Electronic Supplementary Material (ESI) for Biomaterials Science

Electronic Supporting Information for:

Bio-ink for on-demand printing of living cells

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S1. Custom-built inkjet printing system

Xaar-126 piezoelectric inkjet print heads were mounted in a custom frame, positioning them above a modified Reliance RCMS17 Cool Motion stage coupled to a Reliance Cool Muscle servo motor system (Fig. S1). The distance between the substrate and print heads was maintained at 1-2 mm. Motion of the stage was controlled by Cool Works Lite software. Communication with the print heads was facilitated through Xaar XUSB electronics and accompanying software. Stage movement was relayed to the print electronics through an optical strip (1 µm pattern) read by a Renishaw RGH22 read-head. An EFD Ultimus regulator was attached to the laboratory N_2 source to control the pressures applied to print heads.

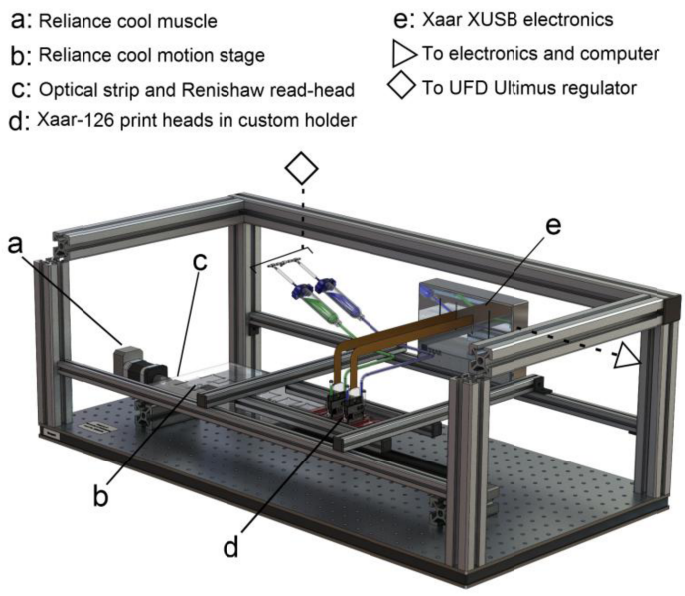

Fig. S1. Schematic of the custom-built inkjet printing system to house Xaar-126 print heads. The system was designed to fit into a standard bio-safety cabinet.

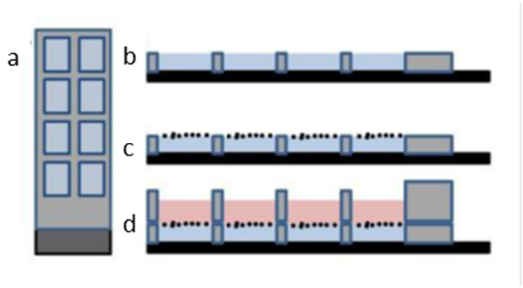

Fig. S2. Schematic of PDMS wells used in cell printing experiments. (a) Top view of PDMS wells adhered to glass slide. (b-d) Side-on view of PDMS adhered to glass slide and completely filled with collagen gel to create a flat surface (b). Cells printed onto the collagen surface (c). Wells created by adhesion of thicker replicate of PDMS on top of first layer and filled with cell culture media (d).

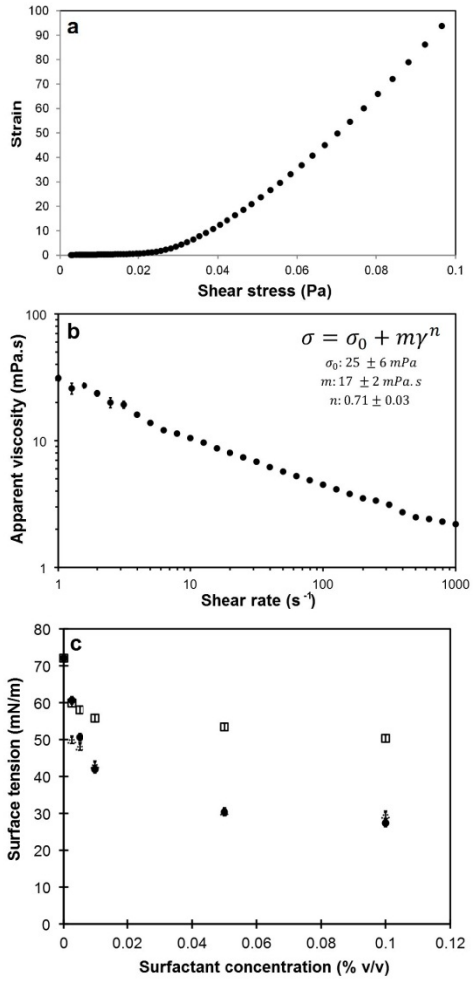

Fig. S3. Rheology and surface tension of bio-ink. (a) Typical result from continuous ramps of shear stress. Apparent yield stress was defined as the intercept between the two linear regions of the curve (b) Shear-dependent viscosity of the bio-ink measured by a stepped ramp of shear rate. Data are combined from numerous measurements (n=7) and the pseudo-plastic behaviour was modelled using the Herschel-Bulkley equation (inset), yielding values for yield stress (σ_0), consistency (m) and rate index (n). Error bars represent the standard error of the mean. (c) Surface tension of 0.05% w/v gellan gum microgel suspensions in DMEM, as a function of added surfactants. Surfactants were Poloxamer 188 (open squares) and the fluorosurfactant Novec FC4430 (closed circles). Solutions containing both surfactants are represented by dotted triangles, where the concentration of Poloxamer 188 was kept constant at 0.1% v/v and the Novec FC4430 concentration was varied. Error bars represent one standard deviation from the mean (n=4).

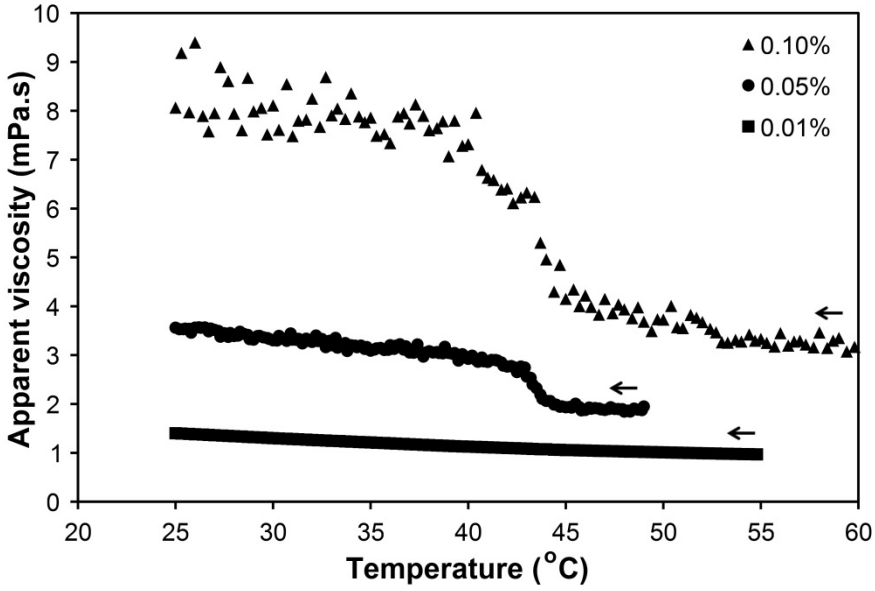

Fig. S4. Viscosity as a function of temperature for gellan gum in cell culture medium on cooling (arrows indicate sweep direction). Gellan gum concentrations were 0.01% (squares), 0.05% (circles) or 0.10% w/v (triangles). Samples were cooled at 1 °C/min with a constant applied shear of 500 s^{-1}.

S2. Cell viability.

Cell viability was assessed by fluorescent live/dead staining using Calcein AM and propidium iodide (both from Molecular Probes). Calcein AM was added at 5 µg/mL and incubated at 37°C for 15 mins in the dark, followed by addition of propidium iodide at 1

μg/mL. Cells were imaged immediately using fluorescence microscopy. Viability was assessed by manual counting live and dead cells using Image J software, or by automated counting with Image Pro software (MediaCybernetics). Comparison of manual and automated counts showed good agreement.

S3. Cell proliferation and differentiation.

Cell proliferation was assessed using the MTS colorimetric assay (Promega). Printed and control cells were seeded into 96 well plates at ~$5x10^3$ cells/well in 100 μL cell culture media. Enzymatic activity was measured after 2, 24 and 48 hrs by adding 20 μL MTS reagent (Promega) to each well, and incubating at 37°C for 90 mins before reading absorbance on a plate reader (SpectraMax 190, Molecular Devices) at 490 nm (formazan product) and 650 nm (background). Absorbance at 24 and 48 hr time points was normalized to the 2 hr time point in order to account for differences in initial cell numbers. Calibration curves ensured that cell numbers were within the linear range of the assay. Printed and control cells were seeded in 96-well plates at ~$1x10^4$ cells/well for differentiation experiments. For C2C12 cells, media was changed gradually to differentiation conditions: the serum content was reduced to 5% FBS after 24 hrs, 2% FBS after 48 hrs and finally to 2% horse serum (HS) after 72 hrs. For PC12 cells media was changed to differentiation conditions, DMEM with 1% HS and nerve growth factor (NGF, 50ng/mL, Invitrogen), after 24 hrs. Differentiation media was changed every 48 hrs. PC12 cells were fixed after 5 days in differentiation media, and C2C12 cells after 4 days in final differentiation media, with 3.7% paraformaldehyde for 10 mins at room temperature (RT, 21°C). For co-cultures of printed C2C12 and PC12 cells on collagen gels, cells were maintained in C2C12 proliferation media (DMEM with 10% FBS) for 24 hrs before being changed to PC12 differentiation media. Cells were fixed as previously described after 8 days in differentiation conditions.

S4. Immunostaining.

C2C12 cells were permeabilised with 50:50 methanol:acetone on ice for 5 mins, and washed with phosphate buffered saline (PBS, Sigma) before blocking solution in 10% donkey serum (DS, Chemicon) with 0.1% v/v Tween-20 (Sigma) for 1 hr at RT. Mouse monoclonal anti-desmin primary antibody (Novocastra) was diluted 1:100 in blocking solution and incubated at 4°C overnight. After two 10 min washes with PBS, cells were incubated for 1 hr at room

temperature in the dark with the secondary antibody Alexa-546 donkey anti-mouse (Invitrogen) diluted 1:1000 in blocking solution. After two further 10 min washes in PBS, DAPI was added at 1:1000 in PBS for 5 mins at room temperature, before replacing with PBS. For differentiated PC12 and C2C12 co-cultures on collagen, the above protocol was used with cells incubated in a mixture of primary antibodies in blocking solution. C2C12 cells were labelled with Cell Signalling rabbit monoclonal anti-desmin (GeneSearch) diluted 1:100, while PC12 cells were labelled with mouse anti-neuronal β-III tubulin (Covance) diluted 1:1000. Secondary antibodies were Alexa-488 donkey anti-rabbit (Invitrogen) and Alexa-546 donkey anti-mouse (Invitrogen). For differentiated PC12 cells on tissue culture polystyrene, cells were stained with a phalloidin dye to avoid lengthy immunostaining protocols that could have detached cells from the substrate. Fixed cells were permeabilised with 0.1% v/v Triton X-100 (Sigma) in PBS for 5 mins at room temperature. After washing twice with PBS, cells were incubated for 20 mins at RT in the dark in Alexa-488 phalloidin (Molecular Probes) at 165 nM in 1% bovine serum albumin in PBS. After two washes, cells were stained with DAPI as above and imaged.