Hybprinter-SAM for Functionally Graded Tissue Engineering Constructs with Patterned Biochemical Signals

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INTRODUCTION: While great strides have been made in the field of tissue engineering thanks to advancements in three-dimensional (3D) bioprinting, there are still limitations in constructing complex tissue engineering constructs (TECs) that may require multiple biomaterials and graded loadings of biofactors. Various instruments have been used to print biomaterials of varying stiffness and viscosity, but to create TECs that better mimic natural tissue, the printing platform should be able to extrude rigid biomaterials to support structural loads, print soft hydrogels to carry biofactors, and deliver patterned biofactors to spatially control biochemical cues for tissue regeneration. In this study, we have developed Hybprinter-SAM, a modular hybrid bioprinting platform that integrates syringe extrusion (SE), molten material extrusion (MME), and acoustic droplet ejection (ADE). This system enables fabrication of functionally graded constructs by combining soft hydrogels and rigid thermoplastics spanning seven orders of magnitude in stiffness, while simultaneously allowing high-resolution, nozzle-free deposition of biological factors. Through optimization of materials, printing fidelity, and biocompatibility, Hybprinter-SAM supports spatial control of both structural organization and localized biochemical cues. As a proof of concept, we engineered a multi-material scaffold mimicking the bone-tendon interface, incorporating patterned fibroblast growth factor-2 (FGF-2) using the ADE module. Precise growth factor localization induced selective upregulation of fibrocartilage-associated transcription factors SOX9 and SCX in human mesenchymal stem cells, demonstrating the system's ability to generate biologically instructive gradients.

METHODS: Hybprinter-SAM system and printing procedure: The system was composed of three modules, including MME, SE, and ADE. MME was used to print thermal plastics such as PCL as the rigid support; SE was used to print soft hydrogel material for cell loading; ADE was used to print biologics such as growth factors in droplet forms. A customized software was developed for the coordination of the three modules in a layer-by-layer fashion. During printing of a single layer, a layer of rigid material was first printed, followed by the printing of hydrogel in the same layer, followed by the deposition of growth factors onto specific spots. The procedure was repeated for the next layers.

Preparation of gelatin-fibrinogen (gelbrin) hydrogel: Briefly, a 15% gelatin (type A; 300 bloom from porcine skin; Sigma, MO) stock was prepared by dissolving in PBS. Simultaneously, fibrinogen Type 1-S (20 mg/mL, Sigma, MO) was dissolved in PBS and incubated at 37 °C for 45 min. The incubated precursor solutions were then mixed 1:1 for final concentrations of 7.5% gelatin, 10 mg/mL fibrinogen. This bioink was cooled at 4 °C for 20 min and brought back to room temperature for 15 min before printing at an environmental temperature of 20 °C. The bioink would be printable for 2 hrs.

Protein local patterning and cell study: Bovine serum albumin (BSA) and fibroblast growth factor-2 (FGF-2) was locally patterned onto the printed polycaprolactone-gelbrin hybrids. Human MSCs were cultured in DMEM media and used in this study. Cell proliferation and differentiation were evaluated.

RESULTS: For bioprinting purposes, the cytocompatibilities of the materials used in this study were first validated. Initially, hMSCs were encapsulated in the hydrogel and printed via the SE module. The cell viability was evaluated through a live and dead assay, which indicated > 95% viability 3 weeks post-printing (**Fig. 1a-b**). The biocompatibility of the whole hybprinting process was further validated. Particularly the MME module operates under high temperatures, which could adversely affect cell viability. To assess this, composite samples of PCL and hMSC-laden gelbrin were printed in a complementary pattern as shown in **Fig. 1c-d**, where the two paths intersect during printing. The cross-sections were of particular concern as the high-temperature PCL strut would make direct contact with the cell-laden hydrogel. Viability analysis was performed post-print (**Fig. 1e-f**), which demonstrated that in the bulk of the hydrogel, cells remained highly viable (>95% viability), with only cells within 200 μm of the PCL strut being affected (68% viability). This can be attributed to the rapid heat dissipation of the hydrogel material.

To achieve a controlled release and localization of biofactors, we developed *in situ* crosslinking during the print process. The rapid crosslinking between fibrinogen and thrombin at each printed layer created a localized high-density crosslinking network which effectively locked in the BSA, resulting in the expected slower release profiles and longer retention (**Fig.2a**). Results show that the printed pattern remained visually distinct after a 3 wk culture (**Fig.2b**) indicating minimal diffusion within the hydrogel. Additionally, both the low (10 nL) and high (50 nL) initial BSA loadings demonstrated a sustained release throughout the course of the study (**Fig.2c-d**). We fabricated a hybrid scaffold laden with hMSCs and FGF-2 to guide differentiation into fibrocartilage with qPCR results confirming successful early-stage differentiation with upregulation of *SCX*, and *SOX9 (*data not shown*)*.

DISCUSSION: The newly developed Hybprinter-SAM system demonstrates unprecedented capability of fabricating complex TECs, by integrating different bioprinting techniques. Using the Hybprinter-SAM system as a manufacturing platform, we are able to create both mechanical and biological gradient at the same time. The results have shown good integration of all three components, as well as excellent cytocompatibility and local differentiation regulation.

SIGNIFICANCE/CLINICAL RELEVANCE: This study has established Hybprinter-SAM as a versatile and scalable hybrid bioprinting platform capable of integrating mechanical heterogeneity with patterned biochemical signaling. These capabilities expand current hybprinting technologies and provide a promising strategy for engineering complex musculoskeletal interfaces and other gradient-dependent tissue.

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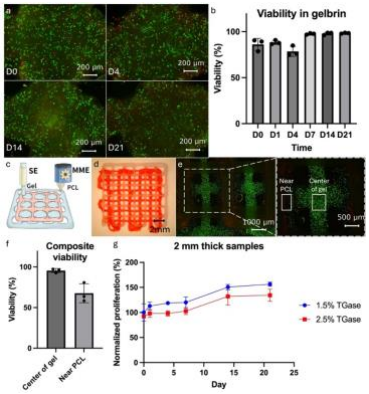

Fig. 1. (a-b) Cell viability study for the hydrogel material. (c-f) Cell viability study for the composite printed sample. (c) Schematic design and (d) printed sample with PCL and hydrogel. (e) Fluorescent picture and (f) quantified viability of the sample. (g) MTS study for proliferation of the printed sample.

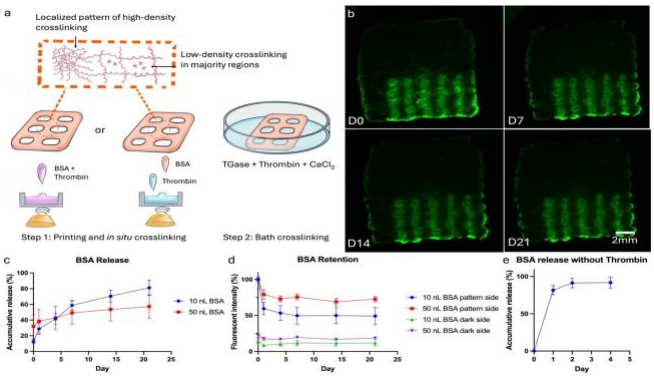

Fig.2 (a) In situ crosslinking strategy for protein immobilization in the gelbrin dual-crosslinking network. (b) Fluorescent images showing controlled release and pattern retention of FITC-BSA printed in droplets of 50 nL. (c-d) Quantified BSA release and retention of in situ crosslinked samples. (e) BSA release of non-in situ crosslinked sample.