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Commercially-relevant orthogonal multi-component supramolecular hydrogels for programmed cell growth

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1 Materials and Methods

DBS-$CONHNH_2$ was synthesised using literature methods and all characterisation data were in agreement with previous reports.[1] Biopolymer heparin was purchased from Calbiochem as heparin sodium salt, from porcine intestinal mucosa with an activity ≥160 units/mg. Self-assembling heparin binder, C16-DAPMA, was synthesised according to previously reported methods, and all characterisation data were in full agreement with those previously published.[2] Polymer gelator (PG) agarose was purchased at Bioreagent grade from Sigma. Mallard Blue was prepared by Stephen M.Bromfield and Ching W. Chan, as previously described.[3]

DBS-$CONHNH_2$ Gel Formation. DBS-$CONHNH_2$ gel was prepared in 10 mM Tris-HCl/ 150 mM NaCl buffer, pH 7.4. 0.5 mL of solvent was added to DBS-$CONHNH_2$ powder (2 mg) and sonicated for 20 minutes. The solution was heated until complete dissolution of the gelator and a transparent solution was observed. The sample was allowed to cool down at room temperature.

DBS-$CONHNH_2$ – Agarose Hybrid Gel Formation. The required weight of agarose (5 mg) and DBS-$CONHNH_2$ (2 mg) were jointly dissolved in 10 mM Tris-HCl/ 150 mM NaCl, pH 7.4 (0.5 mL). After sonicating the mixture, the solution was heated until complete dissolution of the gelators and a transparent solution was observed. The sample was allowed to cool down at room temperature.

DBS-$CONHNH_2$ Gel Formation with Different Concentrations of Heparin and C16-DAPMA. DBS-$CONHNH_2$ powder (2 mg) was mixed with 0.25 mL of 10 mM Tris-HCl/ 150 mM NaCl buffer and sonicated for 20 min. Solutions of heparin or C16-DAPMA were prepared with double the intended final concentration, to allow for subsequent dilution. 0.25 mL of heparin solution was added into the DBS-$CONHNH_2$ solution, followed by heating and shaking until a clear solution was obtained. The sample was allowed to cool down at room temperature. 0.25 mL of 10 mM Tris-HCl/ 150 mM NaCl (pH 7.4) was added to DBS-$CONHNH_2$ powder (2.0 mg) and sonicated for 20 min. A solution of C16-DAPMA and heparin was prepared with three times the intended final concentration, to allow for subsequent dilutions. 0.25 mL of C16-

DAPMA solution was then added to 0.25 mL of heparin solution and stirred to ensure binding. C16-DAPMA/heparin solution (0.25 mL) was added into the DBS-$CONHNH_2$ solution and stirred to ensure mixing. The sample was heated until a clear solution was observed and allowed to cool down at room temperature.

Thermal Stability and T_{gel} Determination. Thermal stability and T_{gel} values for DBS-$CONHNH_2$ were obtained by reproducible tube inversion methodology. All the samples with different concentrations of heparin, C16-DAPMA and heparin-C16-DAPMA aggregates that resulted in gel formation were placed into a thermo-controlled oil bath, with an initial temperature of 25 °C. The temperature was set to rise until 100°C. After each increase of ca. 5°C, the tubes were removed from the bath and turned upside down. The stability of the gels was observed and the T_{gel} was considered as the temperature when the gel started to run down the sides of the vial.

2 Infrared Spectroscopy

DBS-CONHNH$_2$ gel samples were prepared for infrared by removing solvent from the samples under high vacuum. The resulting powder was placed into the infrared spectrometer and the spectra recorded.

Table S1. Concentrations of DBS-CONHNH$_2$, heparin and C16-DAPMA used to record infrared spectra.

Sample	Concentration (mM)
DBS-CONHNH$_2$	8.4
DBS-CONHNH$_2$/Heparin	8.4/0.3
DBSCONHNH$_2$/C16-DAPMA	8.4/1.2
DBS-CONHNH$_2$/Heparin/C16-DAPMA	8.4/0.3/1.2

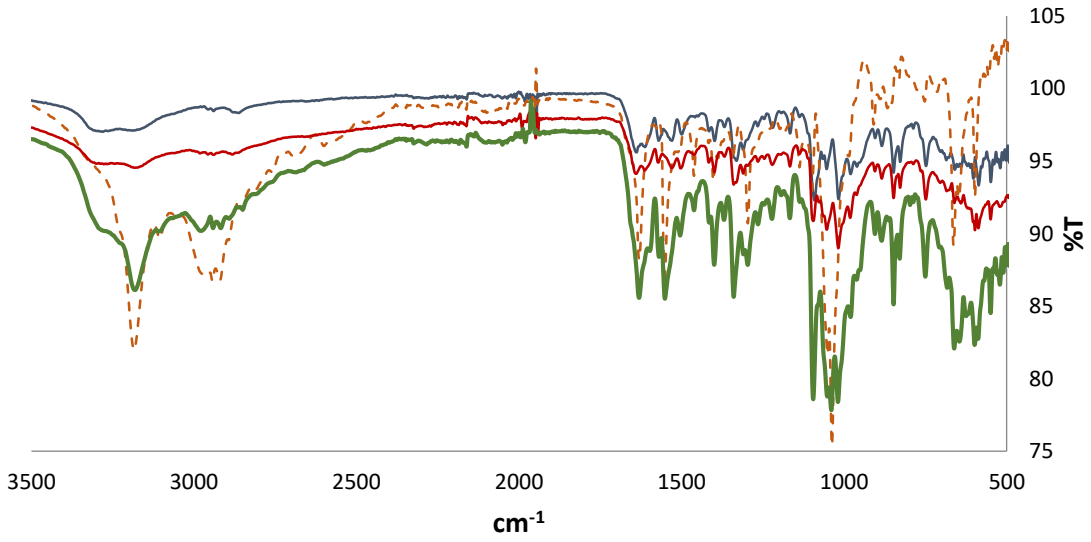

Figure S1. IR spectra of xerogels formed by DBS-CONHNH$_2$ gel (blue line); DBS-CONHNH$_2$ gel with 300 µM of heparin (red line); DBS-CONHNH$_2$ gel with 1200 µM C16-DAPMA (dashed orange line) and DBS-CONHNH$_2$ gel with 1200 µM of C16-DAPMA and 300 µM of heparin (green line).

IR characterization was performed for the gel formed with DBS-$CONHNH_2$ (0.4% w/v) alone and in the presence of 300 µM of heparin, 1200 µM of C16-DAPMA and in the presence of 300 µM of heparin and 1200 µM of C16-DAPMA. By comparing the obtained spectra, it is possible to observe that the expected peaks are all present in the multi-component gel and that the spectrum corresponds to simple overlap of the three compounds. Additionally, the IR spectra of DBS-$CONHNH_2$ with heparin and DBS-$CONHNH_2$ with C16-DAPMA also correspond to the overlap between the typical IR frequencies of each molecule. In respect to the multi-component gel, a strong peak appears at 3182 cm^{-1}, characteristic of N-H stretching. C-H stretches at 2981 and 2943 cm^{-1} are present, as well as C=O stretch at 1629 cm^{-1}. At 1505 and 1406 cm^{-1}, C=C stretches from the aromatic rings of DBS are observed and at 1400 cm^{-1} the presented peak overlaps with the carboxylate peak of heparin. Between 1300 and 1120 cm^{-1} several peaks from the DBS sugar backbone are noticeable. At 1095 cm^{-1} a strong peak is verified and probably corresponding to the overlap of S=O stretch and C-O stretch. Due to the fact that several IR frequencies are common in the three compounds that formed the gel an overlap of the characteristic peaks of each molecule occurred. This suggests that each component can behave independently when the gel is formed and there are no molecular scale interactions between the different components in the multi-component gels.

3 Circular Dichroism Spectroscopy

The DBS-$CONHNH_2$ CD sample was prepared by adding 10 mM Tris-HCl/ 150 mM NaCl (500 µL) to DBS-$CONHNH_2$ (0.6 mg) and sonicating. The sample was heated until a clear solution was formed and 400 µL of sample were immediately transferred to a 1 mm quartz cuvette (incubated in a cell holder at 90 °C) and placed in the spectrometer. The temperature was set to cool down to 20 °C (5 °C/min ramp) while spectra were recorded every 30 seconds for 15 minutes. The same procedure was performed in the presence of 38 µM of heparin, 150 µM of C16-DAPMA and 150 µM of C16-DAPMA/ 38 µM heparin.

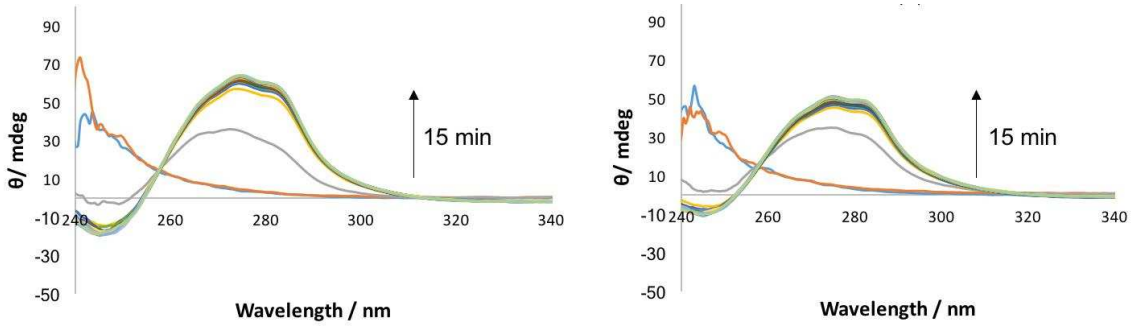

Figure S2. DBS-CONHNH$_2$ in the presence of C16-DAPMA (left) and DBS-CONHNH$_2$ in the presence of C16-DAPMA and heparin (right).

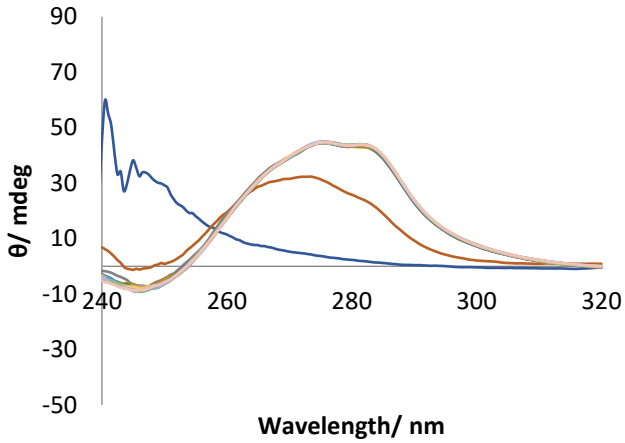

Figure S3. DBS-CONHNH$_2$ in the presence of agarose (0.5% w/v). Spectra were recorded every 60 seconds for 15 minutes.

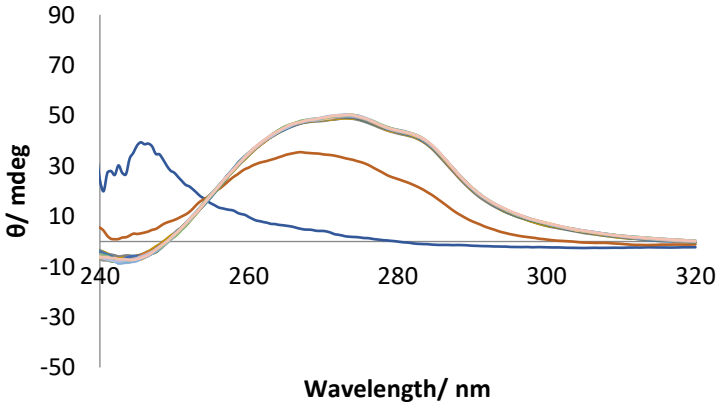

Figure S4. DBS-CONHNH$_2$ in the presence of heparin and agarose (0.5% w/v).

4 Electron Microscopy

DBS-$CONHNH_2$ gel samples for TEM images were prepared in ultra-pure H_2O and images obtained by adding one microspatula of each sample on a copper grid (standard) with Formvar and carbon support film. The excess sample was removed with filter paper then allowed to set for 5 minutes. A negative stain (1% uranyl acetate) was applied to the grid while wet to allow the stain to run across the grid. The grid was left to rest for 30 minutes before taking images.

To obtain SEM images, the gels were freeze fried on pieces of copper shim. After mounting the samples on stubs they were sputter coated with approximately 5 nm of Au/Pd and the images recorded.

Table S2. Concentrations of DBS-$CONHNH_2$, heparin and C16-DAPMA used to record TEM and SEM images of gels prepared in ultra-pure water.

Sample	**Concentration (mM)**
DBS-$CONHNH_2$	8.4
DBS-$CONHNH_2$/Heparin	8.4/0.038
DBS$CONHNH_2$/C16-DAPMA	8.4/0.15
DBS-$CONHNH_2$/Heparin/C16-DAPMA	8.4/0.038/0.15

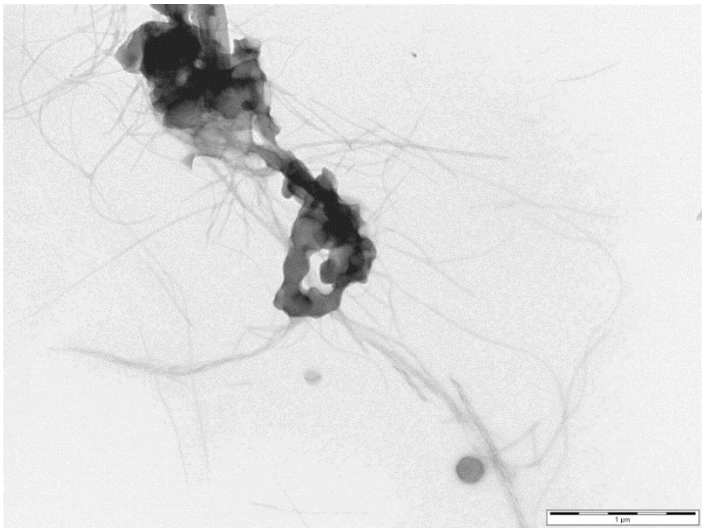

Figure S5. TEM image of DBS-$CONHNH_2$ (0.4% w/v) gel in the presence of C16-DAPMA (150 µM) and heparin (38 µM). Scale bar = 1 µM.

Zoom of Figure S5:

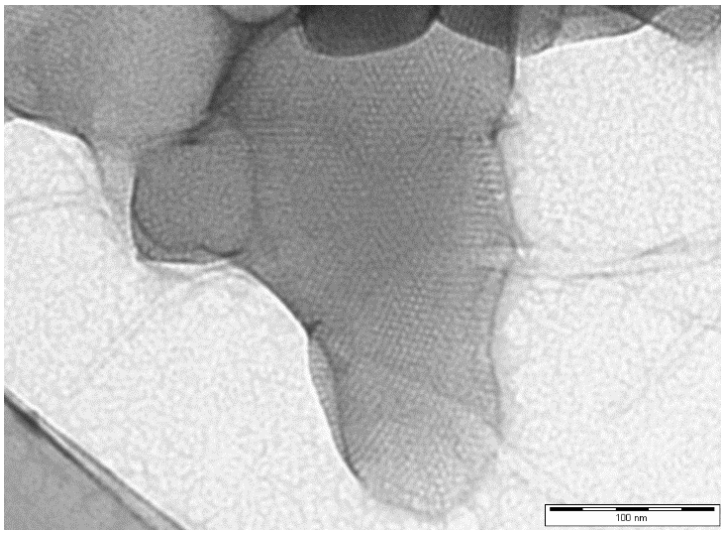

Figure S6. TEM image of the aggregates observed in the DBS-$CONHNH_2$ (0.4% w/v) gel in the presence of C16-DAPMA (150 µM) and heparin (38 µM). Scale bar: 100 nm.

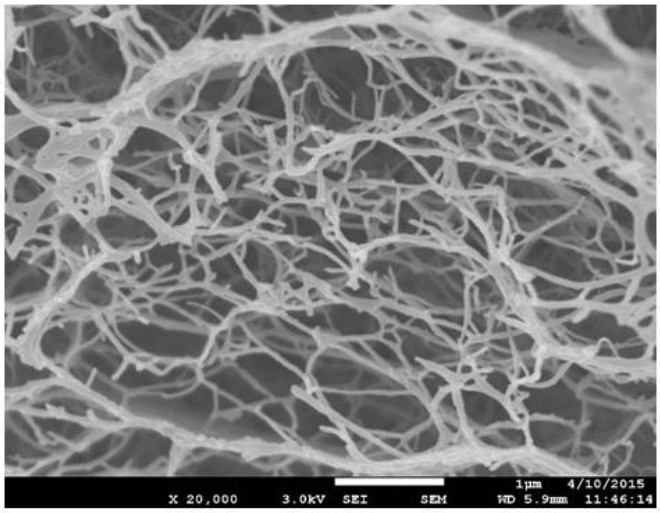

Figure S7. SEM image of DBS-$CONHNH_2$ (0.4% w/v) gel. Scale bar = 1 µm.

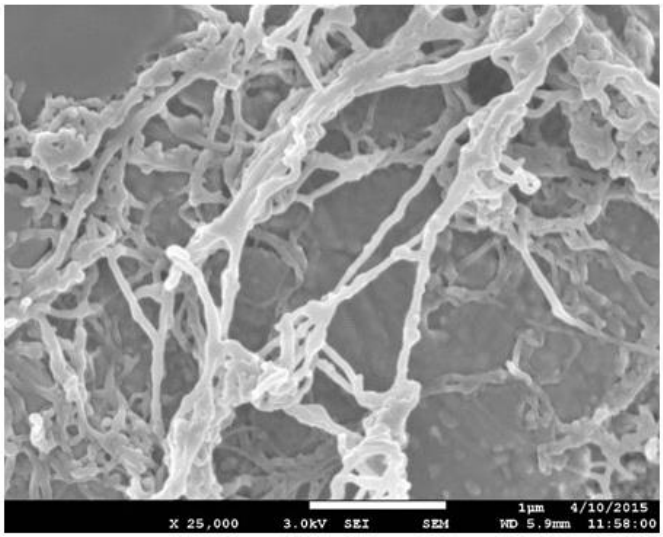

Figure S8. SEM image of DBS-$CONHNH_2$ (0.4% w/v) gel in the presence of heparin (38 µM). Scale bar = 1 µm.

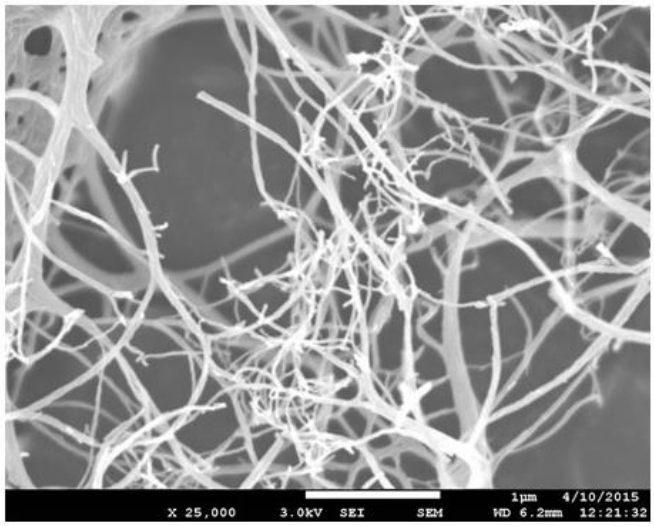

Figure S9. SEM image of DBS-$CONHNH_2$ (0.4% w/v) gel in the presence of C16-DAPMA (150 μM). Scale bar = 1 μm.

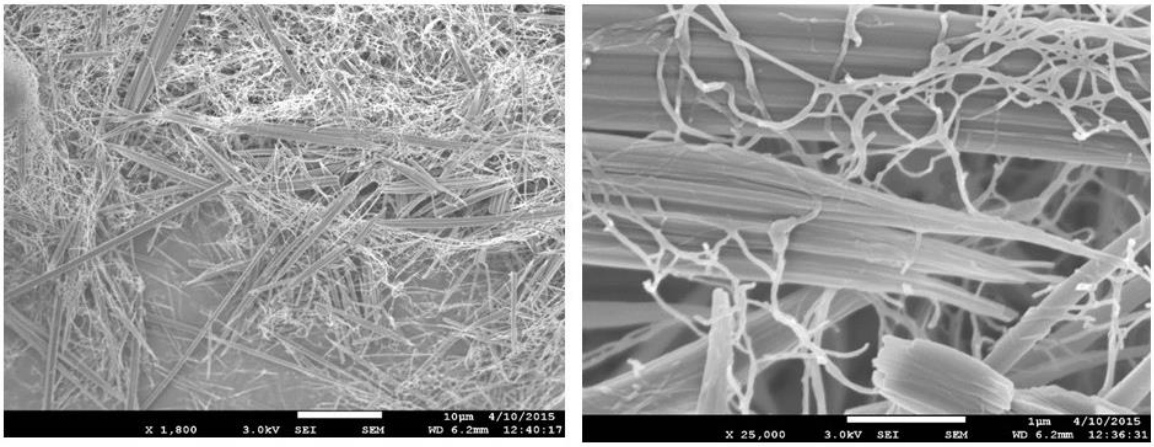

Figure S10. SEM images of DBS-$CONHNH_2$ (0.4% w/v) gel in the presence of C16-DAPMA (150 μM) and heparin (38 μM). Left: Scale bar = 10 μm. Right: Scale bar = 1 μm.

5 Rheology

Rheology measurements were performed in a Malvern Instrument Kinexus Pro+ rheometer. DBS-$CONHNH_2$ (0.4% w/v) hydrogels were prepared in 10 mM Tris-HCl/ 150 mM NaCl buffer (d = 20 mm, h = 0.5 cm) on the lower plate of the equipment with a bottomless vial as template to obtain the intended gel dimensions. The viscoelastic properties were measured by applying dynamic strain sweeps (f = 1 Hz). The measurements were carried out with a parallel geometry plate (d = 20 mm) and a gap of 0.5 mm. The same procedure was performed for DBS-$CONHNH_2$ hydrogels incorporating heparin (1 mM), C16-DAPMA (2 mM), heparin/C16-DAPMA aggregates and agarose (1% w/v).

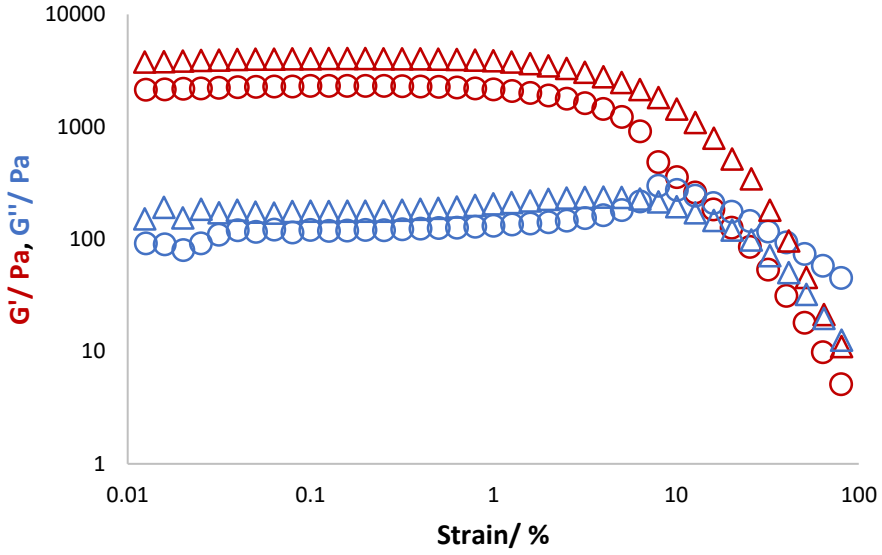

Figure S11. Strain amplitude dependence of the storage modulus (G') and loss modulus (G'') for DBSCONHNH$_2$ gel (○), DBSCONHNH$_2$ gel with heparin (△). Frequency = 1 Hz.

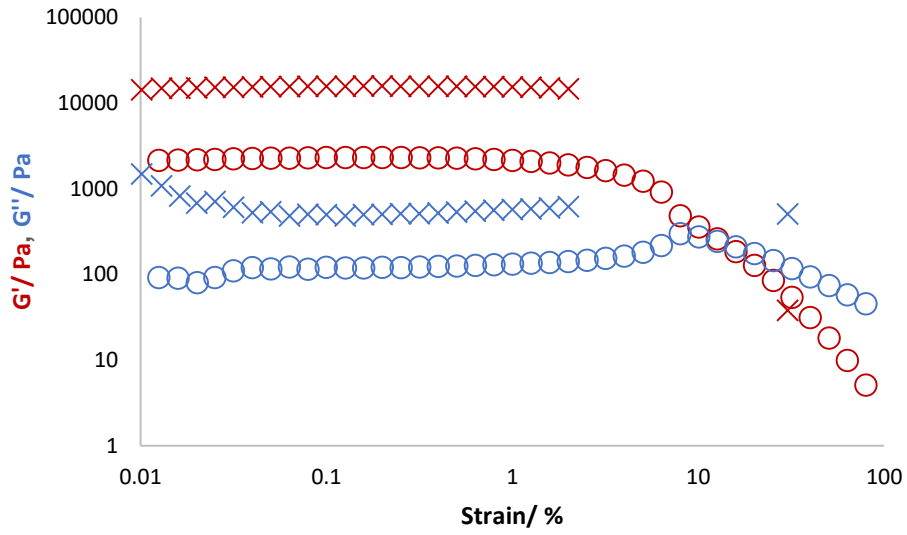

Figure S12. Strain amplitude dependence of the storage modulus (G') and loss modulus (G'') for DBSCONHNH$_2$ gel (○) and DBSCONHNH$_2$ gel with agarose (×). Frequency = 1 Hz.

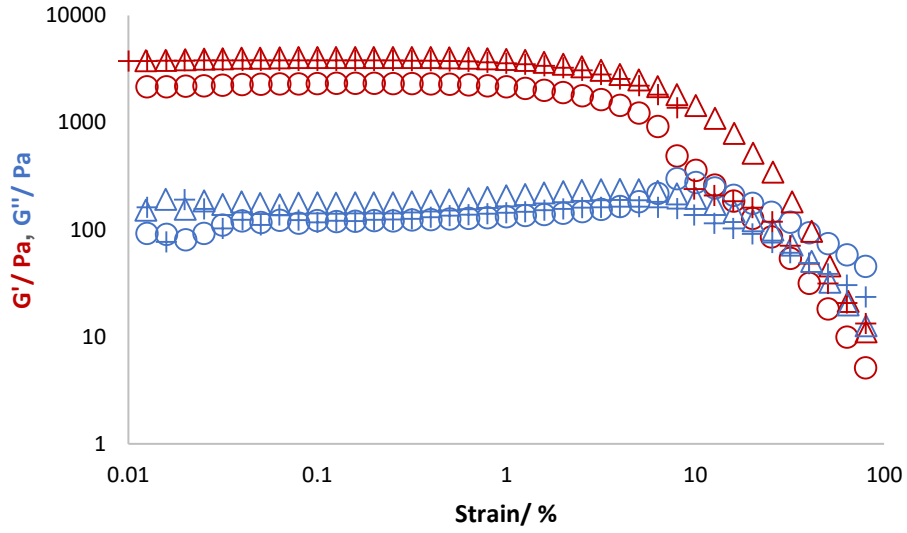

Figure S13. Strain amplitude dependence of the storage modulus (G') and loss modulus (G'') for DBSCONHNH$_2$ gel (○), DBSCONHNH$_2$ gel with heparin (△) and DBSCONHNH$_2$ gel with heparin and agarose (+). Frequency = 1 Hz.

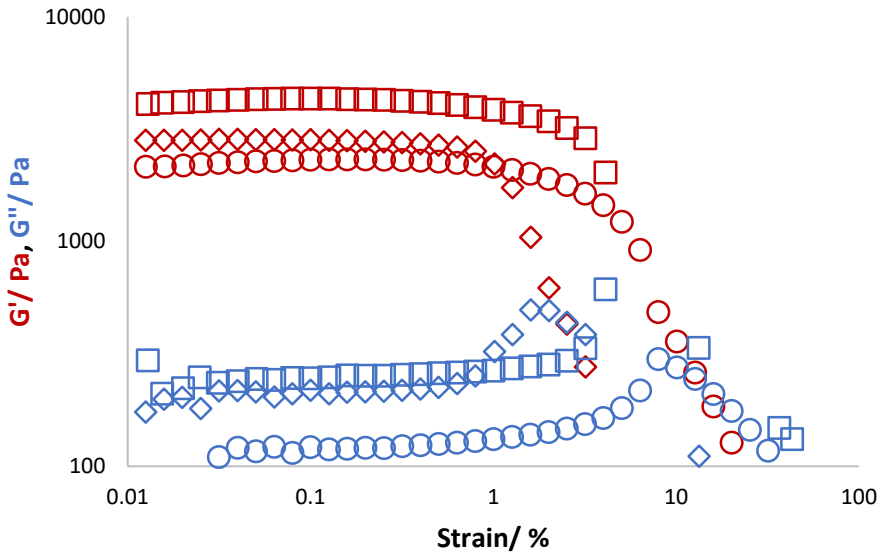

Figure S14. Strain amplitude dependence of the storage modulus (G') and loss modulus (G'') for DBSCONHNH$_2$ gel (○), DBSCONHNH$_2$ gel with C16-DAPMA (◇) and DBSCONHNH$_2$ gel with heparin and C16-DAPMA (□). Frequency = 1 Hz.

6 Release Studies

Release Assay – Surface Release. DBS-CONHNH$_2$ (0.4% w/v) hydrogels (3 mL) containing 1 mM of heparin; 1 mM of heparin and 2 mM of C16-DAPMA; and agarose (0.5% w/v and 1% w/v) with heparin (1 mM) were prepared in 10 mM Tris-HCl/ 150 mM NaCl buffer (pH 7.4), followed by the addition of 1 mL of buffer on top (10 mM Tris-HCl/150 mM NaCl buffer, pH 7.4 or borax/NaOH, pH 10). Aliquots of 65 µL of buffer were collected over time, added into 1935 µL of MalB solution (25.84 µM) and the UV-Vis absorbance recorded. The data were obtained in triplicate. A calibration curve of heparin was obtained by adding 65 µL of known heparin concentrations into 1935 µL of MalB solution and the absorbance spectra obtained.

Release Assay – Cylinder Release. DBS-CONHNH$_2$ (0.4% w/v) hydrogels were prepared with 1% w/v of agarose in 10 mM Tris-HCl/ 150 mM NaCl buffer incorporating 17 mM of heparin. The gel cylinders (approx. dimensions: radius 5 mm, height 14 mm) were removed from the vial carefully using a spatula and placed into a glass jar (100 mL). Buffer solution was added (35 mL, 10 mM Tris-HCl/ 150 mM NaCl) to the jar and aliquots of 65 µL of buffer were collected

over time after gently swirling of the solution and added into 1935 μL of MalB solution (25.84 μM) for UV-Vis absorbance measurement. The data were obtained in triplicate. A calibration curve of heparin was obtained by adding 65 μL of known heparin concentrations into 1935 μL of MalB solution and the absorbance spectra obtained.

7 Cell Growth Studies

Cell Line (3T3 cells: mouse embryonic fibroblasts). Mouse fibroblast 3T3 cells were expanded in a T75 flask with Dulbecco's Modified Eagle's Medium (89% DMEM – high glucose and 3.7 g/L $NaHCO_3$), 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin. To obtain the cells, the medium was removed from the T75 flask and the cells washed with 10 mL Dulbecco's phosphate buffered saline (DPBS) warmed at 37 °C. Trypsin/EDTA (1.5 mL) was added and the cells incubated at 37 °C for approximately 5 min. When cell detachment was observed (by optical microscopy), trypsin was neutralised with 3.5 mL of DMEM, 10% FBS, 1% P/S and the cell suspension collected into a 50 mL tube. The cells were counted in a Neubauer Chamber by mixing 20 μL of cell suspension with 180 μL of trypan blue by optical microscopy.

DBS-$CONHNH_2$ Gel Preparation. DBS-$CONHNH_2$ gel was prepared in 10 mM Tris-HCl/ 150 mM NaCl buffer (pH 7.4). 0.5 mL of buffer was added to DBS-$CONHNH_2$ powder (2 mg). The solution was heated until complete dissolution of the gelator and a transparent solution was observed. The solution was immediately transferred into the well plates (50 μL in each well of the 96 well plates and 100 μL in the inserts). The same procedure was done to prepare the gels with different concentrations of heparin, C16-DAPMA and agarose, by adding a solution of heparin or C16-DAPMA in 10 mM Tris-HCl/ 150 mM NaCl with the appropriate concentration and by weighing agarose together with DBS-$CONHNH_2$ powder.

Metabolic Activity (WST-1 Assay). WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]1,3-benzene disulfonate) was added to each well (10% of the total volume, 5 μL for gels in 96 well plates and 10 μL for gels in transwells, followed by incubation of the plates at 37°C for 2 h. The gels were then disrupted by pipetting them up and down, transferred into Eppendorfs and frozen. Later, the samples were thawed (protecting them from light) and

homogenized. The samples were centrifuged at 13800 rpm for 5 min. The supernatant (80 µL) was collected and added into a new 96 well plate. The absorbance was measured at 440 nm.

MitoTracker Staining. The medium was removed from the culture wells. The plates were incubated with 0.4 µM of MitoTracker Green and 10.8 µM of Hoechst diluted in culture medium, for 90 min at 37°C. The staining solution was removed and the samples washed with Dulbecco's Phosphate Buffered Saline (DPBS) for 30 min at room temperature. DPBS (twice the sample volume) was added to the cells and the images collected from a fluorescence microscope.

Live/Dead Staining. The medium was removed from the culture wells. The plates were incubated with 13.3 µM calcein-acetoxymethyl (AM) and 4.8 µM propidium iodide (PI) in cell culture medium for 60 min at 37°C. The staining solution was removed and the samples washed twice with DPBS for approximately 15 min. Some gels/cells were fixed with 100 µL or 200 µL of 4% paraformaldehyde in PBS (0.01 M, pH 7.4) (96 well plates or 24 well plates respectively) for 1 h. The samples were washed twice with DPBS for ca. 15 min and the images were collected from a fluorescence microscope.

2D Cell Culture: Gels with Cells on Top. Gels were prepared in 96 well plates (50 µL of gel/ well) and sterilised by applying UV light for 20 min. After gelation, the cells were seeded on top of the gels. The desired number of cells (to obtain the following cell density: 50000 cells/mL or 25000 cells/ml after optimisation) was added into a 2 mL Eppendorf and centrifuged at 1800 rpm for 6 min at 25 °C, followed by the removal of the supernatant. The cells were re-suspended in DMEM - 10% FBS - 1% P/S and 100 µL added on top of each gel in the well.

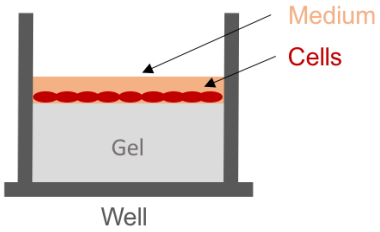

Figure S15. Schematic representation of 2D cell culture performed in 96 well plates.

WST-1 Assay of Metabolic Activity

- *Cell Density: 50000 cells/mL*

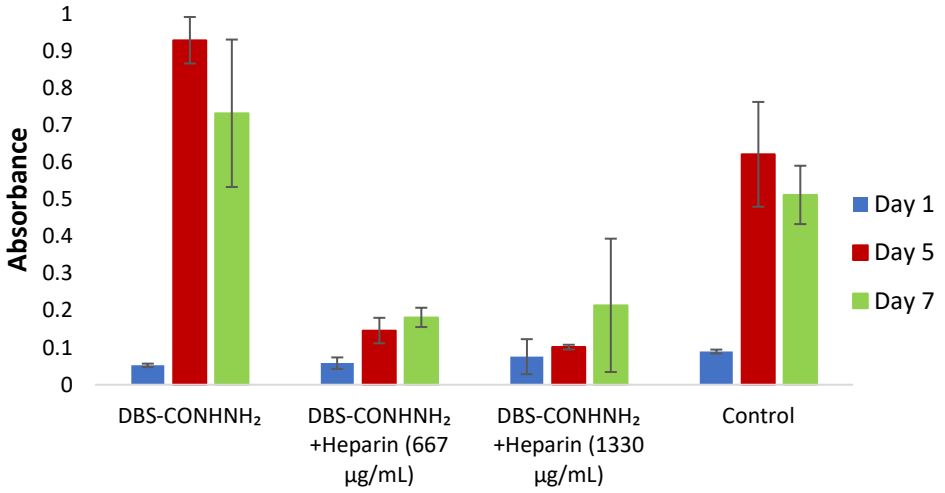

Figure S16. Absorbance of WST-1 reagent at 440 nm with (from left to right): DBS-$CONHNH_2$ hydrogels; DBS-$CONHNH_2$ hydrogels in the presence of heparin (667 µg/mL and 1330 µg/mL, respectively) and control (medium with cells), with cells on top, at day 1, 5 and 7.

- *Cell Density: 50000 cells/mL.* Data presented in main manuscript.

MitoTracker Staining

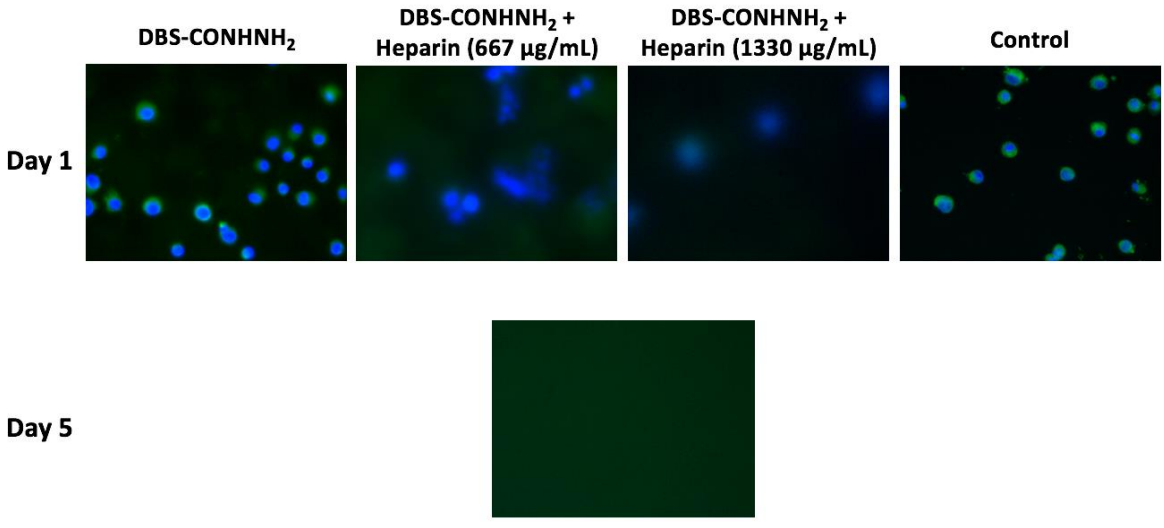

Figure S17. Fluorescence microscopy images of MitoTracker/Hoechst staining of DBS-$CONHNH_2$ hydrogels, DBS-$CONHNH_2$ hydrogels in the presence of heparin (667 µg/mL and 1330 µg/mL) and control (medium with cells), with cells on top, at day 1 and 5. Mag: 20x.

Optical Microscopy

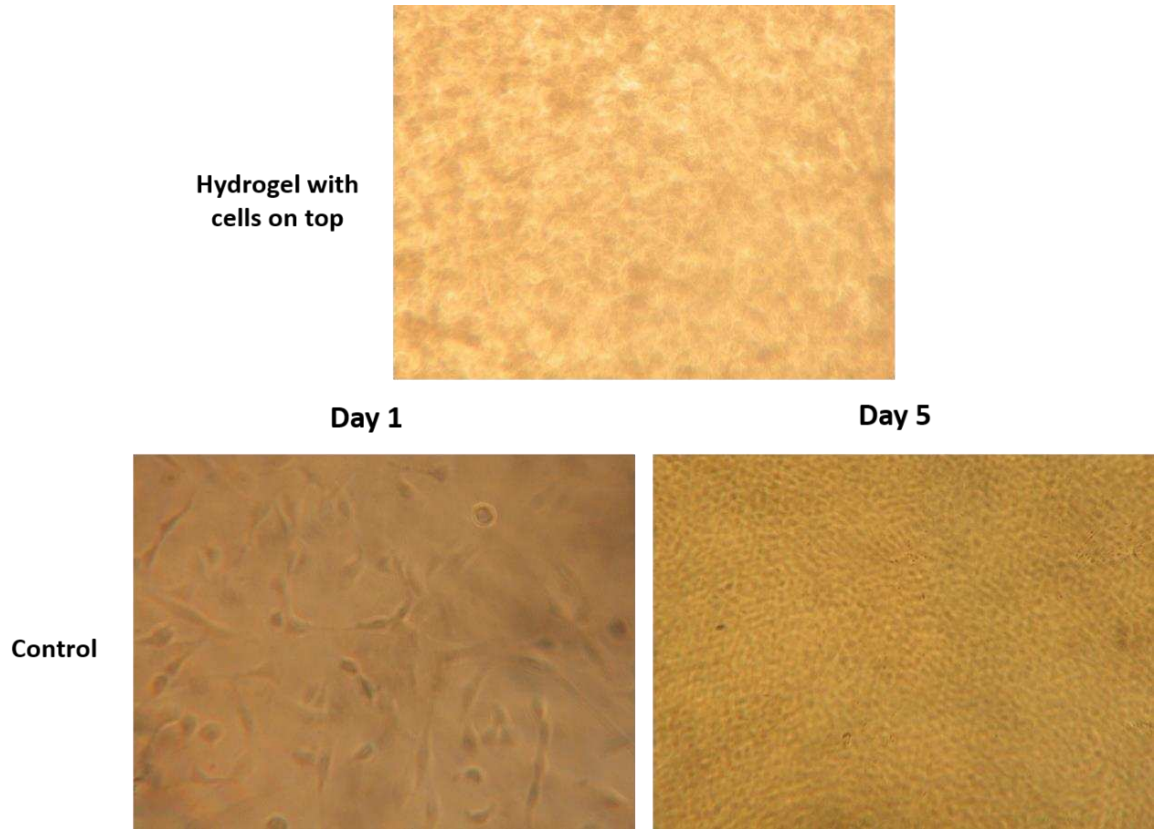

Figure S18. Optical microscopy images of DBS-$CONHNH_2$ hydrogels with cells on top (top) and controls (medium with cells) at day 1 and 5 (bottom). Magnification: 10x.

2D Cell Culture: Gels in Transwells. Gels were prepared in the inserts (100 µL of gel/ insert) and sterilised by applying UV light for 20 min. After gelation, the cells were seeded on the bottom of the wells and both compartments (well and inserts) were filled with medium. The optimised number of cells (cell density: 10000 cells/mL) was added into a 2 mL Eppendorf and centrifuged at 1800 rpm for 6 min at 25 °C, followed by the removal of the supernatant. The cells were re-suspended in DMEM - 10% FBS - 1% P/S and 500 µL added to each well.

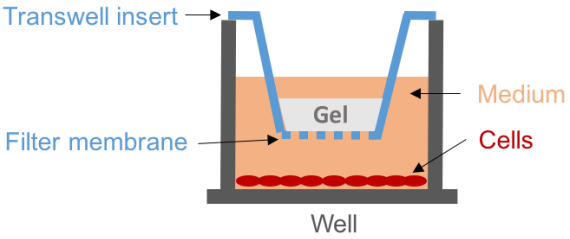

Figure S19. Schematic representation of 2D cell culture performed in transwells.

WST-1 activity

- *Cell Density: 10000 cells/ml*

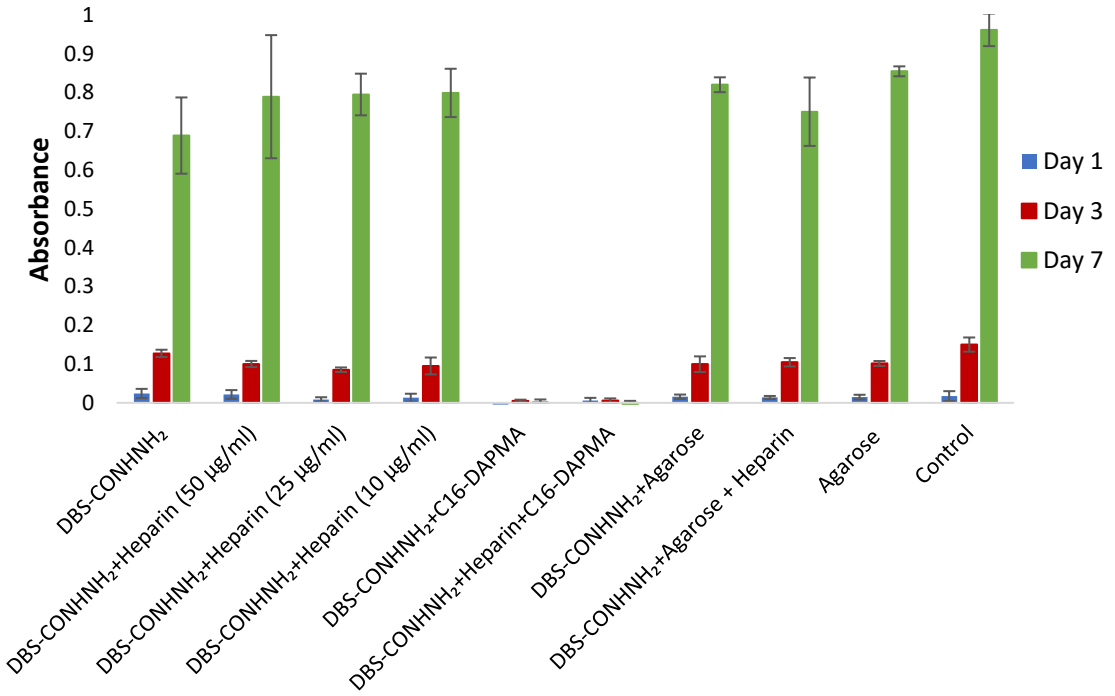

Figure S20. Absorbance of WST-1 reagent at 440 nm with (from left to right): DBS-$CONHNH_2$ hydrogels; DBS-$CONHNH_2$ hydrogels in the presence of heparin (50 µg/mL, 25 µg/mL and 10 µg/mL); DBS-$CONHNH_2$ hydrogels in the presence of C16-DAPMA (140 µg/mL); DBS-$CONHNH_2$ hydrogels in the presence of heparin (50 µg/mL) and C16-DAPMA (140 µg/mL); DBS-$CONHNH_2$ hydrogels with agarose (1% w/v); DBS-$CONHNH_2$ hydrogels with agarose (1% w/v) and heparin (50 µg/mL); agarose (1%) and control (medium with cells), in transwells, at day 1, 3 and 7.

Optical Microscopy

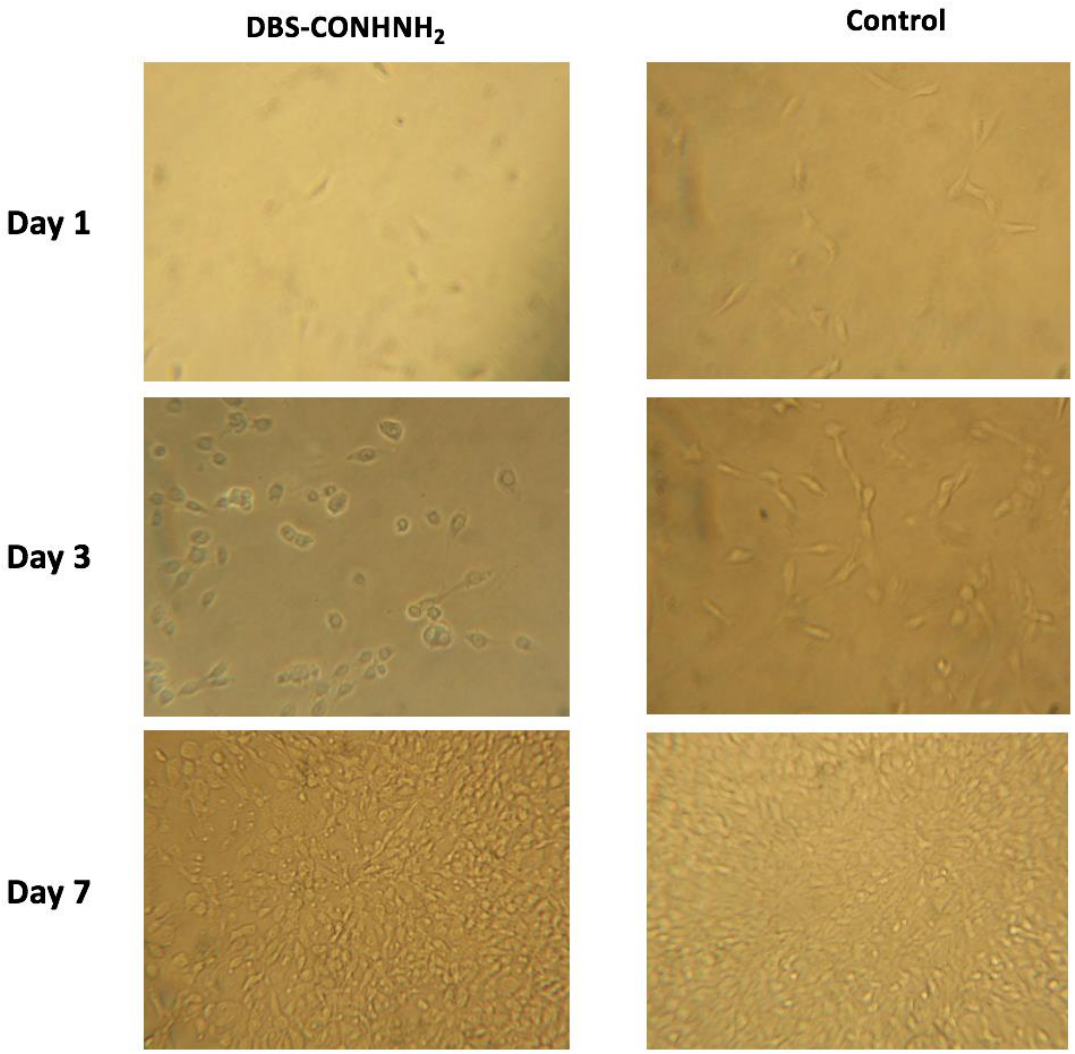

Figure S21. Optical microscopy images of DBS-$CONHNH_2$ hydrogels in transwells, at day 1 3 and 7 and control (medium with cells). Magnification: 20x.

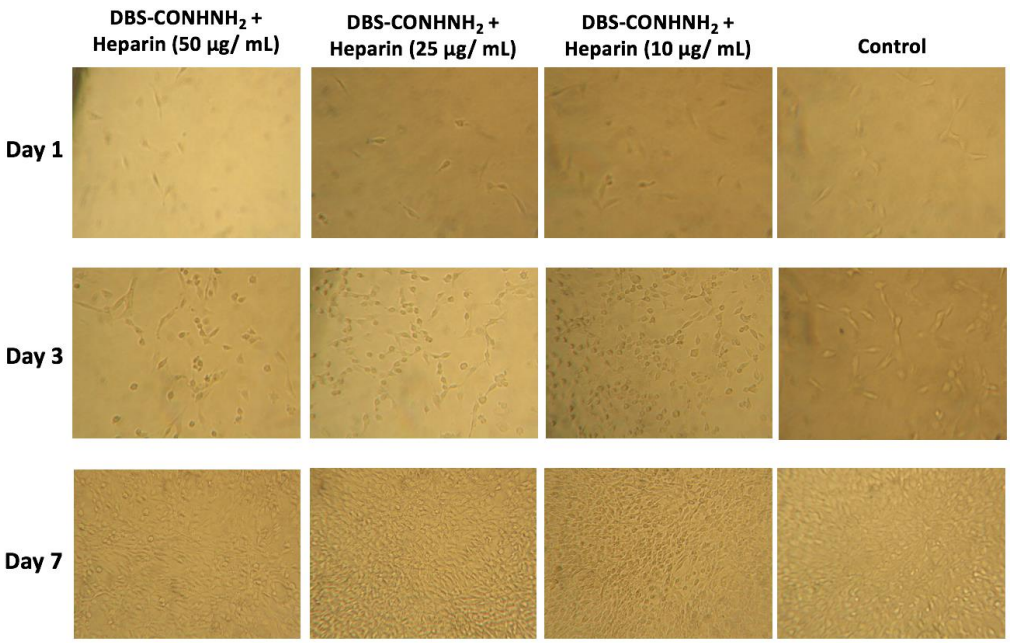

Figure S22. Optical microscopy images of DBS-$CONHNH_2$ hydrogels in the presence of heparin (50 µg/mL, 25 µg/mL and 10 µg/mL) and controls (medium with cells), in transwells, at day 1, 3 and 7. Magnifcation: 20x.

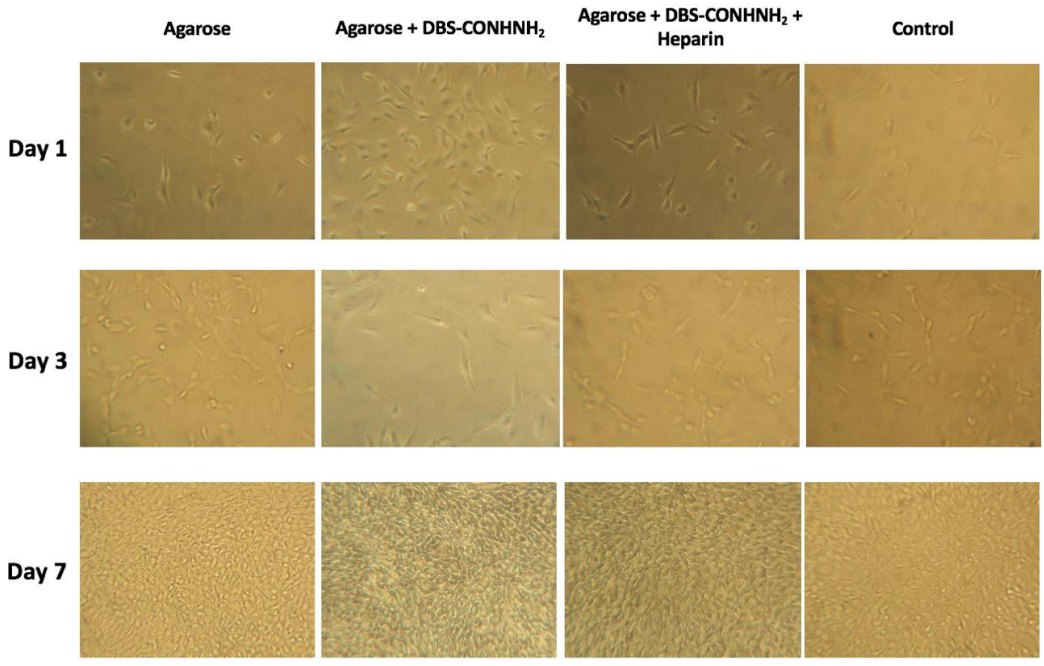

Figure S23. Optical microscopy images of agarose hydrogels, DBS-$CONHNH_2$ and agarose hybrid hydrogels and DBS-$CONHNH_2$ and agarose hybrid hydrogels in the presence of heparin (50 µg/mL) and controls (medium with cells), in transwells, at day 1, 3 and 7. Magnifcation: 20x.

MitoTracker Staining

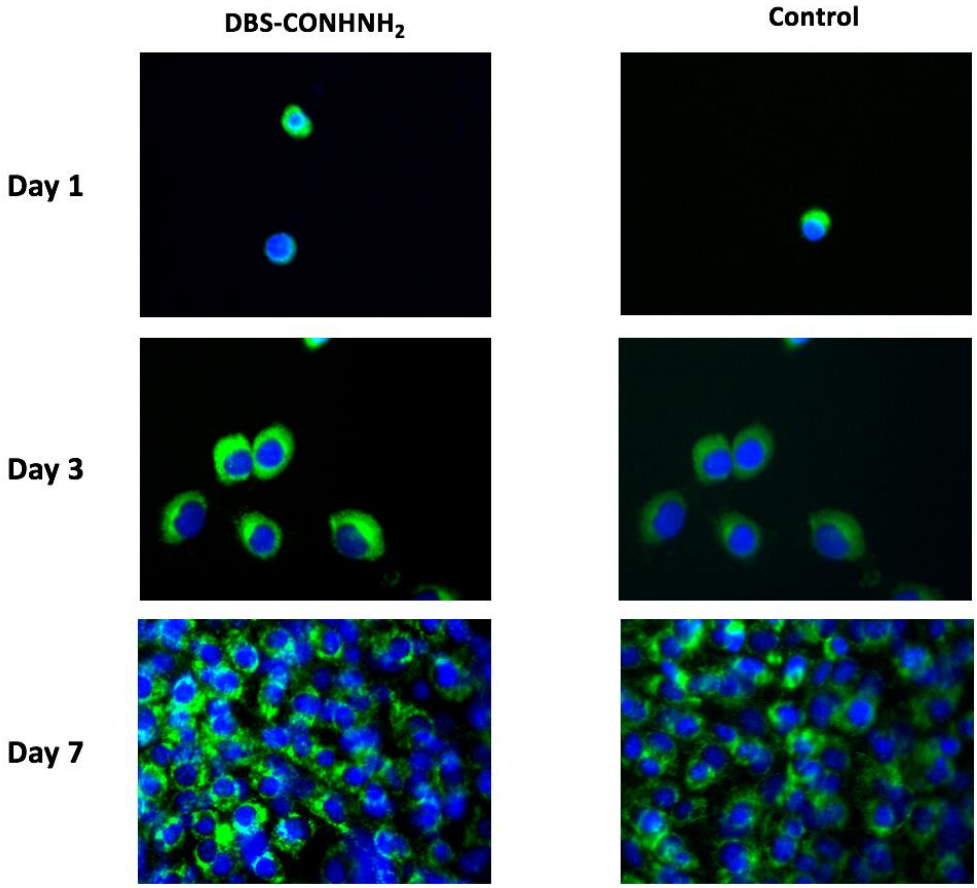

Figure S24. Fluorescence microscopy images of mitotraker/hoechst staining of DBS-$CONHNH_2$ hydrogels (0.4% w/v) and controls (medium with cells), in transwells, at day 1, 3 and 7. Magnification: 40x.

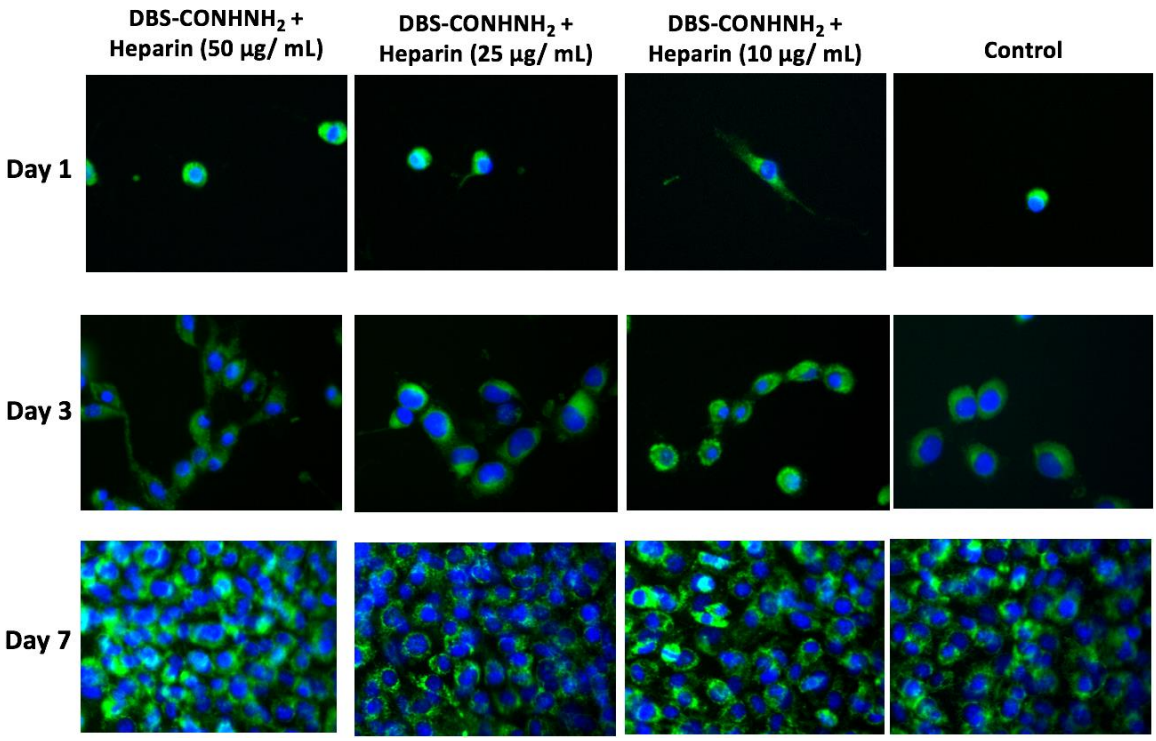

Figure S25. Fluorescence microscopy images of mitotraker/hoechst staining of DBS-$CONHNH_2$ hydrogels in the presence of heparin (50 µg/mL, 25 µg/mL and 10 µg/mL) and controls (medium with cells), in transwells, at day 1, 3 and 7. Magnification: 40x.

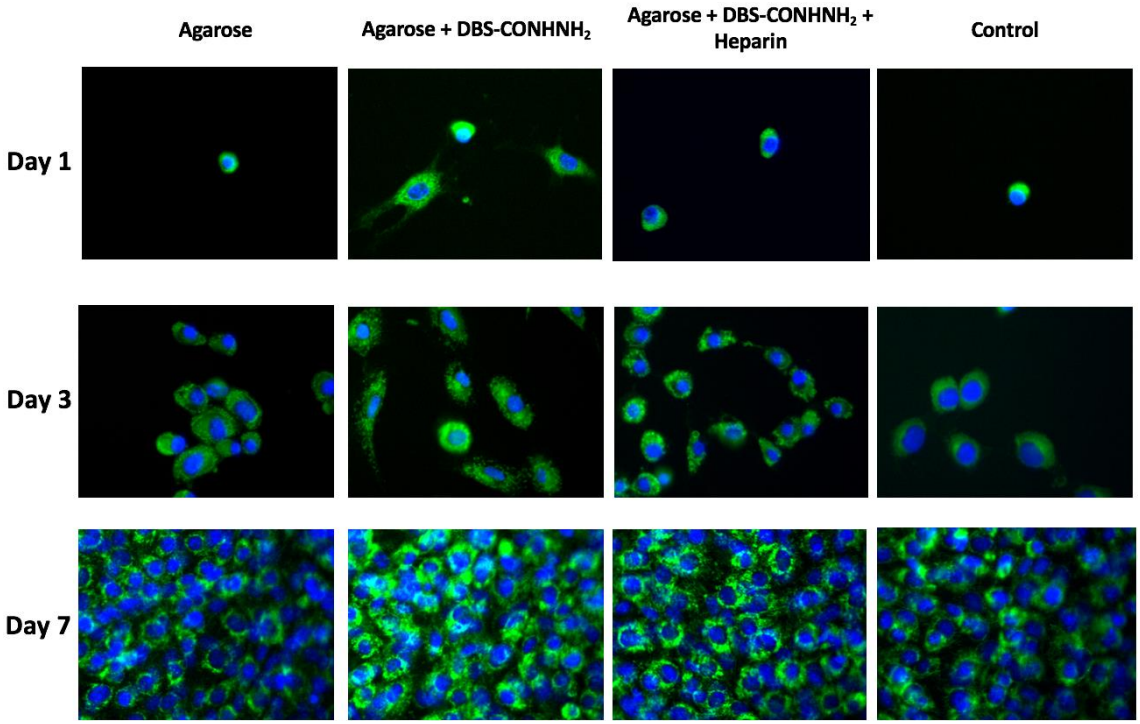

Figure S26. Fluorescence microscopy images of mitotraker/hoechst staining of agarose hydrogels, DBS-$CONHNH_2$ and agarose hybrid hydrogels and DBS-$CONHNH_2$ and agarose hybrid hydrogels in the presence of heparin (50 µg/mL) and controls (medium with cells), in transwells, at day 1, 5 and 7. Magnification: 40x.

Live/Dead Staining

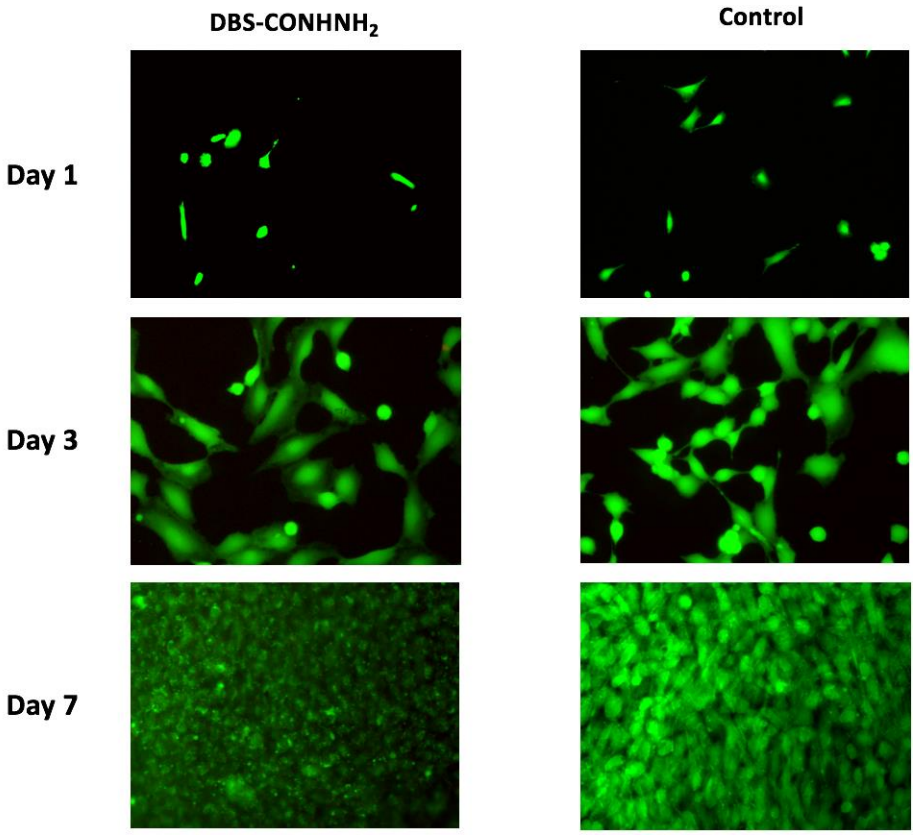

Figure S27. Fluorescence microscopy images of calcein-AM/PI staining of DBS-$CONHNH_2$ hydrogels (0.4% w/v) and controls (medium with cells), in transwells, at day 1, 3 and 7. Magnification: 20x.

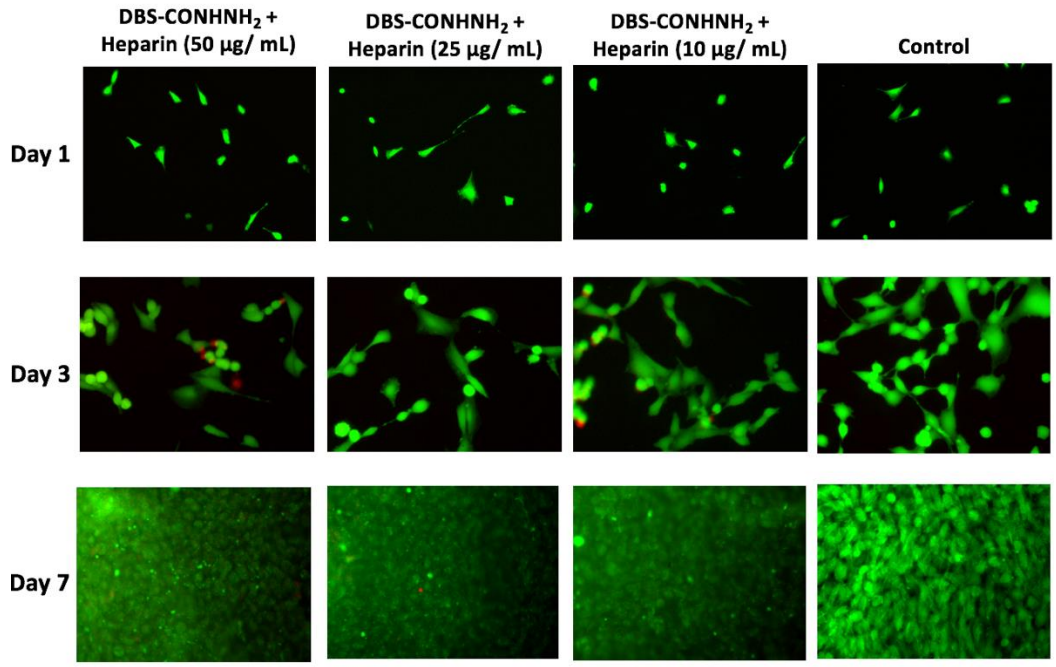

Figure S28. Fluorescence microscopy images of calcein-AM/PI staining of DBS-$CONHNH_2$ hydrogels in the presence of heparin (50 µg/mL, 25 µg/mL and 10 µg/mL) and controls (medium with cells), in transwells, at day 1, 3 and 7. Magnification: 20x.

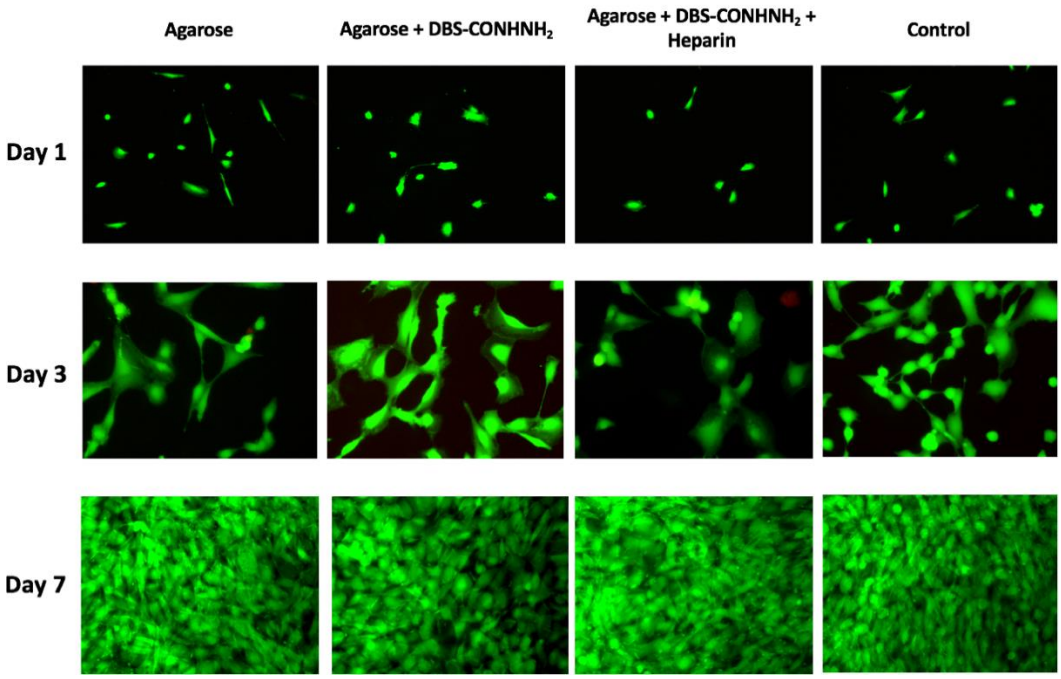

Figure S29. Fluorescence microscopy images of calcein-AM/PI staining of agarose hydrogels, DBS-$CONHNH_2$ and agarose hybrid hydrogels and DBS-$CONHNH_2$ and agarose hybrid hydrogels in the presence of heparin (50 µg/mL) and controls (medium with cells), in transwells, at day 1, 3 and 7. Magnification: 20x.

Migration Assay. Different volumes (100 µL, 80 µL and 60 µL) of DBS-$CONHNH_2$ hydrogels (0.4% w/v) in transwells were prepared as described above. Cells (cell density: 10000 cells/mL) were added on top of the gels (double the volume of the gels). Two different controls were prepared: 200 µL of cells in medium was added to the insert, or 200 µL of cells in medium was added directly to the bottom of the well without the presence of the insert. The inserts were washed with trypsin to detach any cells from the membrane. The presence of cells on the bottom of the wells was followed by optical microscopy over 7 days.

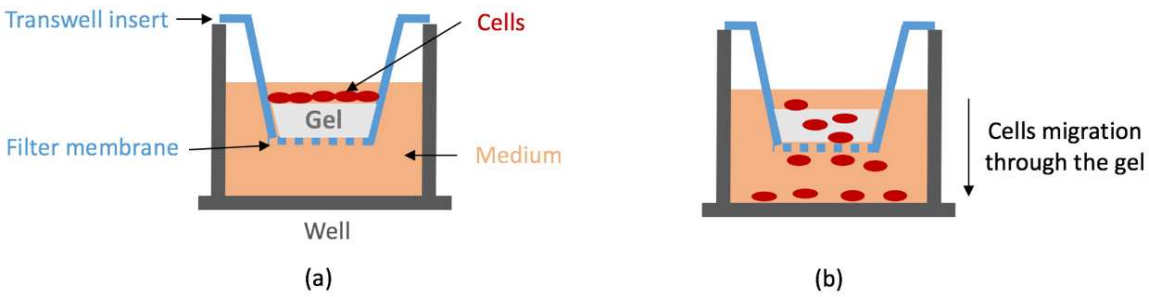

Figure S30. Schematic representation of the cell migration assay. (a) DBS-$CONHNH_2$ hydrogel was formed in the transwell insert and cells were added on top of the hydrogel. Medium was added in the well. (b) Cells migration through the gel network into the well. Adhesion of cells on the bottom of the well can be followed by optical microscopy.

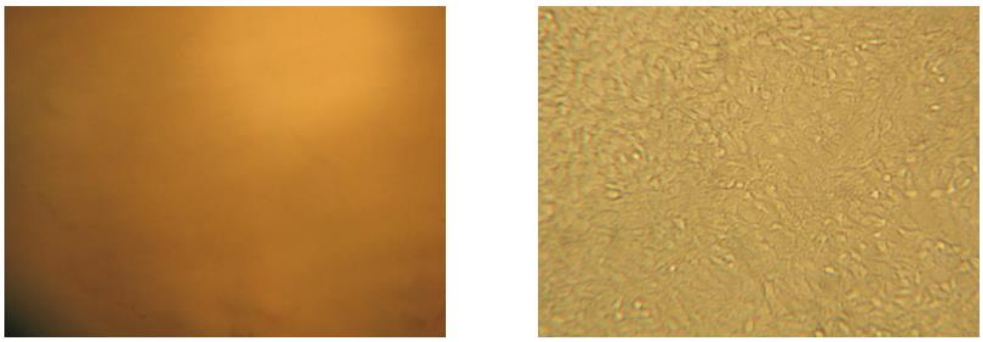

Figure S31. Example of an optical microscope image obtained on the migration assay for the well were DBS-$CONHNH_2$ hydrogels were present (left – no cells present) and control (right - cells added directly into the well and attached to the bottom of the well) after washing the membrane with trypsin, on day 7.

8 References

[1] B. O. Okesola and D. K. Smith, D. K. *Chem. Commun.*, **2013**, *49*, 11164-11166.

[2] L. E. Fechner, B. Albanyan, V. M. P. Vieira, E. Laurini, P. Posocco, S. Pricl and D. K. Smith, *Chem. Sci.* **2016**, *7*, 4653-4659.

[3] S. M. Bromfield, A. Barnard, P. Posocco, M. Fermeglia, S. Pricl and D. K. Smith, *J. Am. Chem. Soc.* **2013**, *135*, 2911-2914.