A Multi-Material Bioink Method for 3D Printing Tunable and Cell-Compatible Hydrogels

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Statement of Purpose: 3D printing has emerged as a promising forefront for tissue engineering due to flexible design capabilities. However, a severe limitation to the growth of bioprinting is the lack of bioinks and the ability to tune mechanical, biological, chemical, and physical properties of the material without compromising "printability"[1]. In this work, we present a single bioink method capable of producing extrudable, soft hydrogels of various formulations in which polymer concentration, material type, degree of cross-linking, and type of cross-linker can all be manipulated. In this method, polymer solutions are lightly cross-linked with a long length chemical cross-linker, a homobifunctional polyethylene glycol (PEG) ending in two reactive groups (PEGX). PEG is an ideal cross-linker since it is commercially available in many physical and chemical variants, permitting versatile cross-linking chemistries that will be necessary for expanding the number of 3D printable bioinks.

Methods and Materials: This ink method consists of mixing polymer and PEGX with or without cells and allowing the mixture to gel within a printing cartridge at 37 °C for 2 hrs. In these experiments, amine-containing polymers including gelatin, fibrinogen, 4 arm PEG amine, gelatin methacrylate, atelocollagen, peptide amphiphiles, and mixtures were cross-linked with a PEGX of activated esters, succinimidyl valerate. To determine printable formulations, phase plots were created by screening varying polymer concentrations against varying PEGX:polymer mass to mass (m:m) ratios (**Fig 1A**). Material "phase" (i.e. solution, soft or robust gel) was determined by tube inversion, and gels were qualitatively assessed for consistency with a spatula. "Robust" gels retained shape whereas "soft" gels were able to be spread. Selected inks (circled, **Fig 1A**) were evaluated by rheology to probe mechanical characterisitics associated with printability. After gels formed, a strain sweep was performed at 10 rad/s. Candidate bioinks were printed with the 3D-Bioplotter (EnvisionTEC) and printed structures were imaged with a camera or stereoscope.Cells studied included HDFs, HUVECs, and hMSCs and were imaged with confocal microscopy and Live/dead and CellTracker stains.

Results: Soft gels found from phase plots were able to be extruded through fine diameter nozzles (200 µm) and were shape-maintaining and self-supporting. This single bioink method was successful at generating several materials and mixtures of printable consistencies (**Fig 1B**). From rheology, soft gels yielded at remarkably high strains ($\gamma_c \geq 1000\%$; **Fig 1C**) while robust gels yielded at lower strains, less than 800%. The critical stress was higher in robust gels ($\sigma_c > 3100$ Pa) compared to soft gels ($\sigma_c < 2200$ Pa). We hypothesize that 3D printing of these hydrogels is possible by local yielding/rupture at the nozzle wall. Soft gels, having low critical stresses, are able to break at the nozzle and extrude, whereas robust gels cannot. This indicates that the critical stress is a measurable parameter that can be correlated to printability.Several candidate bioinks (gelatin, fibrinogen, PEG) from phase plots were 3D printed into well-defined, multi-layer structures (**Fig 1D,E**). Fibrinogen and gelatin were successfully co-printed to demonstrate the ability to spatially organize multiple types of extracellular matrix in one 3D construct (**Fig 1F,G**).Within a co-printed structure of PEG amine and gelatin, cells adhered only to gelatin, demonstrating the ability to control cell adhesion in 3D (**Fig 1H**). Cells may be organized by incorporating cells into the ink for on-demand placement by printing, and by seeding cells into the 3D printed scaffold pores (**Fig 1I**).

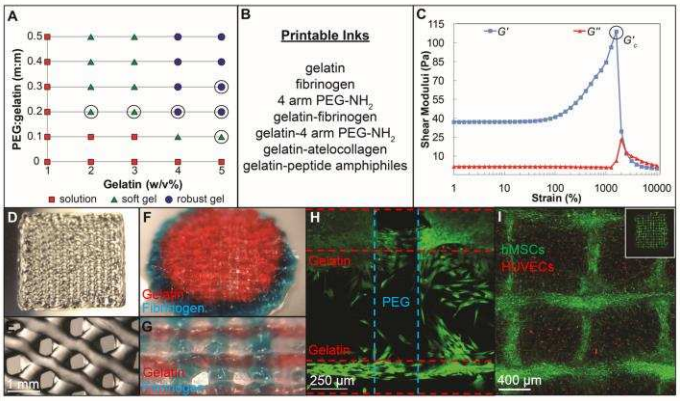

Figure 1: A) an example phase plot, gelatin; B) List of printable inks using PEGX; C) Strain sweep of soft gel; D) printed structure and E) internal structure; F,G) Multi-material printed structures of gelatin and fibrinogen; H) A structure printed of a cell-encapsulating bioink was seeded with another cell type to demonstrate spatial organization of cells; H) Cells seeded onto co-printed gelatin and PEG constructs show preferential cell adhesion to gelatin struts.

Conclusions: We demonstrate a new single bioink method that enables the 3D printing of a variety of different materials. The bioink formulation can also be customized with regards to material type and concentration as well as type and degree of cross-linker to modify mechanical, biological, chemical or physical properties. We envision that the cross-linking reaction can easily be exchanged with other PEGX to further expand the number of 3D printable and cell-compatible materials. We hope that this method will contribute to advancing bioinks to enable biofabrication of compositionally and structurally complex structures and functional tissues.

References: 1) Malda J. Adv Mater. 2013;25(36):5011-28.