Electronic Supplementary Material (ESI) for ChemComm.

Supplementary Information for

Cell-free protein synthesis in hydrogel materials

Materials and Methods

Materials. All materials were purchased from Sigma Aldrich (unless stated otherwise) except; oligos ordered from Eurofins, synthetic gene fragments from Integrated DNA Technologies (IDT) and the EcoFlex kit[1] from Addgene.

Molecular Cloning. The pTU1-A-RFP backbone from the EcoFlex kit was amplified to remove the RFP coding region using 0.5 μM of primers (ESI Table S4), 0.2 mM dNTPs, 5 ng plasmid, 1 × Q5 reaction buffer (New England Biolabs (NEB)) and 1 U of Q5 DNA polymerase in a 50 μL total volume. The amplification solution was heated to 98 °C for 30 s, 98 °C for 10 s and 72 °C for 70 s for 30 cycles before cooling to 4 °C. Purification was performed using a QIAquick PCR purification kit (QIAGEN).

EcoFlex[1] cloning followed the following procedure: 10 ng of PCR fragment from pTU1-A-RFP was combined with 10 ng of pBP-J23100 (promoter), pBP-pET-RBS (ribosomal binding site), pBP_mCherry (gene) and pBP-Bba_B0015 (terminator) with 2 mg mL^{-1} bovine serum albumin, 10 U BsaI, 1 × T4 DNA ligase buffer and 3 U T4 DNA ligase. The combined solution was made up to 15 μL with H_2O and cycled 15 × between 37 °C for 5 min and 16 °C for 10 min before heat inactivation at 50 °C for 10 min and 80 °C for 10 min. The reaction mix was stored at 4 °C until the transformation step.

The EcoFlex reaction product was transformed into One Shot TOP10 chemically competent *E. coli* cells (ThermoFisher) following the manufacturer's protocol. 250 μL transformation solution was plated onto 100 μgmL^{-1} ampicillin selection LB agar plates and incubated at 37 °C overnight. A single colony was picked, grown overnight in 5 mL LB and 100 μg mL^{-1} ampicillin and DNA were purified using a QIAprep Spin Miniprep Kit (QIAGEN). Sequencing was performed (Eurofins, Germany) using the primers in ESI Table S4. Following sequence verification, plasmid purification was performed using a QIAGEN Plasmid Maxi Kit following manufacturers protocol.

Hydrogel preparation. Hydrogels were prepared following one of three methods (Scheme 1). For Method (a), hydrogels were prepared fresh with CF components. For Method (b), hydrogels were prepared in H_2O before freeze drying - volumes may be different to the final required reaction volume (see ESI Table S1 reconstitution % for details). For Method (c), CF components were initially prepared on ice, before freeze drying. To freeze dry hydrogels or reagents, samples were placed at -80 °C for over 30 min then transferred directly to the freeze dryer. Detailed descriptions of hydrogel preparations are provided in ESI Table S5. The water content and percent swelling of each hydrogel is reported in ESI Table S1.

Cell-free protein synthesis. Cell-free protein synthesis reaction composition followed the methods described in 'Hydrogel preparation' and by adding the CF components; 10 μL CF extract (final concentration of 8.9 mg/mL protein prepared following (48)), 25 μL 2 × energy buffer (final

concentration 4.5 mM-10.5 mM Mg-glutamate, 40-160 mM K-glutamate, 0.33-3.33 mM DTT, 1.5 mM each amino acid except leucine, 1.25 mM leucine, 50 mM HEPES, 1.5 mM ATP and GTP, 0.9 mM CTP and UTP, 0.2 mg/mL tRNA, 0.26 mM CoA, 0.33 mM NAD, 0.75 mM cAMP, 0.068 mM folinic acid, 1 mM spermidine, 30 mM 3-PGA, 2 % PEG-8000 (unless stated otherwise), 4 µg plasmid DNA assembled to 50 µL final volume using nanopure H_2O. CF components and hydrogels were combined at room temperature. mCherry fluorescence was measured at 587 nm excitation and 610 nm emission using a Varioskan LUX multimode reader (ThermoFisher). All reactions were performed at 37 °C.

Confocal microscopy. A Zeiss LSM800 Airyscan/Spinning Disk with a DIC (differential interference contrast) was used for confocal microscopy with a 5 x magnification lens. For mCherry analysis a 561 nm laser was utilised, and fluorescence was detected between 610 and 700 nm.

Material Characterisation.

Fluorescein diffusion. Fluorescein was used to assess relative diffusion between a range of agarose % $^{w}/_{v}$. 10 µL of the liquid hydrogels was placed on a glass slide and once set, 0.2 µL 50 µM fluorescein in 1 × PBS (phosphate-buffered saline) was placed at the centre of the gel. Fluorescein diffusion was monitored using a Leica DM6-B Microscope (Leica, U.K.) with a DFC9000GT camera and a Platinum Bright 495 filter and exposed for 1 s per image (n = 3). The fluorescein intensity was measured at the centre of the original fluorescein drop site using ImageJ.[2] Diffusion (D) was calculated following equation (1).

$$D = \frac{I_{5min} - I_{10min}}{5} \quad (1)$$

Rheology. Rheological tests were carried out on a HR-2 Discovery Hybrid Rheometer from TA Instruments equipped with a temperature controller. Rheological experiments were performed in 20 mm parallel-plate geometry using 500 µL of hydrogels (resulting in a gap size of 1.5 mm). The strain and the frequency were set to 1 % and 1 Hz, respectively. For rheological testing of gels expressing proteins, hydrogels were prepared to 500 µL (10 × volumes) in a Petri dish.

Water content. 200 µL of material was prepared to the desired $^{w}/_{v}$ ratio, followed by weighing the material, freeze drying and then re-weighing the dried matrix. The water content of each hydrogel was determined by calculating the % difference between the fresh and freeze-dried material. Each material at each $^{w}/_{v}$ ratio was performed in triplicate.

Swelling capacity. 500 µL of each material was prepared in a Petri dish, allowed to set and weighed to gain initial weights (m_i). The gels were then submerged in H_2O for 2 h, followed by

removing the water and reweighting to determine the swollen weight of the hydrogel (m_s). The swelling ratio (S) was determined following equation (2). Each hydrogel $^{w}/_{v}$ was analysed in triplicate.

$$S = \frac{m_s - m_i}{m_i} x100 \qquad (2)$$

Hydrogel reconstitution

The level of reconstitution that each gel can undergo was determined by; preparing 200 µL of each gel in a 1.5 mL microcentrifuge tube and weighing the combined weight of gel and tube (m_i), lyophilising the gel overnight and reweighing (m_l) followed by the addition of 200 µL H_2O. After 30 min, any unabsorbed H_2O was removed followed by reweighing. The % reconstitution (R) is determined following equation (3). Each hydrogel $^{w}/_{v}$ was analysed in triplicate.

$$R = \frac{m_r - m_l}{m_i - m_l} x100 \qquad (3)$$

Supplementary Figures

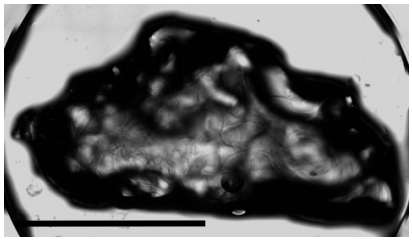

Fig. S1 Confocal microscopy of 0.75 % agarose prepared with cell-free components after 4 h of incubation at 37 °C in the absence of template DNA. Scale bar is 10 mm.

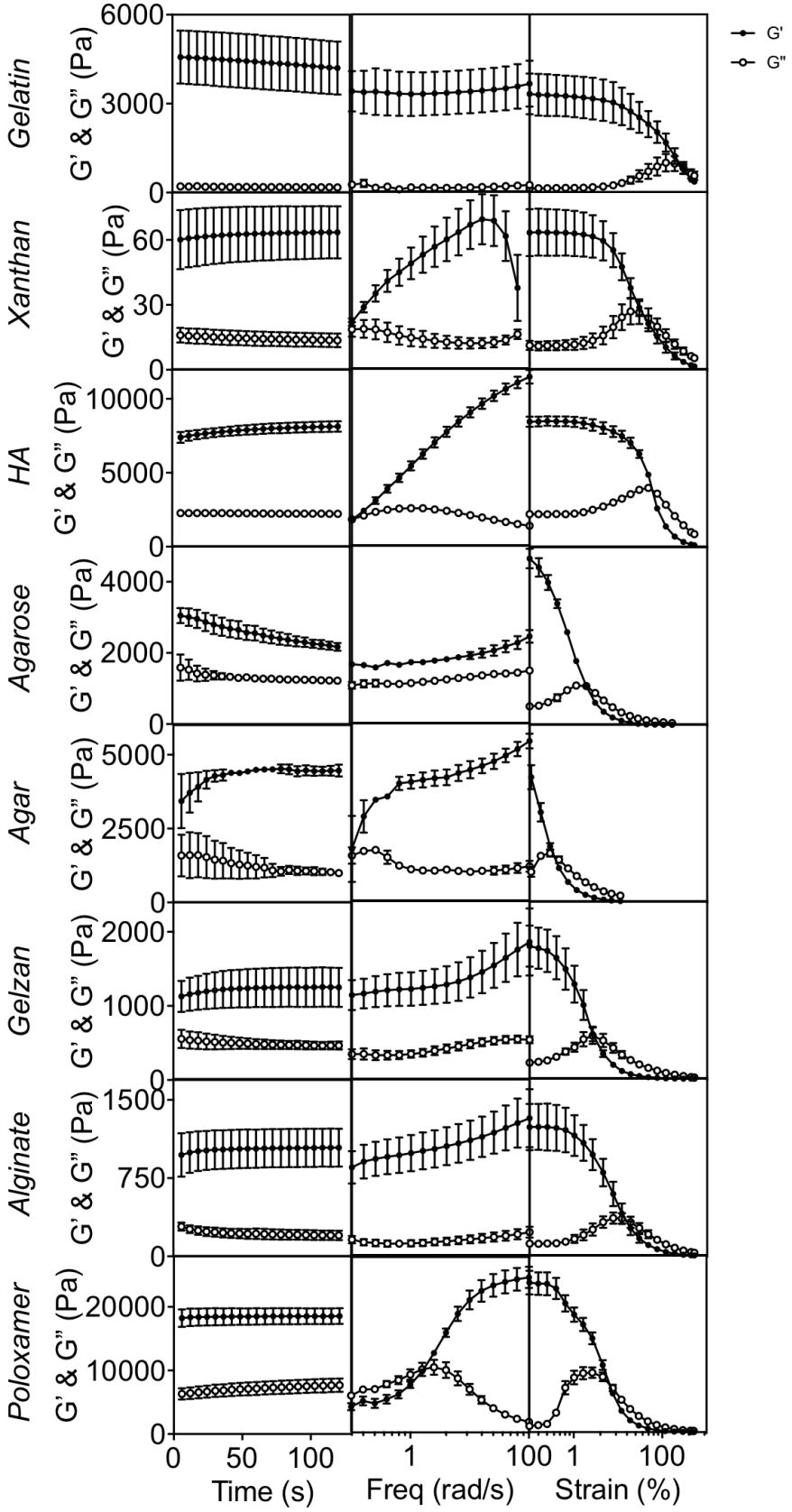

Fig. S2 Rheological analysis of each hydrogel monitoring the storage modulus (G') and the loss modulus (G") over time (left), over a frequency sweep (central) and a strain sweep (right) (*n* = 3, error bars are SE mean).

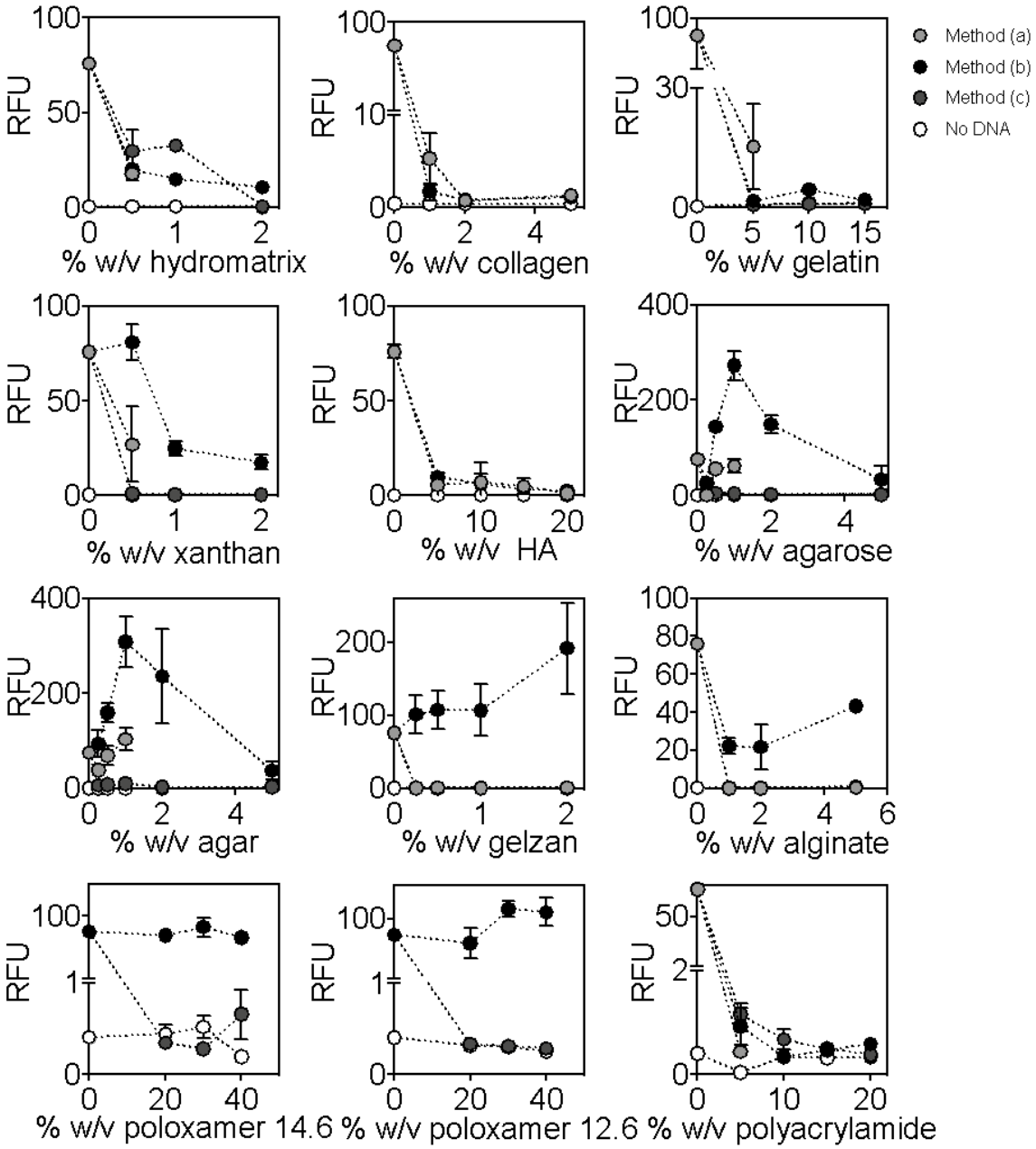

Fig. S3 Cell-free protein synthesis (CFPS) of mCherry in hydrogels prepared either fresh (Method (a)), from freeze-dried hydrogels (Method (b)) or with freeze-dried cell-free reagents (Method (c)) (*n* = 3, error bars are standard error (SE) mean). RFU = relative fluorescence units. The maximum average RFU over a 16 h incubation is plotted.

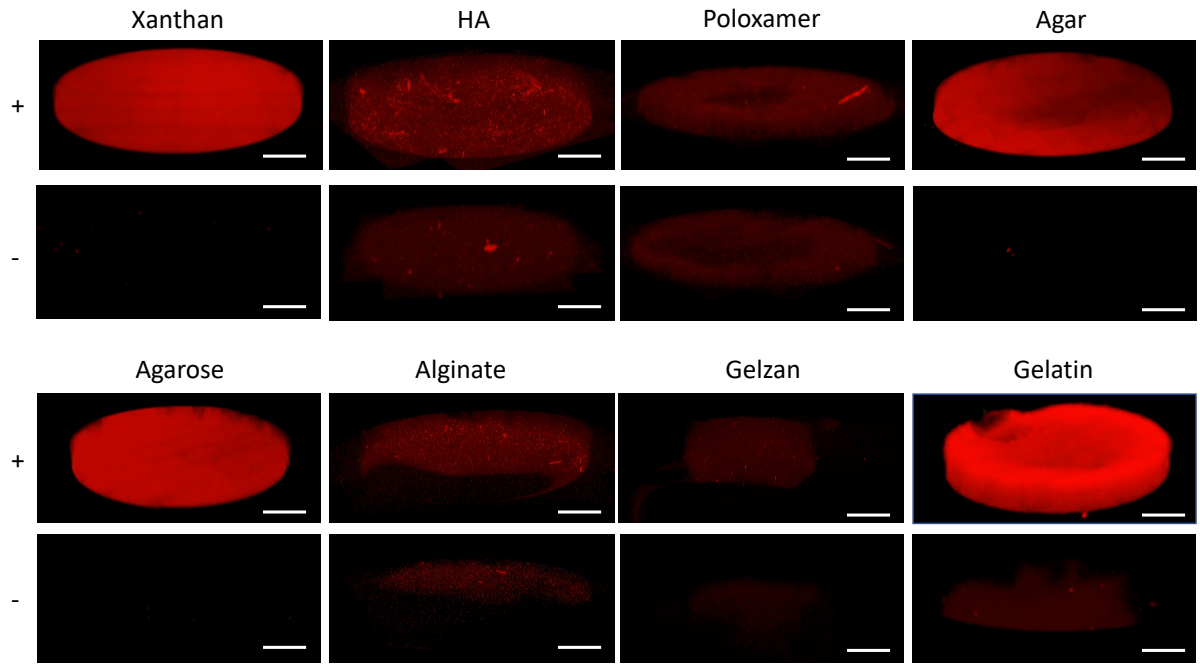

Fig. S4 Confocal microscopy of hydrogels prepared with cell-free reagents in the presence of (top row) and absence of (bottom row) the mCherry template after 4 h of incubation at 37 °C.

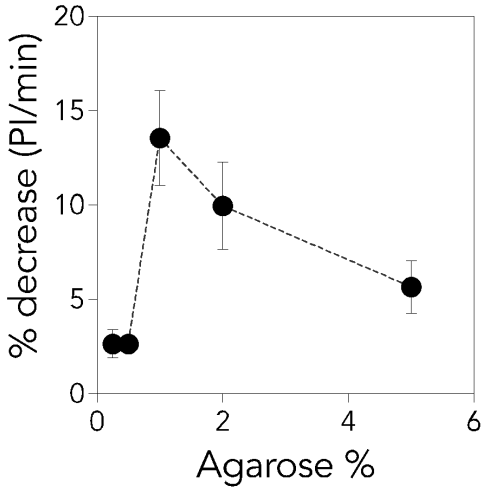

Fig. S5 The % decrease of fluorescein fluorescence intensity over a 5 minute interval to represent diffusion through a range of agarose w/v ratios (*n* = 3, error bars are SE mean). The greater the percentage decrease the faster the signal diffuses through the material.

Supplementary Tables

Table S1 Water content, swelling, diffusion and photos of the hydrogels. Data is described as the mean ± SE mean (*n* = 3) to 3 s.f. All values are a percentage.

Type	Material	% w/v	Water content	Swelling	Reconstitution	Photo
Unstructured	Hyaluronic acid	5.0	95.0 ± 1.00	297 ± 9.07	100 ± 0.00	
		10	91.0 ± 1.53	336 ± 8.74	100 ± 0.00	
		15	86.5 ± 0.296	440 ± 33.2	100 ± 0.00	
		20	84.0 ± 0.371	423 ± 7.22	100 ± 0.00	
	Collagen	1.0	-	-		
		2.0	96.9 ± 0.917	-	81.8 ± 26.8	
		5.0	94.3 ± 2.17	-	120.9 ± 17.9	
	HydroMatrix™	0.5	99.7 ± 0.176	-	100 ± 0.00	
		1.0	99.3 ± 0.546	-	100 ± 0.00	
		2.0	99.0 ± 0.000	-	100 ± 0.00	
	Alginate	1.0	95.4 ± 0.694	-17.2 ± 2.84	36.4 ± 2.24	
		2.0	94.1 ± 0.481	-13.9 ± 2.57	28.6 ± 5.02	
		5.0	91.7 ± 0.865	-13.5 ± 2.56	28.2 ± 2.32	
	Agarose	0.25	100 ± 0.00	0.900 ± 2.10	26.1 ± 0.484	

	0.50	99.3 ± 0.333	8.43 ± 0.0670	33.7 ± 1.52	
	1.0	99.0 ± 0.524	13.3 ± 1.20	40.0 ± 2.96	
	2.0	97.9 ± 0.0333	13.0 ± 3.63	49.3 ± 0.593	
	5.0	95.2 ± 0.153	8.83 ± 0.524	41.8 ± 5.21	
Agar	0.25	99.8 ± 0.418	12.7 ± 3.05	24.1 ± 3.18	
	0.50	99.8 ± 0.689	10.2 ± 1.17	36.1 ± 2.63	
	1.0	99.6 ± 0.219	14.5 ± 2.44	40.7 ± 3.07	
	2.0	99.5 ± 0.669	15.2 ± 1.53	50.1 ± 5.09	
	5.0	94.7 ± 1.30	14.0 ± 2.04	61.0 ± 8.68	
Xanthan Gum	0.5	99.7 ± 0.133	-	100 ± 0.00	
	1.0	99.2 ± 0.252	-	100 ± 0.00	
	2.0	98.2 ± 0.285	-	100 ± 0.00	
Gelatin	2.0	99.3 ± 0.200	18.6 ± 0.841	100 ± 0.00	
	5.0	96.4 ± 0.353	26.5 ± 1.05	39.5 ± 5.83	
	10	92.6 ± 0.351	35.4 ± 1.16	57.8 ± 12.1	

	Gelzan™	0.25	98.9 ± 0.578	-14.9 ± 6.46	50.4 ± 5.89	
		0.50	98.9 ± 0.260	-3.60 ± 1.77	47.8 ± 4.20	
		1.0	97.8 ± 0.498	-2.50 ± 0.503	65.4 ± 8.48	
		2.0	96.6 ± 1.38	2.10 ± 1.80	100 ± 0.00	
Micelle	Pluronic acid F108	20	84.2 ± 0.722	-	29.2 ± 1.87	
		30	75.8 ± 0.260	-	42.2 ± 4.69	
		40	71.1 ± 0.252	-	45.5 ± 0.00	
	Pluronic acid F127	20	81. 9 ± 0.0667	-	35.9 ± 2.00	
		30	77.2 ± 0.153	-	42.1 ± 0.612	
		40	72.4 ± 0.493	-	47.6 ± 2.42	
Structured	Polyacrylamide	5.0	97.1 ± 0.133	142 ± 10.5	30.4 ± 3.76	
		10	91.1 ± 0.152	134 ± 4.58	48.7 ± 5.04	
		15	84.7 ± 0.458	144 ± 10.0	57.0 ± 2.73	
		20	79.3 ± 0.145	139 ± 8.84	67.1 ± 0.524	

Table S2 Rheological characterisation of hydrogels. Data is described as the mean ± SE mean (n = 3) to 3 s.f.

Hydrogel	G' (Pa)	G" (Pa)	Critical Strain (%)
Agar	4460 ± 170	1040 ± 107	0.365 ± 0.0150
Agarose	2250 ± 175	1240 ± 36.4	2.00 ± 0.0432
Gelzan™	1250 ± 374	467 ± 75.2	3.01 ± 0.340
Poloxamer	18500 ± 1780	7480 ± 1480	6.72 ± 0.0377
Xanthan	63.4 ± 17.2	13.8 ± 4.48	39.6 ± 6.43
Alginate	1040 ± 259	203 ± 64.9	14.8 ± 2.81
Hyaluronic acid	8050 ± 484	2240 ± 18.0	60.5 ± 1.79
Gelatin	4250 ± 1270	179 ± 15.5	291 ± 64.5

Table S3 Summary of the hydrogels trialled in this study.

Material class	Material	Method (a) Compatibility	Method (b) compatible	Method (c) compatible
Peptide	collagen	B	B	
	gelatin	B	B	-
	HydroMatrix™	B	B	B
Polysaccharide	alginate	-	B	
	agar	A	A	B
	Xanthan gum	B	A	-
	Gelzan™	-	A	
	hyaluronic acid	B	-	
	agarose	A	A	B
Micelle	poloxamer		A	-
Covalent	polyacrylamide	-	A	-

A = Cell-free protein synthesis performs better than the aqueous system (hydrogel > aqueous)

B = Cell-free protein synthesis detected but at less than the aqueous system (90 % < aqueous)

- = No cell-free protein synthesis detected.

Table S4 Oligonucleotides used in this study

Amplification region	Forward primer (5'-3')	Reverse primer (5'-3')
pTU1-A-	TAGAGTCACACTGGCTCACC TTCGG	GCCAGCTGCATTAATGAATC GGCCAA
Sequencing GOI	GGCGTATCACGAGGCAGAAT TTCAGATA	TTTGAGTGAGCTGATACCGC TCGC

Table S5 Hydrogel preparation methods

Hydrogel	Method (a)	Method (b)	Method (c)
Hyaluronic acid	The powder was weighed directly into the 384-well plate or mould. Fresh CFPS components were pipetted into the well, mixed briefly and allowed to equilibrate with the powder for 30 min at room temperature.	The powder was weighed into 1.5 mL microcentrifuge tubes and made up to the required volume with H_2O. The gel was allowed to equilibrate at room temperature for 1 h before weighing either 50 mg of the gel directly into the 384-well plate or 500 mg into the Petri dish.	N/a
Collagen	For CFPS reactions, collagen was measured directly into the 384-well plate. CF solution was added directed to the solid and allowed to equilibrate for 30 min at room temperature. For characterisation, the material was weighed directly into a Petri dish and made by the slow addition of solution and mixing using a spatula.	For CFPS reactions, collagen was measured directly into the 384-well plate. H_2O was added directed to the solid and allowed to equilibrate for 30 min at room temperature before freeze drying.	N/a
Agarose	The powder was weighed into 1.5 mL microcentrifuge tubes and made up to 1 mL with H_2O at 4 x the final w/v ratio. The tubes were heated to 95 °C for 10 min before cooling to 60 °C to pipette directly with the CF components. Gels were allowed to set for 30 min before analysis.	The powder was weighed into 1.5 mL microcentrifuge tubes and made up to 1 mL with H_2O. The tubes were heated to 95 °C for 10 min before cooling to 60 °C to pipette. Gels were allowed to set for 30 min before freeze drying.	The powder was weighed into 1.5 mL microcentrifuge tubes and made up to 1 mL with H_2O. The tubes were heated to 95 °C for 10 min before cooling to 60 °C to pipette directly onto the freeze-dried CF components. Gels were allowed to set for 30 min before analysis.

Agar and Gelatin	The powder was weighed directly into a glass conical flask, followed by the addition of water at 4 x the final w/v ratio. The mixture was microwaved to dissolve and allowed to cool to 45 °C before pipetting directly with the CF components. Gels were allowed to set for 30 min before analysis.	The powder was weighed directly into a glass conical flask, followed by the addition of water. The mixture was microwaved to dissolve and allowed to cool to 45 °C before pipetting into the appropriate mould for freeze drying.	The powder was weighed directly into a glass conical flask, followed by the addition of water. The mixture was microwaved to dissolve and allowed to cool to 45 °C before pipetting directly onto the freeze-dried CF components. Gels were allowed to set for 30 min before analysis.
Gelzan	Gelzan was prepared by the addition of the required material powder to a conical flask followed by the addition of H_2O, heating to 80 °C and stirring to dissolve. Once dissolved, the solution was pipetted into the CF reaction mixture and the gel was allowed to form for 30 min before transferring to the appropriate vessel.	Gelzan was prepared by the addition of the required material powder to a conical flask followed by the addition of H_2O, heating to 80 °C and stirring to dissolve. Once dissolved, the solution was pipetted into 6 mM $MgSO_4$ (the concentration of $MgSO_4$ found in the CF reaction). The gel was allowed to form for 30 min before freeze drying.	N/a
Alginate	Alginate was prepared by weighing the powder into a conical flask and adding H_2O. The mixture was stirred until dissolved. CF components containing 50 mM $CaCl_2$ were added to a 1.5 mL microcentrifuge tube. Sodium alginate solution was added in a 1:1 $^v/_v$ ratio to the tube by pipetting directly into the $CaCl_2$. After 30 min, the	Alginate was prepared by weighing the powder into a conical flask and adding H_2O. The mixture was stirred until dissolved. 50 mM $CaCl_2$ was added to a 1.5 mL microcentrifuge tube followed by pipetting the sodium alginate solution directly in a 1:1 $^v/_v$ ratio to the tube. After 30 min, the gel was removed using a spatula and	N/a

	gel was removed using a spatula and transferred to either a 384-well plate or vessel for analysis.	transferred to either a 384-well plate or vessel for freeze drying.	
F-108 (mw ≈ 14600 g/mol) and F-127 (mw ≈ 12600 g/mol) pluronic acid	N/a	The polymer powder was weighed into a glass vial and the H_2O was added directly. Vials were cooled to 4 °C and stirred to aid dissolving. Solution was then pipetted to the appropriate vessel and allowed to heat to room temperature to form a gel before rapid freezing and lyophilisation.	The polymer powder was weighed into a glass vial and the H_2O was added directly. Vials were cooled to 4 °C and stirred to aid dissolving. The solution was then pipetted directly onto the freeze-dried CF components and allowed to gel at room temperature before analysis.
Polyacrylamide	Polyacrylamide gels were prepared following the ratios on ESI Table S6 with the addition of CF components. Gels were prepared in 2.0 mL microcentrifuge tubes and pipetted to the appropriate vessel immediately and then allowed to polymerise for at least 60 min at room temperature before analysis.	Polyacrylamide gels were prepared following the ratios on ESI Table S6. Gels were prepared in 2.0 mL microcentrifuge tubes and pipetted to the appropriate vessel immediately and then allowed to polymerise for at least 60 min. Gels were then transferred to dialysis tubing and dialysed for a minimum of 2 h in H_2O. The hydrogel was freeze-dried for future use.	Polyacrylamide gels were prepared following the ratios on ESI Table S6. Gels were prepared in 2.0 mL microcentrifuge tubes and pipetted immediately onto the lyophilised CF components and then allowed to polymerise for at least 60 min at room temperature before analysis.

Table S6 Polyacrylamide gel preparation

	Initial concentration	Vol (µL) 5 %	10 %	15 %	20 %	Final concentration
30 % acrylamide/ bis acrylamide	30	266.7	533.3	800.0	1065.6	5, 10, 15, 20
Tris, pH 7.5	10 mM	480				30 µM
TEMED	66.9 mM	32				1.34 mM
APS	10 %	8				0.05 %
H_2O	-	813.4	545.6	280.0	14.4	-
Total		1600				

References

1. S. J. Moore, H.-E. Lai, R. J. R. Kelwick, S. M. Chee, D. J. Bell, K. M. Polizzi, P. S. Freemont, *ACS Synth. Biol.*, 2016, **5**, 1059.

2. C. A. Schneider, W. S. Rasband, K. W. Eliceiri, *Nat Methods* 2012, **9**, 671.