Electronic Supplementary Material (ESI) for Biomaterials Science.


# Modular mixing of 1,3,5-benzenetricarboxamide supramolecular hydrogelators allows tunable biomimetic hydrogels for control of cell aggregation in 3D

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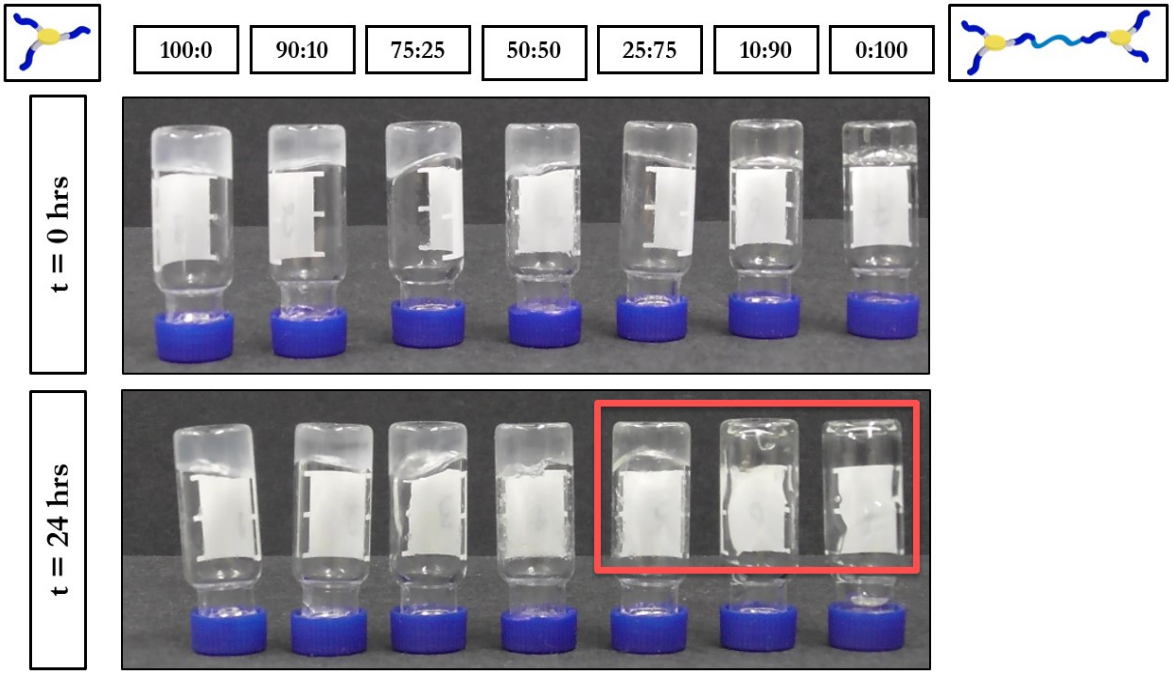


Figure S1. Tuning mechanical properties by modular mixing of BTA and BTA-PEG-BTA: Mixing of BTA and BTA-PEG-BTA allow tweaking of mechanical and viscoelastic properties of hydrogels. On the left is 100:0 BTA architecture and on the right is 0:100 BTA-PEG-BTA architecture with PEG20K connecting two BTA molecule via PEG20K polymer. Gel were made with final 10 wt% and proportion or percentage of BTA and BTA-PEG-BTA were varied. BTA-PEG-BTA flowed and reached to the bottom of the glass vial after 24 hours while 100:0 did not flow at all. Mixed formulation with higher percentage of BTA-PEG-BTA such as 10:90 flowed and also reached to the bottom of the glass vial but with increasing percentage of BTA no flow behaviour was observed until 24 hours.

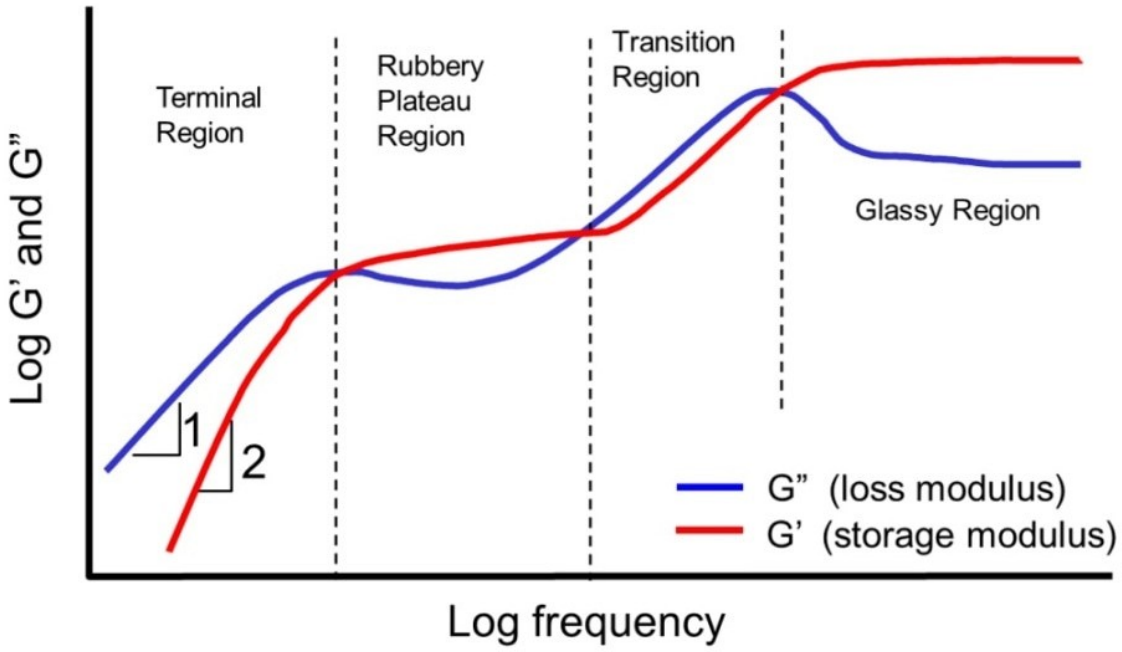


Figure S2. Typical curve for viscoelasticity materials, which shows a transition from terminal region to rubbery plateau going from lower angular frequency to higher angular frequency. In terminal region, loss modulus (G'') is dominant indicating that material behaves dominantly liquid like while in rubbery plateau storage modulus is dominant indicating materials behaves dominantly as solid. Pictures obtained from: AZO MATERIALS website: https://www.azom.com/article.aspx?ArticleID=16985

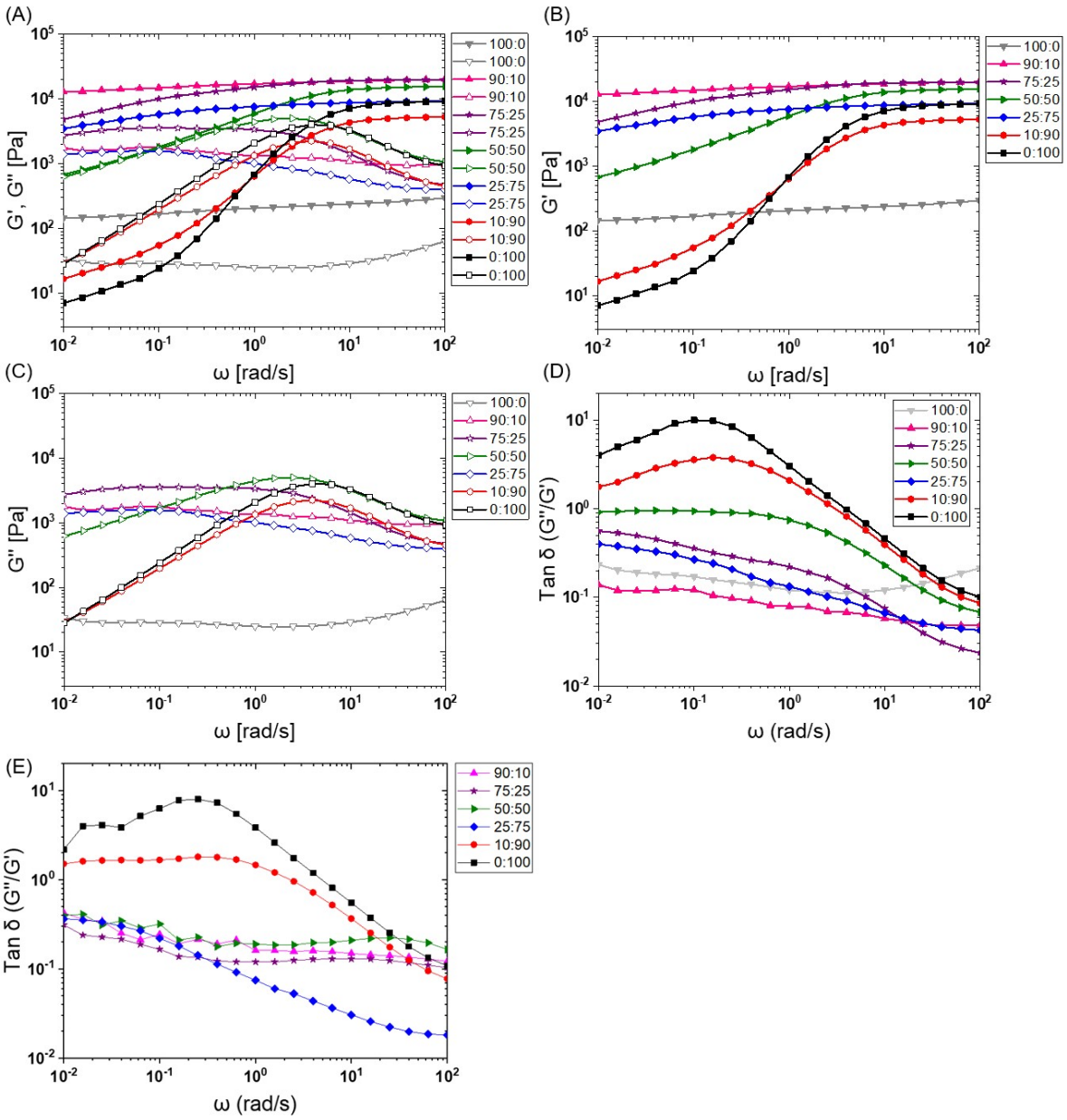


Figure S3. Mechanical and viscoelastic properties of hydrogels: A) Frequency sweep for BTA formulations, B) storage modulus (G') as a function of angular frequency, C) loss modulus (G'') a function of angular frequency and D&E) Tanδ (G''/G') as a function of angular frequency of two independent samples. Tanδ (G''/G') of BTAs showed similar trends in viscoelasticity.

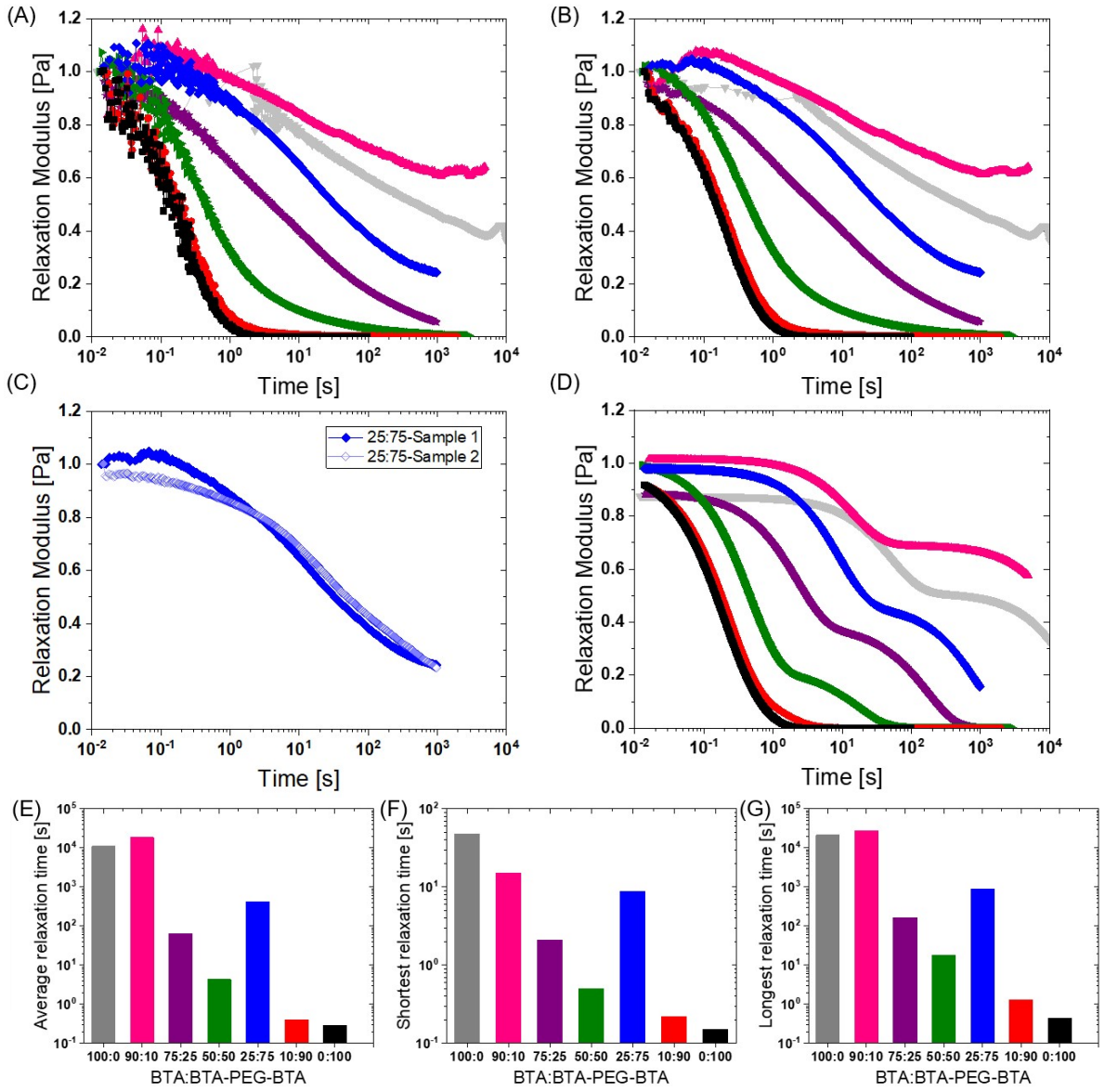


Figure S4. Relaxation modulus curves: A) Normalized relaxation modulus curve for different BTA formulations under constant 1% strain. B) Stress relaxation curves were smoothed by applying smooth function (10-point average) using OriginLab. C) Stress relaxation curve of two independent sample for 25:75, which shows exceptional behaviour and duplicate were performed for confirming stress relaxation behaviour. Both replicates showed similar stress relaxation profile. D) Curves obtained using two Maxwell elements. D) Average stress relaxation times obtained from curve fitting using two Maxwell elements in "D". E) Shortest relaxation time for fast mode of relaxation. F) Longest relaxation time for slow mode of relaxation.

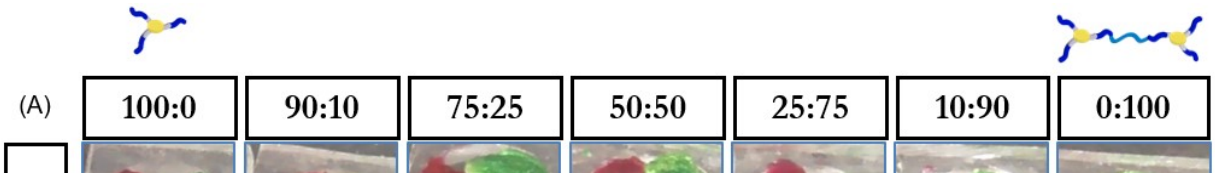

**Table S1:** Stress relaxation times for different BTA formulations. Average, longest and smallest relaxation times are calculated by fitting two mode Maxwell element model. * indicate that sample did not reach half relaxation time.

| ***Formulation*** | ***Average relaxation time (seconds)*** | ***Longest relaxation time (seconds)*** | ***Smallest relaxation time (seconds)*** | $\tau_{1/2}$ **(seconds)** |
|---|---|---|---|---|
| ***100:0*** | 11,000 | 21,600 | 48 | 524 |
| ***90:10*** | **18,500** | **27,300** | 15 | *N/A |
| ***75:25*** | **64** | **165** | 2.1 | 4 |
| ***50:50*** | **5** | **18** | 0.5 | 0.4 |
| ***25:75*** | **417** | **898** | 9 | 33 |
| ***10:90*** | 0.4 | 1 | 0.2 | 0.2 |
| ***0:100*** | **0.3** | **0.4** | 0.2 | 0.1 |

## Cell viability

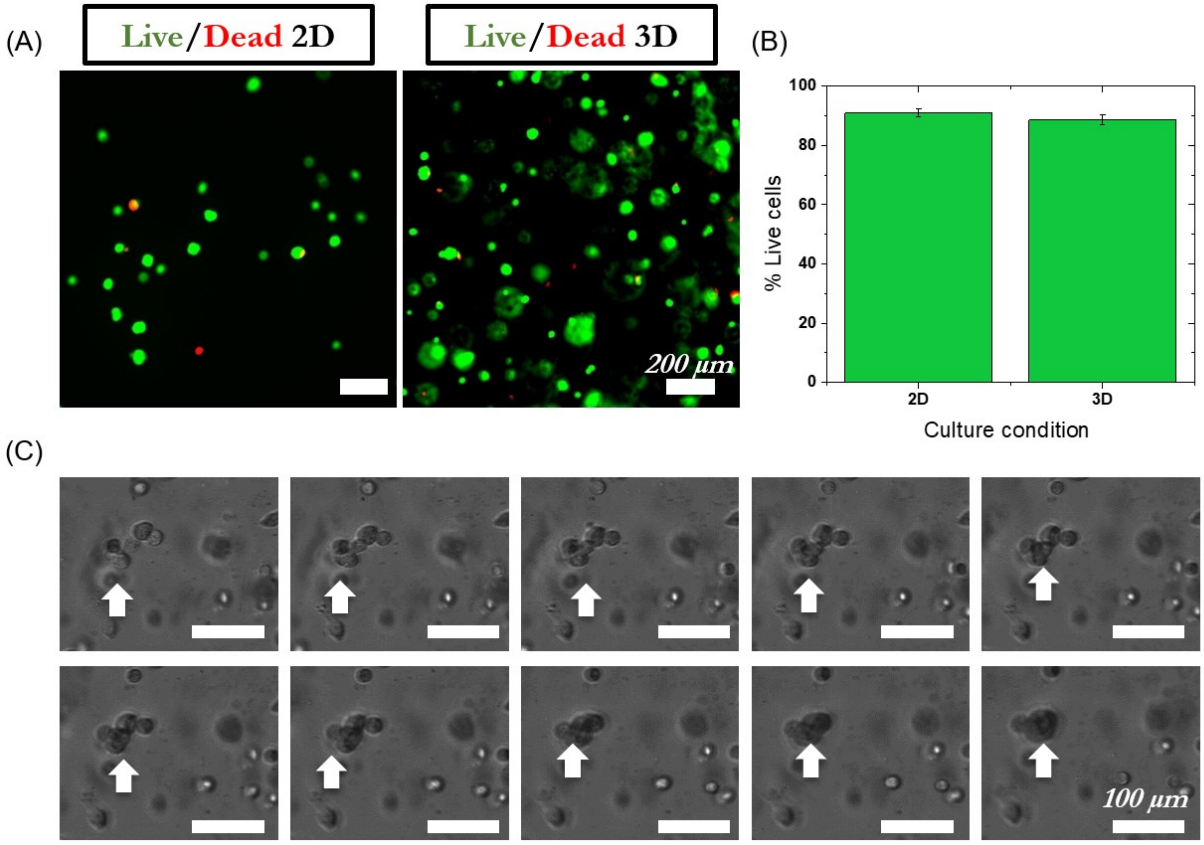


Figure S6. A) Cell viability of Human dermal fibroblasts (HDF) on top (2D) of BTA-PEG-BTA and encapsulated (3D) within BTA-PEG-BTA hydrogels. Green colour represents live cell and red colour represents dead cell. B) % of live cell plotted for 2D and 3D culture conditions, , n=2 biological replicates and imaged at multiple location on top (for 2D) and inside (for 3D) of hydrogel. C) Pictures from time lapse video over 17 hours showing that HDF forming an aggregate within BTA-PEG-BTA gels

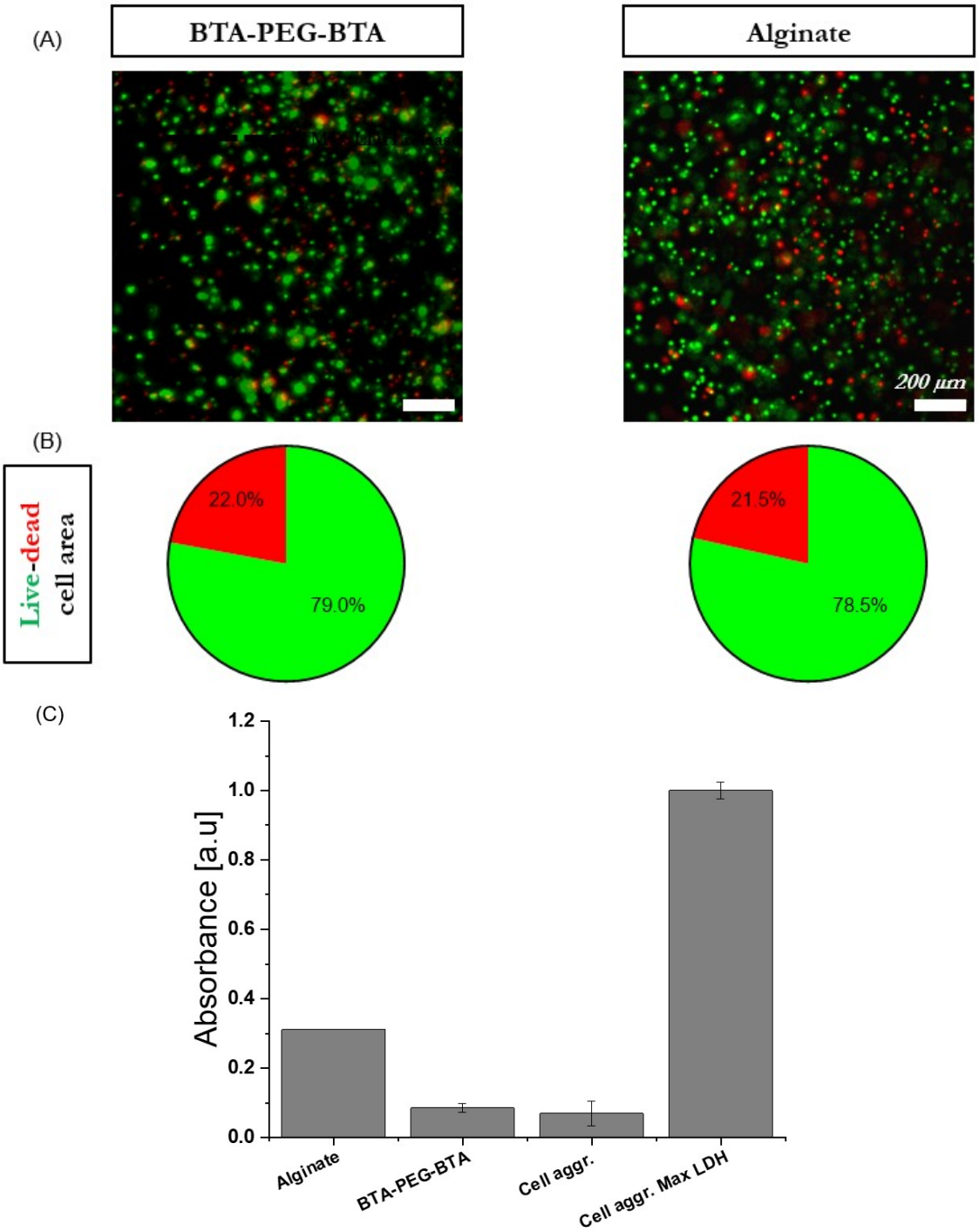


Figure S7. BTA-PEG-BTA and alginate gels were equally effective for culturing chondrocytes. (a) Representative images of chondrocytes (ATDC5) encapsulated within gels after 24 h, stained for calcein (green=live cells) and ethidium homodimer (red=dead cells). Scale bar represents 200 µm. (b) Quantification of the total area of live and dead cells (n = 4 images for each hydrogel condition). (c) Quantification of LDH, a marker of cytotoxicity, after 24 hours of culture. Cell culture data was normalized to the negative control, cell aggregate Max LDH for which LDH absorbance value is 1. For the negative control, lysis buffer was added to

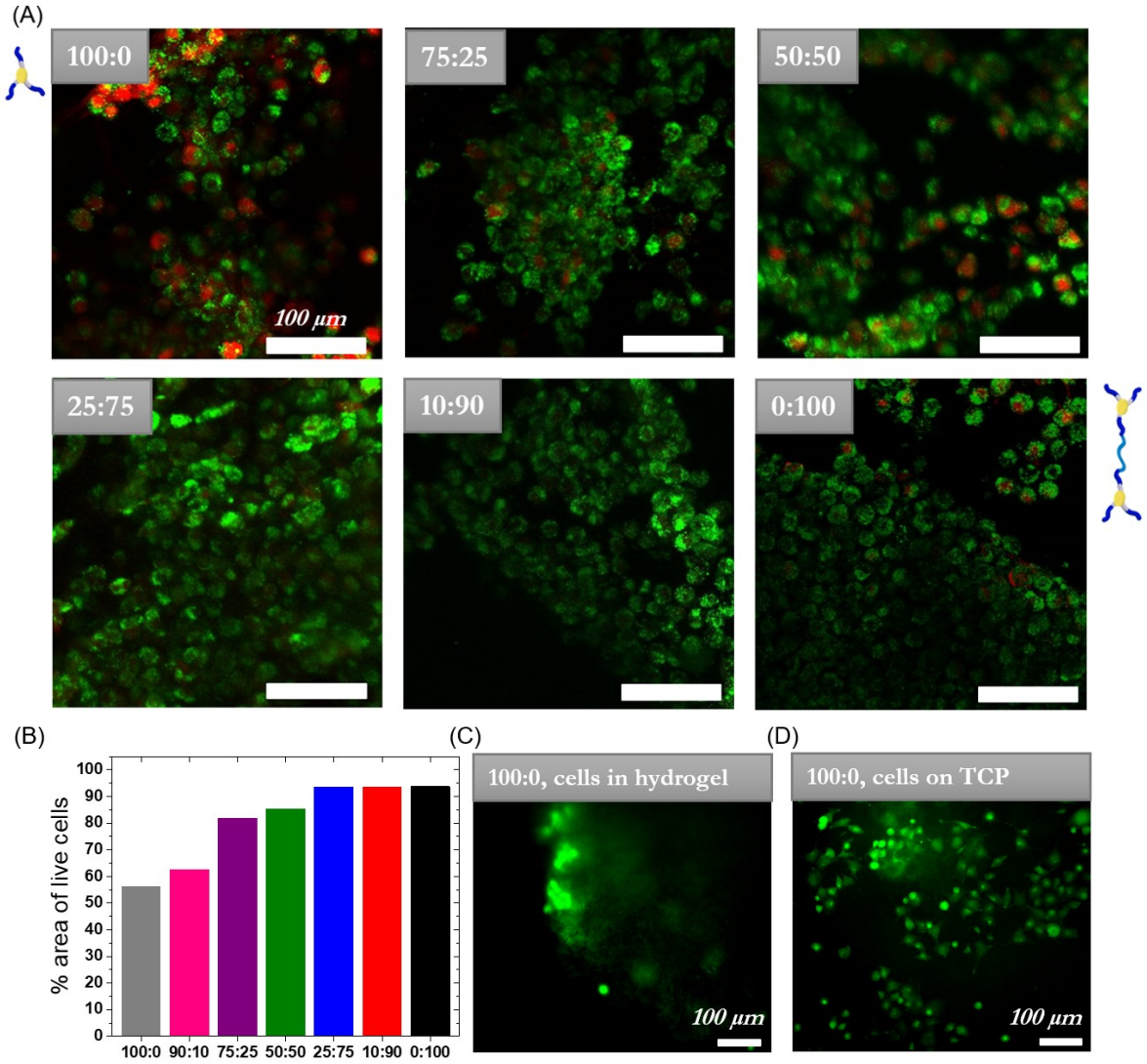


Figure S8. Chondrocytes cultured in chemically dissolved and freeze-dried hydrogels. A) Chondrocytes were cultured for 24 hours and cells were stained for calcein (green=live cells) and ethidium homodimer (red=dead cells. More dead cells were seen in 100:0 compared to other formulations. B) Quantitative analysis of live-dead cell area. C) Chondrocytes, ATDC5, cultured in 100: using self-healing method, green color showed live cells within BTA100:0 hydrogel. D) Not all the cells were retained in the hydrogel due to poor macroscopic self-healing capacity of hydrogel and chondrocytes released from 100:0 hydrogel and attached to tissue culture plastic while hydrogel sit on top (some pieces of the hydrogel were also floating). Chondrocytes attached to tissue culture plastic also stayed alive in presence 100:0 hydrogel

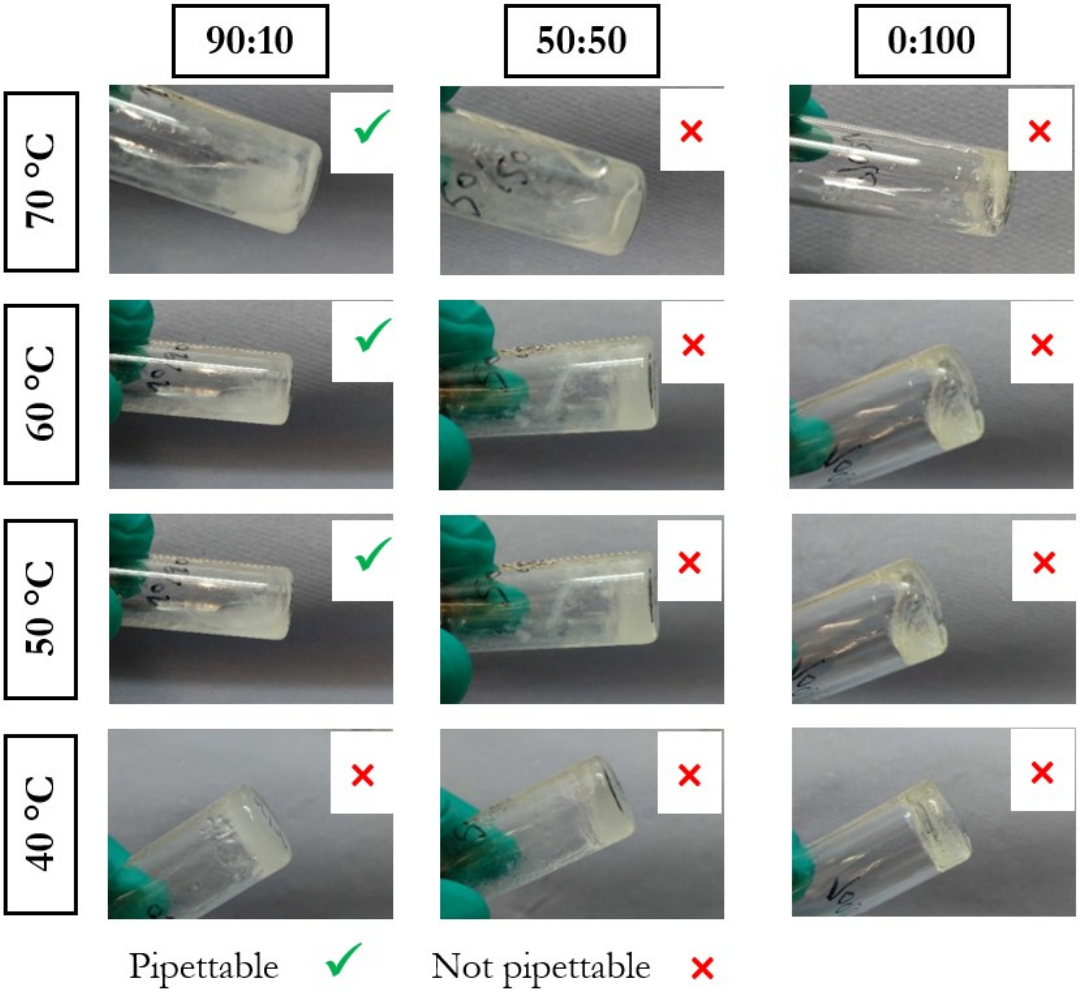


Figure S9. BTA formulation were heated to observe gel to solution transition at different temperatures. We wanted to explore if cells can be encapsulated within gels when gels are in solution form.

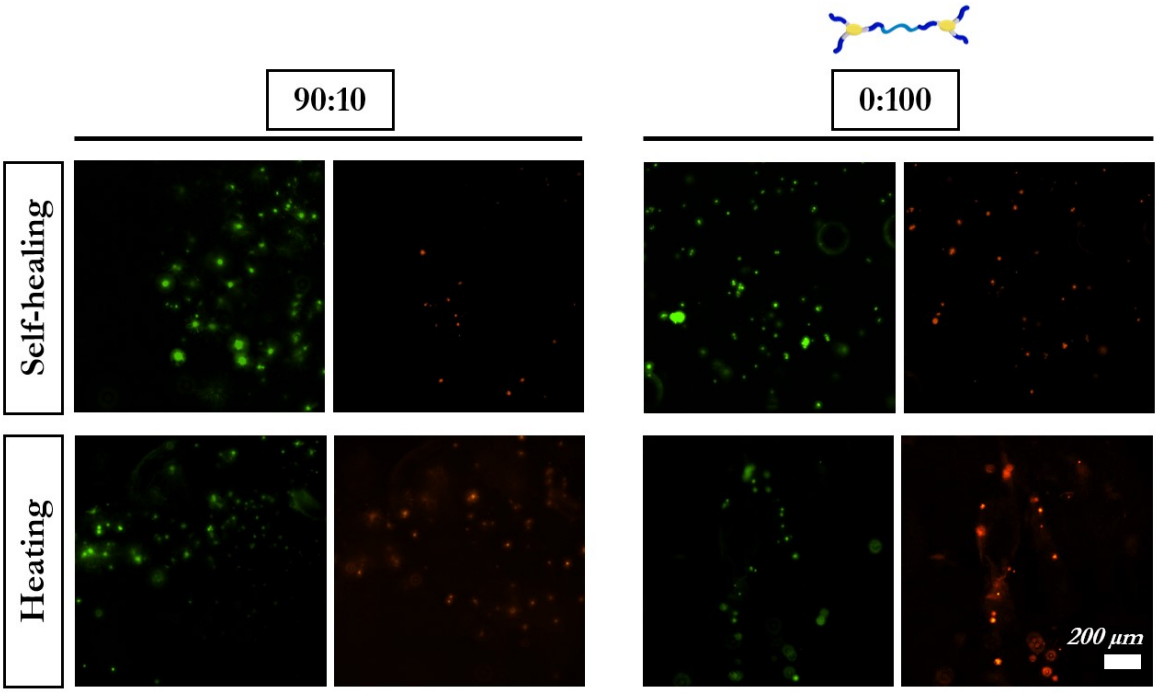


Figure S10. Comparison of viability of cell encapsulation methods. In mechanical method chondrocytes cells suspension were mixed with gel using spatula. Gels was divided into small pieces and then utilizing self-healing capacity cells were encapsulated within gels. In heating method, hydrogels were heated until gel goes to viscous solution (80 °C) and then cell suspension were mixed. Upon cooling, viscous solution will again form the gel. We were expecting heating method will provide more uniform cell mixing in gels without compromising cell viability. We did not observe improved cell viability and rather it lead to new challenges. Heating of the gel was done in glass vial since plastic well plate does not support. For 0:100 we have a very narrow window of few seconds of mixing and pipetting gel from the glass vial into well plate since it goes to gel very quickly upon cooling. For 90:10 we have a little broad window of mixing; however upon introducing gels into well plate and during process of gelation, significant number of cells settle down at the bottom of the well. Heating method did not provide any additional benefit of uniform encapsulation and we stick with mechanical mixing and utilizing self-healing capacity of hydrogels to mix to

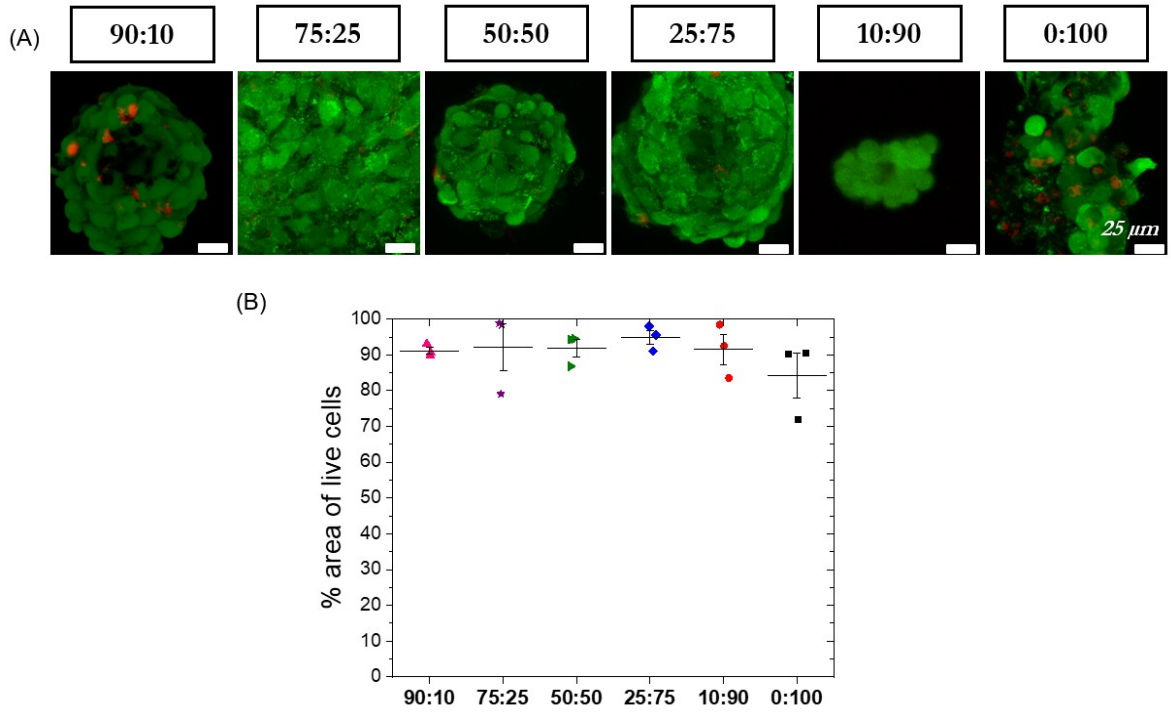


Figure S11. A) Cell viability using live-dead assay. ATDC5 chondrocytes cultured in BTA formulations for 7 days and then stained for calcein and ethidium homodimer, which stain live (green) and dead (red) cells respectively. All formulation showed high cell viability after 7 days in culture.

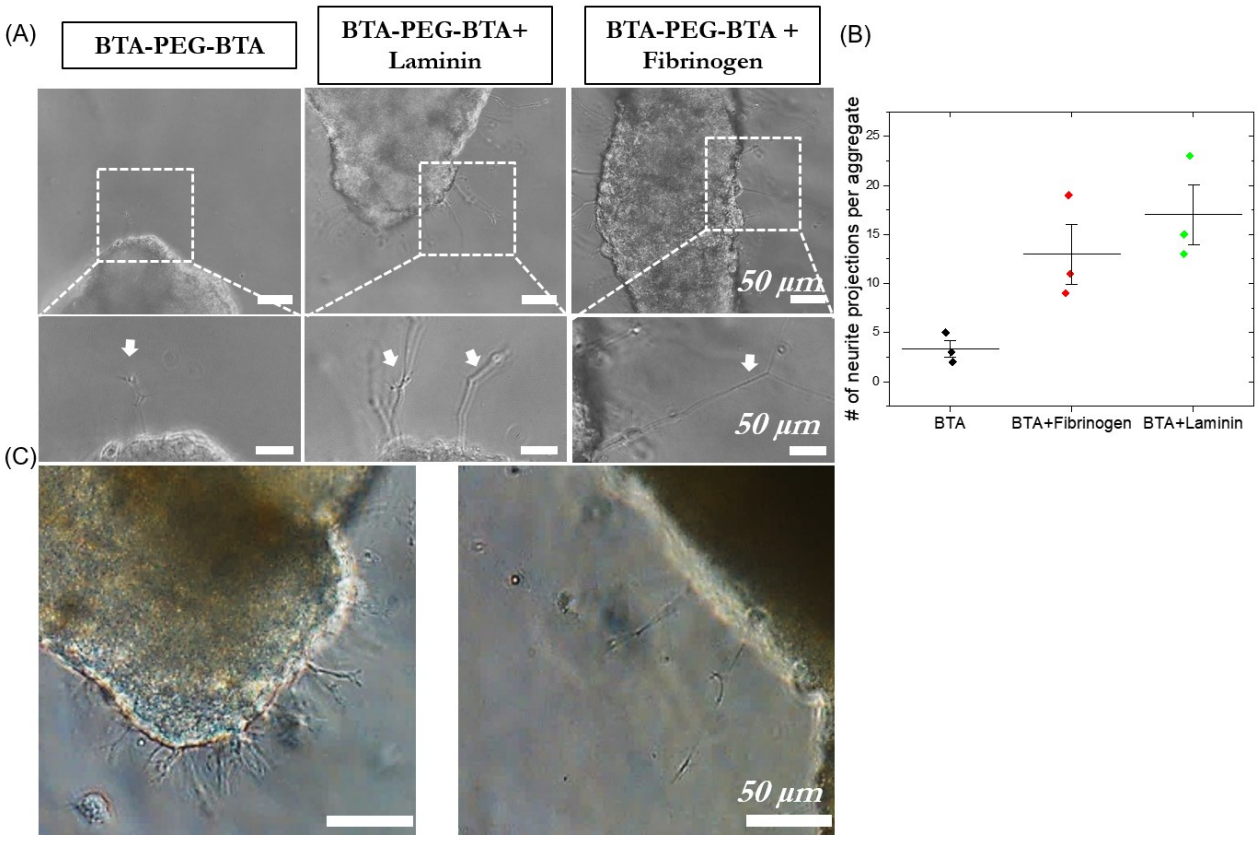


Figure S12. Nerve cell growth in BTA-PEG-BTA gels. A) PC12 nerve cell growth in BTA-PEG-BTA gels after 4 days in culture. Addition of laminin and fibrinogen enhanced outgrowth of nerve cells within BTA-PEG-BTA gels. B) Numbers of neurite growing out of single cell aggregate, three cell aggregate were analysed per condition, n=3 biological replicates. C) Dorsal root ganglion (DRG) cells growth in BTA-PEG-BTA hydrogel after 2 days in culture showing neurites outgrowth in BTA-PEG-BTA, n=2 biological replicates.

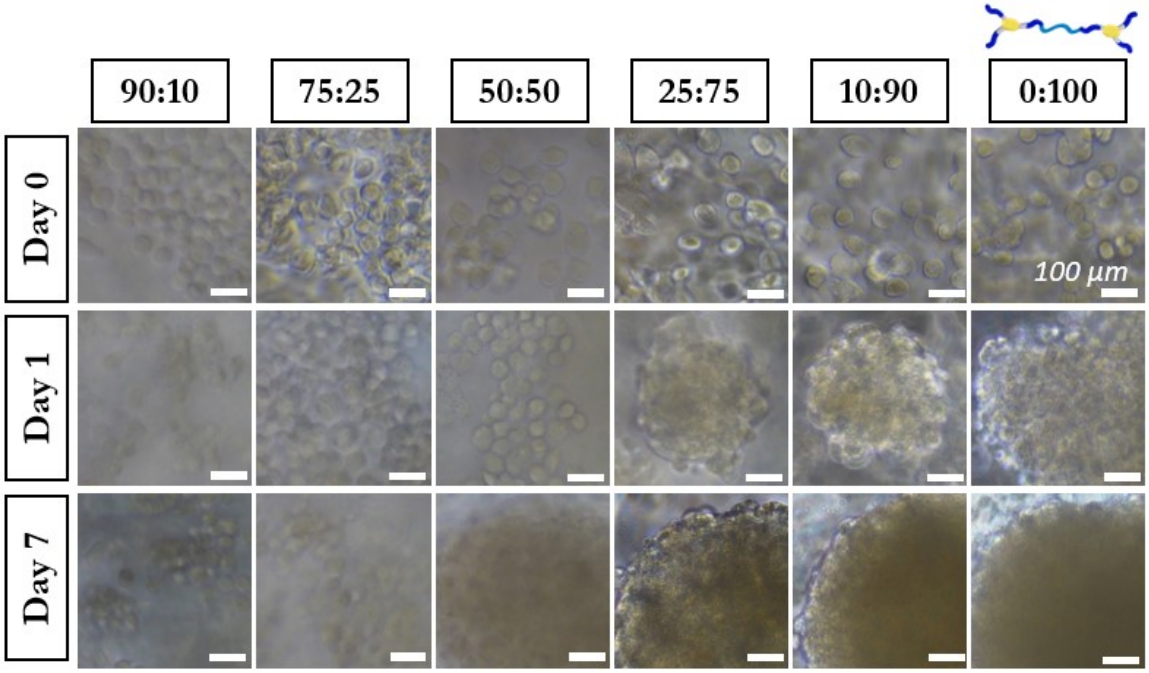


Figure S13. ATDC5 chondrocytes cultured in BTA formulations. Cell for aggregates within all gels with faster and bigger aggregates in hydrogels with greater percentage of BTA-PEG-BTA. ATDC5 chondrocytes form smaller aggregates in hydrogels with greater percentage of BTA.

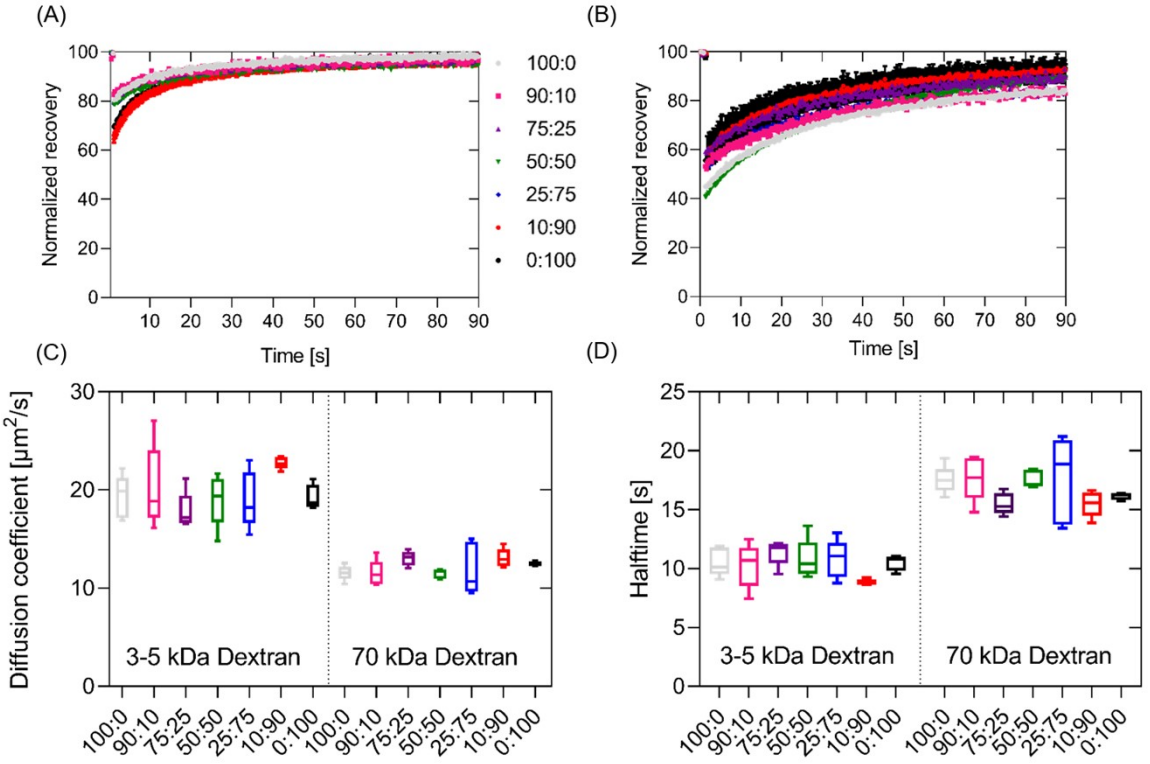


Figure S14. FRAP experiment. Recovery curves after photo bleaching were obtained for A) 3-5 kDa and B)70 kDa FITC-dextran. C) Diffusion halftimes were calculated from the normalized recovery curves and D) diffusion coefficients were calculated via the Soumpasis equation.