Development of a Novel Hybprinter-SAM for Functionally Graded Tissue Engineering Constructs with Patterned and Localized Biochemical Signals

Jiannan Li[1†], Carolyn Kim[1, 3†], Hossein V. Alizadeh[1], Shreya Garg[1, 2], Arnaud Bruyas[1], Peng Zhao[1], Isadora S. D. Passos[1, 3], Chi-Chun Pan[1], Andrea S. Flores Pérez[1,2], Mark A. Skylar-Scott[2], Sungwoo Kim[1], Yunzhi P. Yang[1, 2, 4*]

[1]Department of Orthopaedic Surgery, School of Medicine, Stanford University, Stanford, CA 94305, USA

[2]Department of Bioengineering, School of Engineering, Stanford University, Stanford, CA 94305, USA

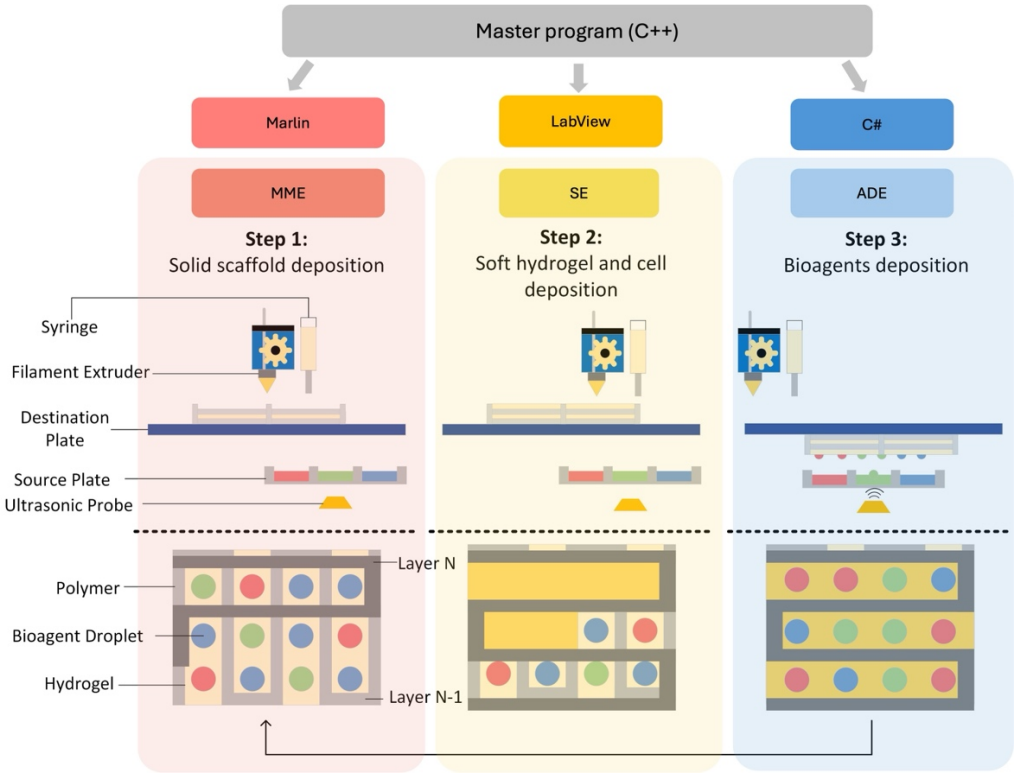
[3]Department of Mechanical Engineering, School of Engineering, Stanford University, Stanford, CA 94305, USA

[4]Department of Material Science and Engineering, School of Engineering, Stanford University, Stanford, CA 94305, USA

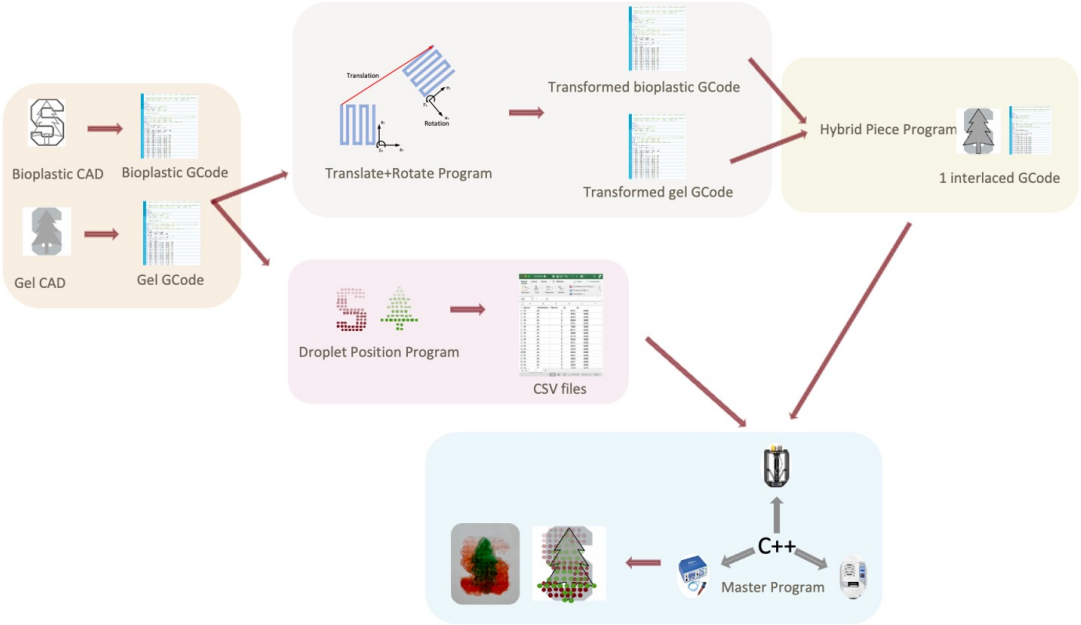
[†]These authors contributed equally to this work.

[*]Corresponding author. Email: ypyang@stanford.edu.

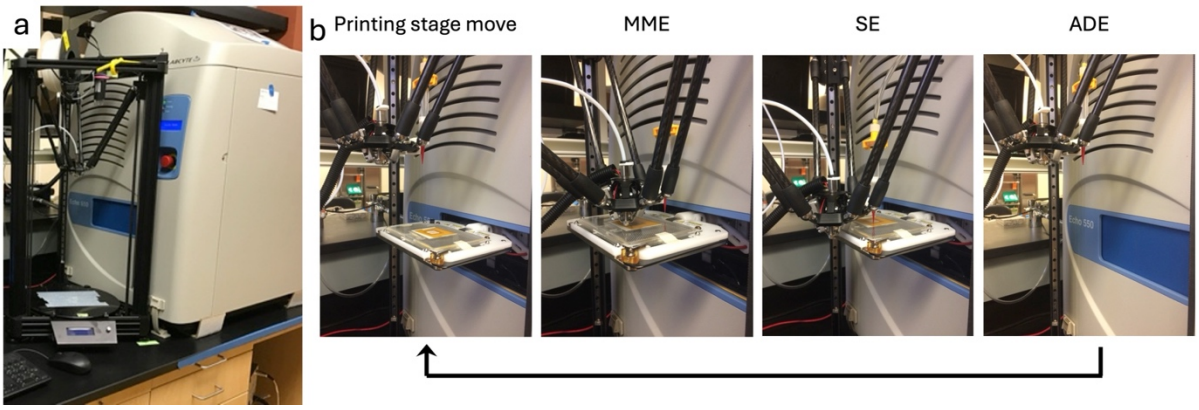
Supplementary Information



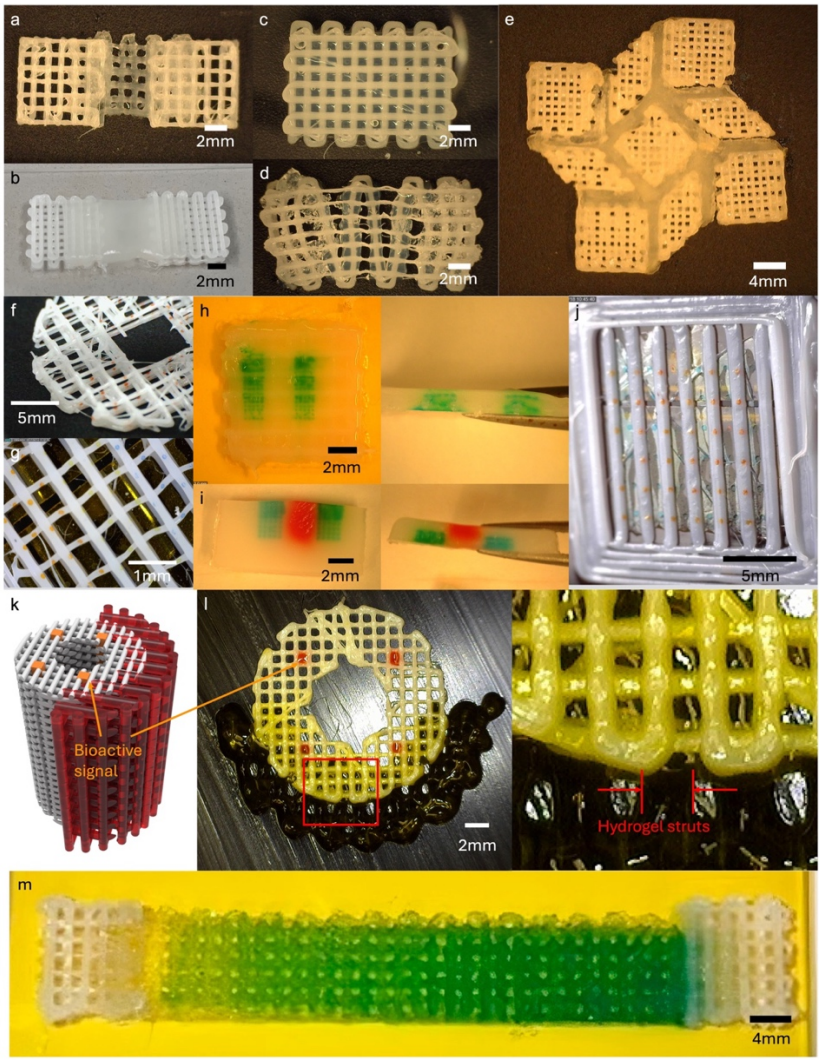
Supplemental Figure S1. Working principle of hybrid printing comprising molten material extrusion (MME), syringe extrusion (SE) and acoustic droplet ejection (ADE). All three modules are coordinated by custom developed software program, and they follow a layer-by-layer fabrication protocol in the order of MME→SE→ADE, resulting in an engineered construct comprising rigid polymeric scaffold, soft hydrogel scaffold, and aqueous-phase bioreagents.



Supplemental Figure S2. Process flow of hybprinting design and fabrication including CAD design, slicing, coordinate system transformation, droplet pattern generation, and final master programming integration.



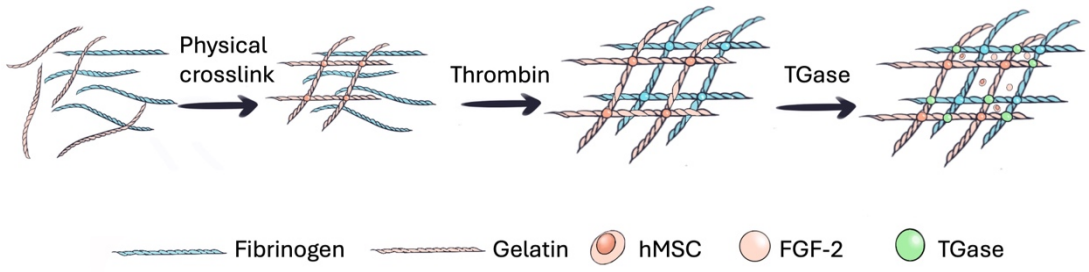
Supplemental Figure S3. (a) Assembly of Hybprinter-SAM. (b) Actual pictures showing process flow for Hybprinter-SAM, following a layer-by-layer fabrication protocol in the order of MME→SE→ADE.



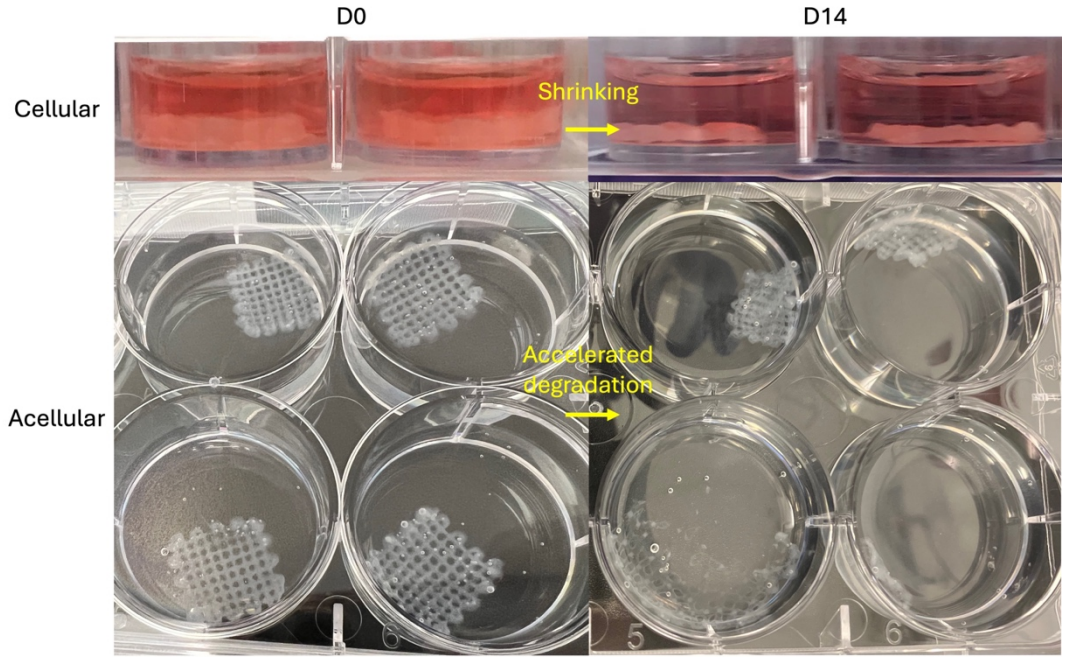
Supplemental Figure S4. (a-e) Hybrid soft-rigid tissue constructs of hydrogel and PCL that can withstand robust mechanical manipulation. (f, g) ADE deposition of droplets onto MME printed PCL struts. (h, i) Hydrogels with biological signal patterns. (j-l) Soft-rigid hybrid constructs with multiple biological factors (demonstrated by dyes) with different patterns, enabled by Hybprinter-SAM. (m) Printed sample of the soft-rigid construct with biological gradient, represented by color.

Materials/Module	Printing parameters	Values
PCL/MME	Printing speed	5 mm/s
	Printing temperature	120 °C
	Extrusion rate	100%-400%
	Layer height	0.2 mm
	Strut size	150-400 μm
Gelbrin/SE	Printing speed	2 mm/s
	Printing temperature	20 °C
	Extrusion pressure	10-20 psi
	Layer height	0.2 mm
	Strut size	300-800 μm
Reagent/ADE	Droplet size	2.5 nL
	Spot size	85-280 μm

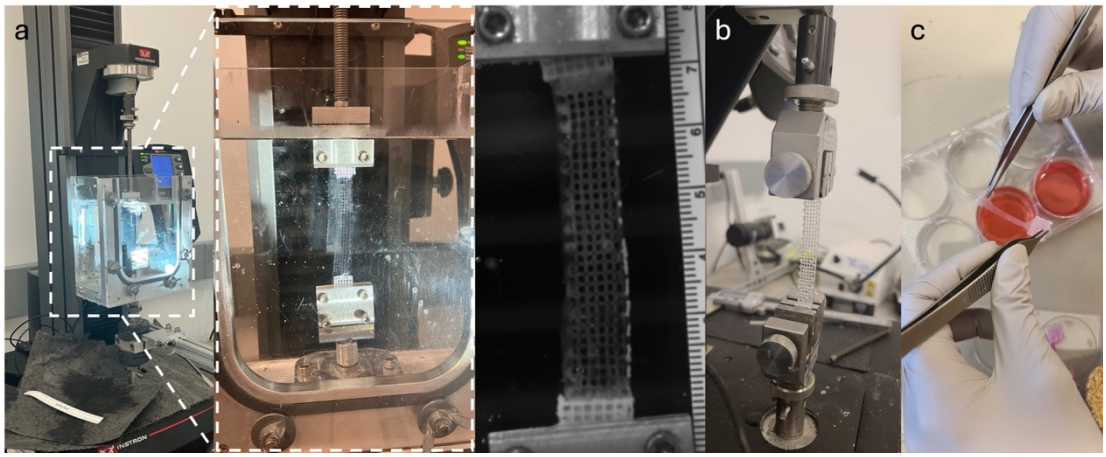
Supplemental Table S5. Important printing parameters for Hybprinter-SAM. Note that extrusion rate for PCL was a parameter set for controlling the actual strut size printed with MME.



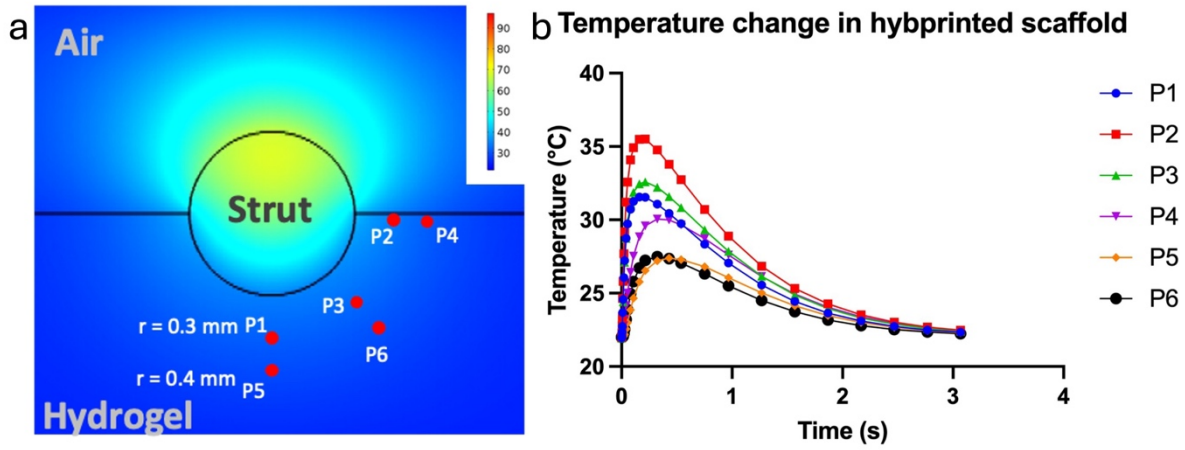
Supplemental Figure S6. Crosslinking mechanism of gelbrin hydrogel network: Gelatin first underwent temperature dependent physical crosslinking before printing; upon printing, thrombin first crosslinked fibrinogen into fibrin, followed by Ca^{2+}-dependent TGase crosslinking of gelatin and fibrin.



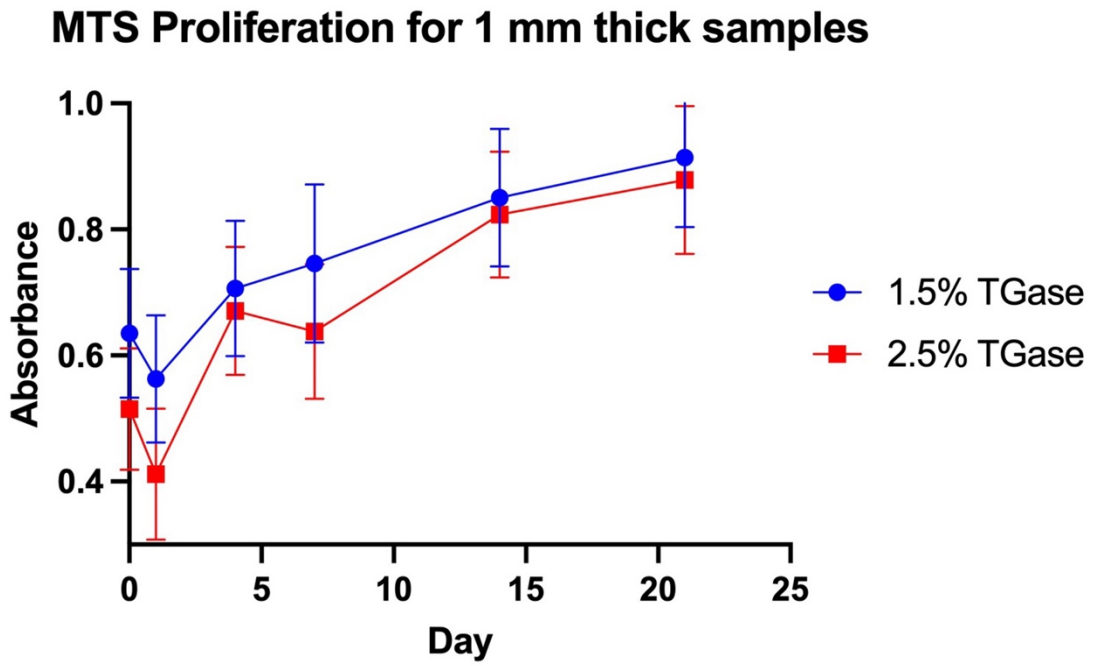
Supplemental Figure S7. Difference during 14-day culturing between cellular and acellular scaffolds, where cellular scaffolds shrank yet maintained its structural integrity, and acellular scaffolds almost fully degraded and dissembled.



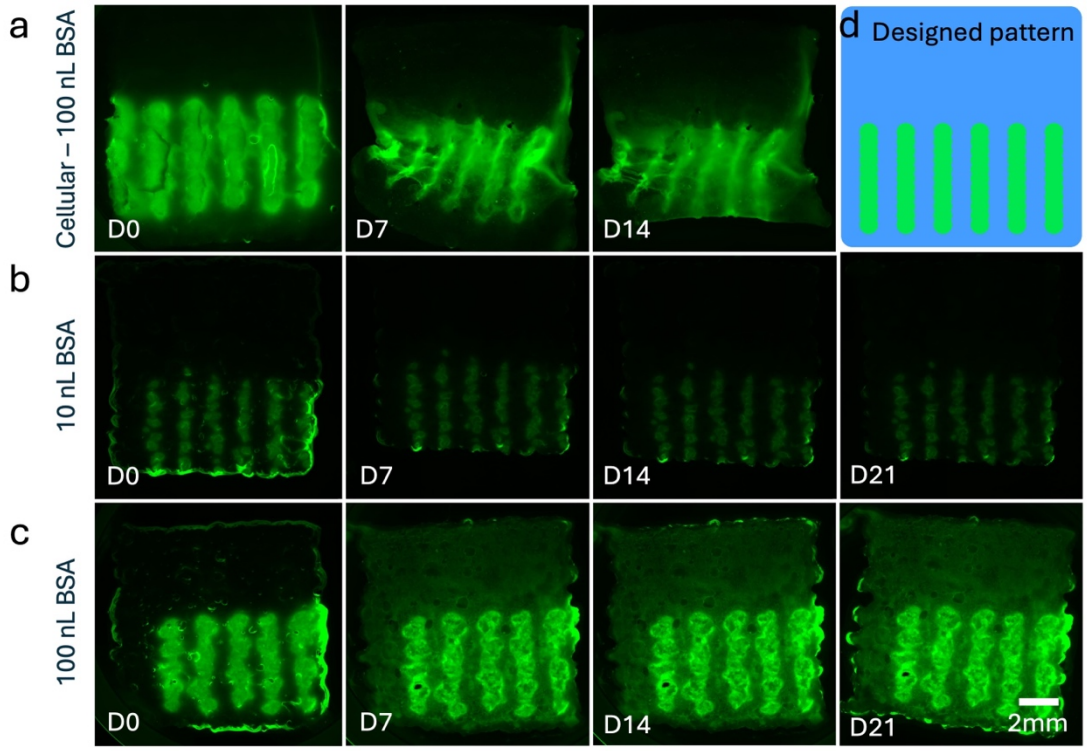
Supplemental Figure S8. Mechanical measurement setup for the hybprinted scaffold. (a) Measurement setup in water bath. (b) Measurement setup in air. (c) Gel deformation in air affecting measurement.



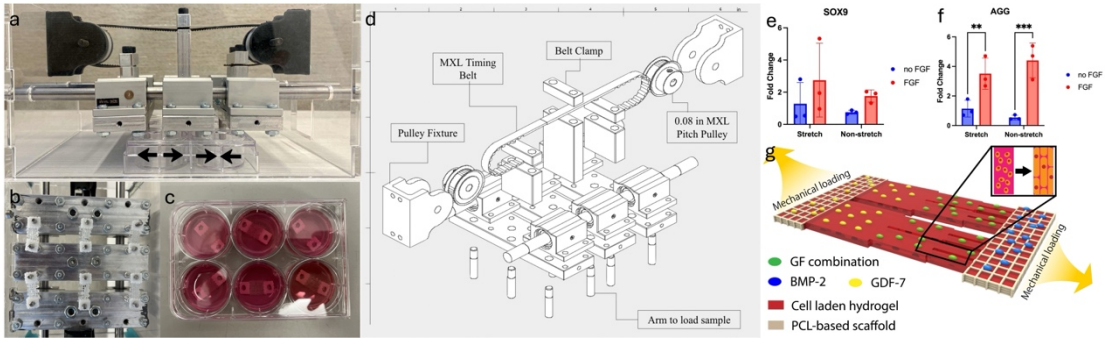
Supplemental Figure S9. COMSOL simulation of temperature change in the hybprinted scaffold. (a) Simulation design. (b) Simulation results for probes at different locations.



Supplemental Figure S10. MTS proliferation study for 1 mm thick samples.



Supplemental Figure S11. (a-c) Fluorescence retention of patterned FITC-BSA onto SE printed gelbrin scaffold. (a) Cellular condition, 100 nL BSA droplet size. (b) Acellular condition, 10 nL BSA droplet size. (c) Acellular condition, 100 nL BSA droplet size. (d) Conceptual design of the pattern.



Supplemental Figure S12. (a) Side view of the bioreactor compatible with a 6-well plate. (b-c) Samples in a 6-well plate loaded onto the bioreactor. (d) An exploded schematic of the bioreactor's hardware and working mechanism. (e-f) qPCR expression of (e) SOX9 and (f) AGG, (e) COL1 and (f) AGG for comparison of mechanically stretched and non-stretched groups at D7. (g) Design for high-throughput screening comprising mechanical stimuli, soft-rigid material integration, combinatorial growth factors loading to study bone-tendon regeneration, where one may deposit BMP-2 for bone development, GDF-7 for tendon development, and screen different growth factors combinations for fibrocartilage development.

Mechanical Stimuli Type	Physiological Strain/Rate	Relevant Frequency
Static Tension	5%	NA
Slow Tension	0.1 mm/day	NA
Cyclic Tension	11%	0.5 - 1 Hz
Cyclic Tension-Compression	11%	0.5 - 1 Hz

Supplemental Table S13. Mechanical stimulus types and rates that can be provided by the custom designed bioreactor.

Gene	FW	RV
ACTB	CACCATTGGCAATGAGCGGTTC	AGGTCTTTGCGGATGTCCACGT
SOX9	GACTTCCGCGACGTGGAC	GTTGGGCGGCAGGTACTG
SCX	CCCAAACAGATCTGCACCTTC	GCGAATCGCTGTCTTTCTGTC
COL1	GGACACAGAGGTTTCAGTGGT	GCACCATCATTTCCACGAGC
COL2	TGGGGCCTTGTTCACCTTTGA	CGAGGCAACGATGGTCAGCC
AGG	TCGAGGACAGCGAGGCC	TCGAGGGTGTAGCGTGTAGAGA

Supplemental Table S14. Information of the primers used for qPCR.