Multi-component hybrid hydrogels – understanding the extent of orthogonal assembly and its impact on controlled release

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1 Materials and Methods

1.1 Materials Used. Low-molecular-weight gelator (LMWG) DBS-COOH was synthesised according to our previously reported methods, and all characterisation data were in full agreement with those previously published.\(^1\) 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.\(^3\)

1.2. Spectrometric Methods. Infrared spectra were recorded on Perkin Elmer Spectrum Two FT-IR spectrometer. Kinetic studies were performed on a Bruker AV500 spectrometer (\(^1\)H 500 MHz) and a Jasco J810 CD spectrophotometer. UV-Vis absorbance was recorded on a Shimadzu UV-2401PC spectrophotometer. Thermal stability was recorded on a Huber Ministat 230 circulator oil bath. TEM and SEM images were obtained by Meg Stark at Biology Technology Faculty, University of York, using a FEI Tecnai 12 Bio TWIN operated at 120 kV for the TEM images and a JEOL JSM-7600F operated at 3 kV for the SEM images. Rheology studies were performed on a Kinexus Pro+ rheometer from Malvern instruments.

1.3. Gel Preparation. DBS-COOH gel preparation. DBS-COOH hydrogels (0.2% wt/vol) were prepared by adding 1 mL of 10 mM Tris-HCl/150 mM NaCl buffer into 2 mg of gelator, followed by 10 minutes of sonication. 30 µL of 0.5 M NaOH\(_{(aq)}\) was added to dissolve all solid. The DBS-COOH solution was transferred to a vial with GdL (8 mg, 44.9 mM), followed by
shaking to dissolve. The vial was allowed to stand overnight. DBS-COOH gel (0.2% wt/vol) was prepared with different concentrations of heparin in 10 mM Tris-HCl/150 mM NaCl. 0.5 mL of 10 mM Tris-HCl/150 mM NaCl was added to DBS-COOH powder (2.0 mg) and sonicated. 30 μL of 0.5 M NaOH(aq) was added to dissolve all solid. Solutions of heparin were prepared with double the concentration of the intended final concentration, to allow for subsequent dilution. The DBS-COOH solution was transferred to a vial with GdL (8 mg, 44.9 mM), followed by shaking to dissolve and 0.5 mL of heparin solution was immediately added and stirred to ensure mixing. The vials were allowed to stand overnight. The same procedure was followed to prepare the gels with different concentrations of C16-DAPMA and heparin/C16-DAPMA aggregates. For gels with different concentrations of gelator the amount of NaOH(aq) and GdL added was adjusted accordingly. *Agarose gel preparation.* Heparin solutions with the intended concentration were prepared in 10 mM Tris-HCl/150 mM NaCl buffer and added to a known weight of agarose. Solutions were heated to 90°C in a thermoregulated oil bath to dissolve agarose, then left to stand at room temperature for gelation to occur. *DBS-COOH + Agarose gel preparation.* For 3 mL of gel, heparin solution (3 mL, 1mM in 10 mM Tris-HCl/150 mM NaCl buffer) was added to DBS-COOH (6.0 or 60.0 mg, to give 0.2 or 2.0 % wt/vol respectively), and dissolved using NaOH(aq) (30 μL, 0.5 M for 0.2% wt/vol DBS-COOH or 5 M for 2.0% wt/vol DBS-COOH), sonicating for 10-15 minutes. The solution was then transferred to a vial containing agarose (15.0 or 30.0 mg, to give 0.5 or 1.0% wt/vol respectively), heated to 90 °C in a thermoregulated oil bath to dissolve, cooled to 50°C and transferred to a vial containing GdL (24.0 mg for 0.2% wt/vol DBS-COOH, or 240.0 mg for 2.0% wt/vol DBS-COOH). Solutions were shaken to dissolve GdL, and then left to stand overnight for gelation to occur. When formed, all gels in this study had final pH values ca. 5.
1.4. $T_{gel}$ Determination

$T_{gel}$ values were observed by reproducible tube inversion methodology. All the samples with different concentrations of heparin and heparin-C16-DAPMA aggregates that resulted in gel formation and were placed into a thermo-controlled oil bath, with an initial temperature of 25°C. The temperature was set to rise to 100 °C. At each increase of 5 °C, approximately, 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 began to run down the sides of the vial.

1.5. Infrared Characterisation

Infrared spectra of DBS-COOH gel (0.2% wt/vol) with 300 µM of heparin and DBS-COOH gel with 300 µM of heparin and 800 µM of C16-DAPMA were recorded. Gels were prepared as described above. After gel formation, the solvent of the samples was removed under high vacuum and the resulting powders placed into the infrared spectrometer.

1.6. NMR Characterisation of Kinetics

DBS-COOH gel was prepared by adding $D_2O$ (0.7 mL) to DBS-COOH (1.4 mg) and sonicating. 20 µL of NaOH$_{aq}$ (0.5 M) was added to dissolve all solid and DMSO (1.4 µL) was added as an internal standard. The solution was then transferred to a vial containing GdL (5.6 mg, 44.9 mM), followed by shaking. The sample was then immediately transferred to a NMR tube and placed in the spectrometer, with spectra recorded every 30 minutes for 10 hours. The kinetics of DBS-COOH gel formation with 300 µM heparin were obtained by adding $D_2O$ (0.5 mL) to DBS-COOH (2.0 mg) and sonicating. 30 µL of NaOH$_{aq}$, 0.5 M, was added to dissolve all solid and DMSO (2.0 µL) was added as an internal standard. The solution was then transferred to a vial containing GdL (8 mg, 44.9 mM), followed by shaking. 0.5 mL of heparin (600 µM) in
D$_2$O was added to the DBS-COOH solution, stirred and immediately transferred to a NMR tube and placed in a spectrometer, with spectra recorded every 30 minutes for 10 hours. The same procedure was performed for the kinetics of DBS-COOH with 300 µM of heparin and 800 µM of C16-DAPMA.

1.7. Circular Dichroism Characterisation of Kinetics

DBS-COOH sample was prepared by adding 10 mM Tris-HCl/150 mM NaCl (500 µL) to DBS-COOH (0.2 mg) and sonicating. 5 µL of NaOH$_{(aq)}$ (0.5 M) was added to dissolve all solid and then the solution was transferred to a vial containing GdL (1 mg), followed by shaking. 400 µL of sample were immediately transferred to a CD cuvette (1 mm pathlength) and placed in the spectrometer, with spectra recorded every 5 minutes for 5 hours and a further reading after 6 hours. The same procedure was performed in the presence of 38 µM of heparin and 150 µM of C16-DAPMA/38 µM heparin.

1.8. Electron Microscopy

DBS-COOH gel samples for TEM images were prepared in ultra-pure H$_2$O and obtained by adding one microspatula of each sample on a copper grid (standard) with Formvar and carbon support film. The excess of 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-dried on bits of copper shim. After mounting the samples on stubs they were sputter coated with approximately 5 nm of Au/Pd and the images were recorded.
Table 1. Concentrations of DBS-COOH, C16-DAPMA and heparin used to recorded TEM and SEM images of gels prepared in ultra-pure H$_2$O.

| Sample                      | Concentration (mM) |
|-----------------------------|--------------------|
| DBS-COOH                    | 4.5                |
| DBS-COOH/Heparin            | 4.5/0.038          |
|                             | 4.5/0.30           |
| DBS-COOH/Heparin/ C16-DAPMA | 4.5/0.038/0.15     |
|                             | 4.5/0.30/0.80      |

1.9. Rheology Characterization

DBS-COOH hydrogels (0.2% wt/vol) were prepared as described above 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), dynamic frequency sweeps (Y = 0.1%) and temperature variation (f = 1 Hz, Y = 0.1%). 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-COOH hydrogels incorporating heparin (1 mM), C16-DAPMA (2 mM), heparin/C16-DAPMA aggregates and agarose (1% wt/vol).

1.10. Release Assays

Release Assay - Aliquots

3 mL of DBS-COOH hydrogels (0.2, 2, 5 and 10% wt/vol), agarose hydrogels (1.0, 2.5, 5.0, 7.5 and 10% wt/vol), DBS-COOH hydrogels (0.2% wt/vol) containing 1 mM of heparin and 2 mM of C16-DAPMA and DBS-COOH hydrogels (2% wt/vol) with agarose (0.5% wt/vol and
1% wt/vol) and heparin (1 mM) were prepared in 10 mM Tris-HCl/150 mM NaCl buffer, followed by the addition of 1 mL of buffer on top. 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 - Gel Cylinder**

DBS-COOH hydrogels (2% wt/vol) were prepared with 1% wt/vol 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 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 swirled the solution and added into 1935 µL of MalB solution (25.84 µM) for UV-Vis measurement. 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 absorbance spectra obtained.

**Geometry Calculation for Release Assays**

For the different approaches to heparin release experiments, we can determine surface area/volume ratios as follows:

Sample 1 (release within vial): Surface area (top circle of gel) = \(\pi r^2\), Volume = 3 cm\(^3\)
\[ r = 0.9 \text{ cm}, \text{ therefore, surface area} = 2.545 \text{ cm}^2 \]
Surface Area:Volume Ratio = \(\frac{2.545}{3} = 0.85 \text{ cm}^{-1}\)
Sample 2 (release from gel cylinder): Surface area (full surface of cylinder) = \(2\pi r^2 + 2\pi rh\),
Volume = 1 cm\(^3\)

\(r = 0.5\) cm, \(h = 1.27\) cm therefore surface area = \(1.571 + 3.990 = 5.561\) cm\(^2\)
Surface Area:Volume Ratio = 5.6 cm\(^{-1}\)

Performing release using gel cylinders increases the Surface Area:Volume ratio 6.5-fold compared with using gels-in-vials, by the combination of decreasing the total gel volume and exposing all surfaces of the gel to the receiving buffer solution.

1.11. NMR Study of DBS-COOH Breakdown on pH Variation

\(\text{D}_2\text{O} (1\ \text{mL})\) was added to DBS-COOH (2.0 mg) and sonicated for 5 minutes. \(\text{NaOH}_{(\text{aq})} (30\ \mu\text{L}, 0.5 \text{ M})\) was added and the solution sonicated for 5 minutes to dissolve all solid. \(\text{DMSO} (2.0\ \mu\text{L})\) was added to act as an internal standard, and the solution transferred to a vial containing heparin (0.68 mg, to give 1 mM). The solution was then transferred to a vial containing agarose (5.0 mg), heated to 90°C in a thermoregulated oil bath to dissolve agarose, cooled to 50 °C, transferred to a vial containing GdL (8.0 mg) and shaken to dissolve. A sample (0.7 mL) was then immediately transferred to an NMR tube and placed in the spectrometer to record an initial spectrum. After leaving the NMR tube overnight for DBS-COOH to fully gel, another spectrum of each gel was recorded. \(\text{NaOH} (20.74\ \text{mg})\) was dissolved in \(\text{D}_2\text{O} (1\ \text{mL}, \text{to give } 0.52 \text{ M})\), and a sample of this solution (150 \(\mu\text{L}\)) added to the top of each gel in the NMR tube. Spectra were recorded of the gel periodically until all the DBS-COOH was again deemed mobile (~ 70 h).
2 Images of Gels

Figure S1. DBS-COOH (0.2% wt/vol) gel formation with 37.7 µM; 50 µM; 100 µM; 150 µM; 300 µM; 400 µM; 500 µM (from left to right) of heparin demonstrating that gels still form in the presence of heparin. Vial dimensions: Diameter – 1 cm; Height – 4 cm; Gel height – 1 cm; Gel volume – 1 mL.

Figure S2. DBS-COOH (0.2% wt/vol) gel formation with 150 µM; 300 µM; 400 µM; 500 µM; 600 µM; 700µM; 800 µM; 900 µM; 1000 µM (from left to right) of C16-DAPMA demonstrating the disruptive effect of the self-assembling multivalent micellar system. Vial dimensions: Diameter – 1 cm; Height – 4 cm; Gel height – 1 cm; Gel volume – 1 mL.
3 Infra-Red Spectroscopy

Figure S3. Infrared spectra of DBS-COOH with heparin (300 µM) xerogel (red line) and DBS-COOH in the presence of both heparin (300 µM) and C16-DAPMA (800 µM) xerogel.

Figure S4. Infrared spectrum of DBS-COOH
Figure S5. Infrared spectrum of C16-DAPMA.

Figure S6. Infrared spectrum of heparin
Figure S7. Percentage of gelator immobilisation (representing gel formation) over time as monitored by NMR spectroscopy, for DBS-COOH (0.2% wt/vol) (blue circles); DBS-COOH (0.2% wt/vol) with heparin (300 µM) (red triangles) and DBS-COOH (0.2% wt/vol) in the presence of both heparin (300 µM) and C16-DAPMA (800 µM) (green squares).
5 CD Studies of Nanoscale Assembly

Figure S8. Kinetic studies of the growth of DBS-COOH fibrillar network over time, using CD spectroscopy. (a) DBS-COOH (0.02% wt/vol); (b) DBS-COOH (0.02% wt/vol) in the presence of heparin (38 µM) and (c) DBS-COOH (0.02% wt/vol) in the presence of C16-DAPMA (150 µM) and heparin (38 µM). In the presence of heparin (b), the spectrum reaches saturation at 260 nm – for this reason, kinetic studies used data extracted at 255 and 265 nm.

Figure S9. HT Data from CD spectroscopy extracted at 255 nm over time, indicative of no change as the CD band at 255 nm associated with DBS-COOH assembly grows in over time.
Figure S10. CD intensity at 255 nm plotted against time for DBS-COOH (0.02% wt/vol) (blue circles); DBS-COOH (0.02% wt/vol) with heparin (38 µM) (red triangles) and DBS-COOH (0.02% wt/vol) in the presence of both heparin (38 µM) and C16-DAPMA (150 µM) (green diamonds).

Figure S11. CD intensity at 265 nm plotted against time for DBS-COOH (0.02% wt/vol) (blue circles); DBS-COOH (0.02% wt/vol) with heparin (38 µM) (red triangles) and DBS-COOH (0.02% wt/vol) in the presence of both heparin (38 µM) and C16-DAPMA (150 µM) (green diamonds).
6 Additional Electron Microscopy Images

Figure S12. TEM image of DBS-COOH (0.2% wt/vol). Scale bar = 200 nm.

Figure S13. TEM image of DBS-COOH (0.2% wt/vol) in the presence of heparin (38 mM).

Scale bar = 100 nm.
Figure S14. SEM image of agarose (0.5% w/v). Scale bar = 1 µm.

Figure S15. SEM image of DBS-COOH gel (0.2% w/v) in the presence of both heparin (38 µM) and C16-DAPMA (150 µM). Scale bar = 1 µm.
Figure S16. TEM image of DBS-COOH gel (0.2% w/v) in the presence of both heparin (38 µM) and C16-DAPMA (150 µM). Scale bar = 200 nm.
7 Rheology

Figure S17. Strain amplitude dependence of the storage modulus ($G'$, red) and loss modulus ($G''$, blue) for DBS-COOH gel (0.2% wt/vol) (○), DBS-COOH gel (0.2% wt/vol) with heparin (1 mM) (△) and DBS-COOH gel (0.2% wt/vol) with both heparin (1 mM) and C16-DAPMA (2 mM) (□). Frequency = 1 Hz.

Figure S18. Frequency dependence of the storage modulus ($G'$, red) and loss modulus ($G''$, blue) for DBS-COOH gel (0.2% wt/vol) (○), DBS-COOH gel (0.2% wt/vol) with heparin (1 mM) (△) and DBS-COOH gel (0.2% wt/vol) with heparin (1 mM) and C16-DAPMA (2 mM) (□). Amplitude strain = 0.1%.
Figure S19. Strain amplitude dependence of the storage modulus ($G'$, red) and loss modulus ($G''$, blue) for DBS-COOH gel (2% wt/vol) (○).

Figure S20. Frequency dependence of the storage modulus ($G'$, red) and loss modulus ($G''$, blue) for DBS-COOH gel (2% wt/vol) (○).
Figure S21. Strain amplitude dependence of the storage modulus (G’, red) and loss modulus (G’’, blue) for DBS-COOH gel (0.2% wt/vol) (○), DBS-COOH gel (0.2% wt/vol) with agarose (1% wt/vol) (◇) and DBS-COOH gel (0.2% wt/vol) with both agarose (1% wt/vol) and heparin (1 mM) (△). Frequency = 1 Hz.

Figure S22. Frequency dependence of the storage modulus (G’, red) and loss modulus (G’’, blue) for DBS-COOH gel (0.2% wt/vol) (○), DBS-COOH gel (0.2% wt/vol) with agarose (1% wt/vol) (◇) and DBS-COOH gel (0.2% wt/vol) with agarose (1% wt/vol) and heparin (1 mM) (△). Amplitude strain = 0.1%
Figure S23. Strain amplitude dependence of the storage modulus (G’, red) and loss modulus (G”, blue) for agarose gel (1% wt/vol) (○).

Figure S24. Frequency dependence of the storage modulus (G’, red) and loss modulus (G”, blue) for agarose gel (1% wt/vol) (○).
Heparin Release Experiments

Figure S25. DBS-COOH hydrogel (0.2% wt/vol) containing 1 mM of heparin with buffer on top for monitoring the release of heparin.

Figure S26. Heparin release from DBS-COOH hydrogel (0.2% wt/vol) with heparin (1 mM) (▲) and DBS-COOH hydrogel (0.2% wt/vol) with heparin (1 mM) interacting with C16-DAPMA (2 mM) (■).
Figure S27. Heparin release from 2% wt/vol (●), 5% wt/vol (●) and 10% wt/vol (○) DBS-COOH hydrogels containing heparin (1 mM).

Figure S28. Heparin release from DBS-COOH hydrogel (2% wt/vol) with heparin (1 mM) in the absence of agarose (▲), with 0.5% wt/vol of agarose (○) and 1% wt/vol of agarose (■).
Figure S29. Assay design for heparin release from hybrid gel cylinders containing DBS-COOH (2.0% wt/vol), agarose (1.0% wt/vol) and heparin (17 mM). 1 mL gels were prepared in small sample vials. The gel cylinders were then transferred to large glass jars and 35 mL buffer was added. 65 μL of buffer was removed in aliquots at timepoints, and added to 1935 μL MalB and a UV-Visible spectrum recorded.

9 References

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