 mM by Davis et al. *J. Am. Chem. Soc*. 2014, 136, 12596−12599)

10. Cytotoxicity assay

The reported results in **Fig. S10** represent the average of three individual MTS tests, where at least 6 replicates were performed for each type of hydrogel. Aliquots of 20, 90, 100 µl of hydrogel samples containing 0.5 mg guanosine were distributed in 96 well plates and incubated with 100 µl DMEM culture medium for one hour in order to better adjust the hydrogels for cell culture. The BDBA-K hydrogel samples were previously treated for 2 hours with 3xTAEMg^{2+} buffer solution, in order to adjust the pH value. NHDF cells were seeded at a density of 5 $x10^3$ cells/well, in 96 well plates, on top of the hydrogel samples. After 20 hours 20 µL of CellTiter 96® Aqueous One Solution reagent were added to each well, and the plates were incubated for another 4 hours before reading the result. Absorbance at 490 nm was recorded with a plate reader (EnSight, PerkinElmer).

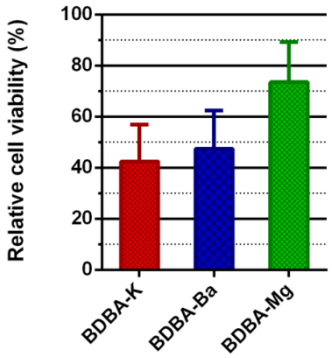

Figure S10: Cell viability by MTS assay on BDBA-K, BDBA-Ba and BDBA-Mg hydrogels. The reference sample (100%) is considered for cell viability on culture medium.

In our cytotoxicity experiments we have used the ISO 10993-5 standard, which recommends a quantitative evaluation of the cytotoxicity of a material after 24 to 72 hours using a colorimetric method such as MTT/MTS assay. The test item is considered

non-cytotoxic if the percentage of viable cells is equal to or greater than 70% of the untreated control, as our results show for the BDBA-Mg gel. (International Organization for Standardization: Biological evaluation of medical devices - Part 5: Tests for in vitro cytotoxicity Geneva, Switzerland; 2009:1–34. [ISO 10993–5]).

Author Contributions

The manuscript was written through contributions of all authors. / All authors have given approval to the final version of the manuscript.