Sensing in 3D Printed Neural Microphysiological Systems

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ABSTRACT

The research presented in this dissertation supports the overall goal of producing sensor functionalized neural microphysiological systems to enable deeper fundamental understandings of disease pathology and to provide drug screening and discovery platforms for improved clinical translation. Towards this goal, work addressing three broad objectives has been completed. The first objective was expanding the manufacturing process capabilities for hydrogels and tissues through augmentation of the 3D printing systems and developing novel modeling capabilities. The second objective was to expand the palette of available materials which exhibit the rheological properties required for 3D printing and the mechanical and biological properties required for neural tissue culture. The third objective was to develop sensing capabilities for both monitoring and control of the manufacturing process and to provide non-destructive assessment of microphysiological systems in real-time to quantify the dynamics of disease progression or response to treatment.

The first objective of process improvement was addressed both through modification of the 3D printing system itself and through modeling of process physics. A new manifold was implemented which enabled on-the-fly mixing of bioprinting inks (bioinks) to produce
smooth concentration gradients or discrete changes in concentration. Modeling capabilities to understand the transport occurring during both the processing and post-processing windows were developed to provide insight into the relationship between the programmed concentration distribution and its temporal evolution and stability. Vacuum-based pick-and-place capabilities for integration of prefabricated components for sensing and stimulation into the printed hydrogel constructs were developed. Models of the stress profiles, which relate to cell viability, within the printing nozzle during extrusion were produced using parameters extracted from rheological characterization of bioinks.

The second objective was addressed through the development hydrogel bioinks which exhibited yield stresses without the use of rheological modifiers (fillers) to enable 3D printing of free-standing neural tissue constructs. A hybrid bioink was developed using the combination of a synthetic polaxamer with biomacromolecules present in native neural tissue. Functionalization of the biomacromolecules with catechol or methacrylate groups enabled two crosslinking mechanisms: chelation and UV exposure. Crosslinked gels exhibited moduli in the range of native neural tissue and enabled high viability culture of multiple neural cell types.

The third objective was addressed through the characterization and implementation of physical and electronic sensors. The resonance of millimeter-scale dynamic-mode piezoelectric cantilevers submerged in polymer solutions was found to persist into the gel phase enabling viscoelastic sensing in hydrogels and monitoring of sol-gel transitions. Resonant frequency and quality factor of the cantilevers were related with the viscoelastic properties of hydrogels through both a first principles approach and empirical correlation.
Electrode functionalized hollow fibers were implemented as impedimetric sensors to monitor bioink quality during 3D printing. Impedance spectra were collected during extrusion of cell-laden bioinks and the magnitude and phased angle of the impedance response correlated with quality measures such as cell viability, cell type, and stemness which were validated with traditional off-line assays.
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GENERAL AUDIENCE ABSTRACT

The research presented in this dissertation supports the overall goal of producing sensor functionalized neural microphysiological systems to enable deeper fundamental understandings of disease pathology and to provide drug screening and discovery platforms for improved clinical translation. Microphysiological systems are miniaturized tissue constructs which strive to mimic the complex conditions present in-vivo within an in-vitro platform. By producing these microphysiological systems with sensing functionality, new insight into the mechanistic progression of diseases and the response to new treatment options can be realized. Towards this goal, work addressing three broad objectives has been completed. The first objective was expanding the manufacturing process capabilities for hydrogels and tissues through augmentation of the 3D printing systems and developing novel modeling capabilities. The second objective was to expand the palette of available materials which exhibit both the properties required for 3D printing and the mechanical and biological properties required for neural tissue culture. The third objective was to develop sensing capabilities for both monitoring and control of the manufacturing process and to provide non-destructive assessment of microphysiological systems in real-time to quantify the dynamics of disease progression or response to treatment. Through these efforts higher
quality microphysiological systems may be produced benefitting future researchers, medical professionals, and patients.
DEDICATION

For my late father, Philip Haring, who taught me to be curious, work hard, and make the most of the time that we have.
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I would like to first gratefully acknowledge my advisor, Prof. Blake Johnson for his continued support, motivation, and mentorship throughout the duration of my studies. His passion for teaching and discovery made this work possible. I appreciate the many discussions we’ve had around research, career goals, and life.

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ATTRIBUTION

Prof. Blake N. Johnson: (Virginia Tech) Assistant professor in the Department of Industrial and Systems Engineering has overseen the work reported in this dissertation in its entirety.

Prof. David Dillard: (Virginia Tech) Professor in the Department of Biomedical Engineering and Mechanics contributed insight and guidance to the viscoelastic modeling and understanding in Chapter 9.

Prof. Zhenyu Kong: (Virginia Tech) Professor in the Department of Industrial and Systems Engineering contributed insight and guidance in timeseries analysis of sensor response in Chapter 9.

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Prof. Xiaoting Jia: (Virginia Tech) Assistant professor in the Department of Electrical and Computer Engineering provided insight into impedance spectroscopy and knowledge of functional fiber manufacturing in Chapter 8.

Prof. Harald Sontheimer: (Virginia Tech) Professor in the School of Neuroscience provided insight into neurobiology and cell biology in Chapters 2, 6, 7, and 8.

Prof. Jia-Qiang He: (Virginia-Maryland College of Veterinary Science) Associate Professor in the Department of Biomedical Sciences and Pathobiology provided insight into stem cell behavior and provided stem cells used in Chapter 8.

Prof Kenneth J. Oestreich: (Ohio State College of Medicine) Professor of Microbial Infection and Immunity provided conceptualization for particle/cell trapping in chapter 5.
Dr. Assad Khan: (Virginia Tech) Former graduate student in Prof. Liu’s group synthesized and characterized plasmonic nanoparticles used in Chapter 3.

Dr. Emily G. Thompson: (Virginia Tech) Former graduate student in Prof. Sontheimer’s group cultured and analyzed cells used in Chapters 6, 7, and 8.

Yuxin Tong: (Virginia Tech) Graduate student in Prof. Johnson’s group provided assistance with FEA modeling in Chapter 4 and 5.

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**Figure S3. 1** Highlight of the range of silver nanoprism sizes used in this study for plasmonic ink formulations. TEM images of silver nanoprisms with diameters of 15 ± 4 nm (a), 28 ± 6 nm (b), 39 ± 7 nm (c), 49 ± 3 nm (d), 53 ± 5 nm (e), 58 ± 4 nm (f), 71 ± 13 nm (g), and 79 ± 11 nm (h).

**Figure S3. 2** UV-Vis spectra of F-127 (a) and PEGDA (b) inks used in Figures 3.2e and f.

**Figure S3. 3** UV-Vis spectra of uncrosslinked PEGDA inks.

**Figure S3. 4** Peak absorbance intensity for 15 and 79 nm nanoparticles in PEGDA and Pluronic F-127 inks as a function of nanoparticle concentration. The concentration at dilution factor = 1.0 corresponds to the concentration used for graded plasmonic constructs shown in the main text.

**Figure S3. 5** UV-Vis spectra of the pure and blended F-127 inks used in Figure 3.2.

**Figure S3. 6** Schematic of the UV-Vis measurement used to generate the 2D absorbance heat map shown in Figure 3.2. Absorbance spectra were acquired across the 3D printed sheet at 10 equally distributed points (represented schematically by crosses).

**Figure S3. 7** a) Schematic of the 3D UV-Vis measurement principle corresponding to data shown in Figures 3.3 and 3.4. b) Schematic of the path of incident light along the 3D constructs (solid line) that occurs by rotating the sample through the angle θ during the measurement (start of the path corresponds to low angle values; dashed line shows scan direction).
**Figure S3.** Absorbance spectra of the blue filter (A) and red band-pass filter (B) inks used for the functionally graded plasmonic night vision contact lens shown in Figure 3.5.

**Figure 4.1** Schematic showing the concept of programming temporal release profiles of individual actives from a single polypill by controlling their spatial distributions (i.e., concentration profiles). Microextrusion 3D printing processes enable the fabrication of polypills containing multiple actives distributed with varying concentration profiles, and was leveraged here to provide a systematic study of the relationship between programmed spatial distribution and temporal release profile.

**Figure 4.2** Microextrusion printing of polypills with varying concentration profiles and analysis of concentration profile dynamics throughout processing and post-processing intervals. A) Top-down photographs of the 3D printed hydrogel Pluronic F-127 pills containing core-shell, multilayer, and gradient concentration profiles acquired after the 90-min processing interval (i.e., microextrusion 3D printing process). A red dye facilitates the measurement of radial concentration profile using image processing techniques. B) Top-down photographs of the same 3D printed Pluronic F-127 pills shown in (A) acquired after the 12-hr post-processing interval (i.e., drying-based solidification process). C) Dimensionless radial concentration profile corresponding to the dashed lines in (A) and (B) showing the dynamics of programmed concentration profiles (G-code) throughout pill processing (hydrogel) and post-processing (solid).

**Figure 4.3** Finite element modeling of mass transfer in 3D printed pills during the processing (i.e., 3D printing) interval. A) Schematic of the computational approach for
modeling mass transfer in 3D printed parts, such as polypills, throughout the processing interval as a series of transient simulations.  

B) Results from the finite element model described in (A) showing the predicted two-dimensional concentration profiles of active in the radius \( r \)-height \( h \) plane after the processing interval for the core-shell, multilayer, and gradient structures \( c_{\text{max}} = 12.5 \text{ mg/mL} \).  

C) Radial concentration profile corresponding to the dashed lines in (B) showing both the dynamics of programmed concentration profiles throughout the pill processing interval and the effect of height and position on concentration profile dynamics \( h_1 = 2.25 \text{ mm}, h_2 = 4.25 \text{ mm}, h_3 = 6 \text{ mm} \).

Figure 4. 4 Finite element modeling of mass transfer in 3D printed pills during the post-processing \((i.e.,\text{ drying})\) interval.  

A) Predicted change in two-dimensional concentration profiles in the \( r-\theta \) plane after the post-processing interval for the core-shell, multilayer, and gradient structures \( c_{\text{max}} = 12.5 \text{ mg mL}^{-1} \).  

B) Radial concentration profiles corresponding to the dashed lines in (A) highlighting concentration profile dynamics throughout the post-processing interval for the different spatial distributions.

Figure 4. 5 Dissolution studies of 3D printed pills in aqueous solution.  

A) Top-down micrographs of dissolving 3D printed Pluronic F-127 pills at various time points throughout the dissolution process \((t = 0, 100, 200,\text{ and } 300 \text{ min})\).  

B) Dissolution profile of the 3D printed Pluronic F-127 pills in DI water \((n = 6 \text{ for } \text{pH} = 7; n = 3 \text{ for } \text{pH} = 3)\).

Figure 4. 6 Temporal release profiles of single active-containing pills with core-shell, multilayer, and gradient concentration profiles.  

A) Schematic of capped metformin hydrochloride pills with varying concentration profiles. Temporal release profiles of metformin hydrochloride pills containing core-shell (B), multilayer (C), and gradient (D)
distributions (n = 3 for each pill type). Control pills contained a uniform distribution of metformin hydrochloride. Core-shell, multilayer, and gradient concentration profiles enable programming of delayed, pulsed, and constant linear release profiles in 3D printed pills containing a single active (here metformin hydrochloride).

**Figure 4.7** Temporal release profiles of polypills with core-shell, multilayer, and gradient concentration profiles. **A** Schematic of a stacked polypill containing interior glyburide, acarbose, and metformin layers and drug-free caps. Temporal release profiles of glyburide **(B)**, acarbose **(C)**, and metformin **(D)** from the polypills. Control pills contained a uniform distribution of the actives. Core-shell, multilayer, and gradient concentration profiles enable programming of delayed, pulsed, and constant linear release profiles in 3D printed polypills (here, three commonly used oral agents for treatment of type 2 diabetes - metformin hydrochloride, acarbose, and glyburide).

**Figure S4.1** Measurements of pill shrinkage during the solidification process. Measurement of pill shrinkage during solidification was measured by acquiring diameter and height measurements before and after drying with digital calipers. Pills shrank 13% and 30% in diameter and height, respectively. Measurements on pills before drying also confirmed that the AM process accurately produced the programmed pill dimensions, yielding pills within 100 µm of the programmed diameter and height (i.e., that defined by the tool path). Measurements correspond to n = 3 different pills.
**Figure S4. 2** Photograph of two 3D printed solid Pluronic F-127 polypills released from the substrate following the post-processing (i.e., drying) interval. The solid pills could withstand handling forces without fracture or crumbling.

**Figure S4. 3** A) Schematic of the programmed concentration profiles (i.e., those described by the tool path) in 3D printed pills. B) Predicted two-dimensional concentration profile of metformin hydrochloride in the r-h plane for core-shell, multilayer, and gradient structures following the post-processing interval corresponding to data shown in Figure 4 of the main text ($c_{\text{max}} = 12.5 \text{ mg mL}^{-1}$).

**Figure 5. 1** Additive manufacturing concept for fabrication of 3D microfluidic MEMS devices. A combination of 3D printing and robotic embedding facilitates the integration of orthogonal in-plane and out-of-plane piezoelectric transducers, functional 3D printable materials, and microfluidic channels. In-plane and out-of-plane piezoelectric transducers facilitate the trapping of continuously flowing particles in microfluidic channels in transverse and lateral directions.

**Figure 5. 2** a) Highlight of the seven fabrication steps (I-VII) for the 3D printed acoustofluidic device including 3D printing and embedding processes. Each step shows in vertically descending order: the assembly schematic, a photograph of the device during the fabrication step, and the height profile of the device after completion of the step. b) Cross-sectional schematic (top) and photograph (bottom) of the device.

**Figure 5. 3** a) Experimentally measured and simulated electrical impedance response of the 3D printed acoustofluidic devices over 0 – 20 MHz and highlight of experimentally measured
impedance (Z) and phase angle (φ) characteristics from four resonant modes that exhibit strong impedance-coupling. b) Photograph of the embedded PZT chip highlighting the acoustic source. c) Calculated 3D mode shape and displacement profile (dashed line) for each of the resonant modes shown in terms of the transverse displacement. d) Secondary transducer configuration for sensing of acoustic waves generated by the embedded PZT chip showing the corresponding voltage signal generated in the secondary transducer (Vs,p-p) at each mode. e) Comparison of the voltage generated in the secondary acoustic transducer with the maximum total displacement (Dmax) calculated using finite element simulations for each resonant frequency (fn). Also shown is the fast Fourier transform of the measured voltage signal shown in (d).

**Figure 5. 4** a) Schematic of droplet-based flow visualization studies using the in-plane piezoelectric transducer. Acoustic waves generated by the robotically embedded in-plane transducer propagate into the droplet producing pressure oscillations (P) and streaming flow (v) that exert forces on suspended particles (diameter = D). b) Micrographs of the suspended particle systems under excitation at each mode of the 3D printed acoustofluidic device show distinct regimes of trapping and streaming behavior for particles ranging from 0.8 – 70 µm in size. c) Fluorescence micrographs and particle distribution plots of multi-particle systems under excitation at each mode of the 3D printed acoustofluidic device show size-selective separation of particles and mode-dependent separation profiles.

**Figure 5. 5** a) Schematic of a 3D microfluidic MEMS device containing two orthogonal piezoelectric chips (one in-plane and one out-of-plane). b) Concept of orienting piezoelectric transducers with both the in-plane and out-of-plane components of a microchannel using
additive manufacturing to facilitate manipulation of continuously flowing particles (e.g., whole cells). c) Vacuum-based robotic embedding principle associated with integration of the in-plane piezoelectric transducer. d–e) Adhesion-based robotic embedding principle associated with integration of the out-of-plane piezoelectric transducer. f) Photographs of the device before and after printing of Ag interconnects to the out-of-plane piezoelectric transducer. g) Electrical impedance spectra of the in-plane and out-of-plane piezoelectric transducers over the 0 – 20 MHz frequency range.

**Figure 5.6** a) Schematic of the fabrication steps associated with integration of a 3D printed microchannel in between the in-plane and out-of-plane piezoelectric transducers with corresponding photographs. b) Schematic showing the microchannel orientation with respect to the integrated piezoelectric transducers and corresponding photograph after dissolution and washout of the printed eutectic Gallium-Indium. c) Schematic showing the principle of exciting both the in-plane and out-of-plane transducer during continuous flow of suspended particles through the microchannel. Flow visualization studies showing continuously flowing 6 µm particles in the presence of stimulation from the in-plane transducer (d), presence of stimulation from the out-of-plane transducer (e), presence of simultaneous stimulation from both transducers (f), and absence of acoustic stimulation (g) (white arrow indicates the direction of flow).

**Figure S5.1** a) Multi-material pick-and-place 3D printing process for fabrication of ceramic-based electronics consisting of a print stage, extrusion nozzles, suction-based pick-and-place
tool, 3-axis robot, vision system, and pressure regulators.  

**b) Magnified view of the print heads and pick-and-place tool.**

**Figure S5.2** Magnified view of the profilometry data of 3D printed MEMS devices during the printing process showing: the 3D printed Ag conductive pathway (**a**), the robotically placed PZT chip on top of the Ag conductive pathway (**b**), the 3D printed thermosetting epoxy anchor (**c**), and the second 3D printed Ag conductive pathway on top of the PZT chip (**d**).

**Figure S5.3** Calculated transverse displacement profiles in the width direction (dashed line) of the PZT chip upon excitation at each resonant mode (**a**–**d**) and corresponding displacement profiles (**e**–**h**).

**Figure S5.4 a)** Representative micrographs comparing the accuracy and precision of PZT chip placement via the robotic pick-and-place approach vs. hand positioning.  

**b)** Schematic showing the percent error in chip placement relative to a target location (*e.g.*, a chip that is centered on a printed trace of adhesive material).  

**c)** Experimental data showing the chip placement accuracy and precision of each approach corresponding to panel (**b**).

**Figure 6.1 a)** Neural tissue bioink design for biomimicry and processability. Native extracellular matrix components of neural tissue were combined with a synthetic polymer to achieve biomimicry of neural tissue chemistry and mechanical properties as well as desirable rheological properties for microextrusion 3D bioprinting of soft, free-standing neural tissues.  

**b)** The slightly crosslinked hydrogel network creates a highly printable bioink capable of forming free-standing structures.  

**c)** The printed bioink could be chelated or
photocured to produce a cured chemically and mechanically biomimetic 3D bioprinted neural tissue.

**Figure 6.2** Gelation temperatures of the bio-inspired PF127-based neural bioinks obtained from DSC show an inverse relationship with PF127-SH content.

**Figure 6.3** Rheological data of for the chelating (a and c) and photocuring (b and d) neural bioink formulations that contain 2 wt% DC-Gel and 5 wt% DC-Gel-MA, respectively, 1 wt% DC-HA, and varying concentrations of PF127-SH ranging from 6 – 15 wt%. Stress sweeps (a and b) show the yield stress as indicated by the sharp drop in relative modulus. Frequency sweeps (c and d) show the shear thinning fluid behavior as indicated by the decrease in complex viscosity with shear rate.

**Figure 6.4** a) Velocity profiles calculated using finite element analysis associated with continuous bioink microextrusion through a 400 µm nozzle diameter. b) Fraction of the nozzle in which the bioink experiences relatively low shear stress.

**Figure 6.5** a) Schematic showing two curing pathways: photocuring of formulations containing methacrylated gelatin in the presence of a photoinitiator through UV light exposure, and chelation of dopamine groups with iron (III). b) Compressive elastic modulus results of inks cured through UV exposure or chelation show an increasing modulus with increasing PF127-SH content. The chelating bioinks were relatively softer than photocured bioinks.

**Figure 6.6** Printed bioinks (cross-hatched structure (panels a and c) and the Virginia Tech logo (panels b and d); height = 2 and 4 mm, respectively) shown from prismatic (a and b) and top-down views (c and d). e) Percent alamarBlue (% AB) reduction in the bioprinted
Schwann cell-laden tissue constructs cured through chelation or photocuring. Alginate was used as a control. On all days, n = 4 samples per bioink were sacrificially tested.

**Figure 6.7** Fluorescence micrographs of 3D bioprinted neural and glial tissue constructs using the gelatin-hyaluronic acid-Pluronic F-127 (Gel-HA-PF127) bioink containing rodent Schwann cells (a), human glioma cells (b), and rodent neuronal cells (c) after seven days in culture.

**Figure 6.8** Schematic of slight crosslinking through covalent bonding of dopamine (DA) conjugated gelatin (DC-Gel) and hyaluronic acid (DC-HA) with thiolated Pluronic F-127 (PF127-SH) through a thiol (SH) – catechol reaction resulting in a micelle based hydrogel with slightly crosslinked biomacromolecules (note: DA contains a catechol group).

**Figure S6.1** Fluorescence micrographs (10x) of Schwann cell- (a, d, g), glioma cell- (b, e, h), and neuronal (c, f, i) cell-laden bioprinted tissue constructs on days 1 (a-c), 3 (d-f), and 7 (g-i).

**Figure 7.1** 3D printed migration assays for high-throughput study of spatially-distributed chemotactic signals (e.g., spatially-opposing sources). a) Schematic of fabrication via 3D printing into 35 mm petri dishes. b) Schematic of cell seeding and chemoattractant loading steps via manual pipetting. c) Schematic of a migration assay design with multiple spatially-distributed sources for studying the effect of spatially-opposing chemotactic signals on glioblastoma migration. d) Dimensions of the device highlighting the cell-seeding chamber (radius = 3 mm), microchannel (5 mm long), and chemoattractant-loading chamber (1 mm
radius). e) Photograph of a device printed in a 35 mm petri dish. Micrographs of the cell-seeding chamber (f), microchannel (g), and chemoattractant-loading chamber (h). i) Photograph demonstrating the potential for multiplexing, showing the integration of three devices in a 35 mm dish (all single-source designs).

Figure 7. 2 Sensitivity analysis of chemoattractant gradient characteristics using FEA simulations, including the magnitude of the concentration gradient at the migrating cell front and the chemoattractant penetration time. a) 3D and top-down schematics showing the simulation domain, including the cell-seeding chamber, microchannel, and chemoattractant-loading chamber (8 mm dashed line indicates line plot location corresponding to data in panel d). b) Schematic showing the temporal evolution of the spatial concentration profile. c) Surface plots of FEA simulations using a basis of 50 ng/mL fibroblast growth factor (FGF) loaded in a 1.5% alginate hydrogel at t = 4, 8, 24, and 48 hr (C_{max} = 1.3 μM (4 hr), 1.1 μM (8 hr), 740 nM (24 hr), and 540 nM (48 hr)). d) Line plots corresponding to panels a and c showing the concentration profiles in the device at 4, 8, 24, and 48 hr. The center of the cell-seeding chamber and the entrance to the chemoattractant-loading chamber correspond to the positions of x = 0 and 8 mm, respectively.

Figure 7. 3 Fibroblast migratory response to a FGF gradient. a) Schematic of fibroblast chemotaxis due to FGF gradients during wound healing. b) Migration velocity relative to the negative control for FGF loading concentrations (C_{load}) of 5, 10, 15, 25, 50, and 100 ng/mL showing an optimal concentration at 50 ng/mL. c) FEA results showing the temporal evolution of the FGF concentration in the cell-seeding chamber (location indicated by the red
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Figure 7.4 Glioblastoma migratory response toward single sources of chemoattractants in the absence of competing gradients. a) Schematic describing the spatially-opposing chemotactic signals that glioblastoma cells encounter in vivo through exposure to spatially-varying vascular microenvironments. b) Migration velocity relative to a negative control in which EGF was absent for loading concentrations of 25, 50, 75, and 100 ng/mL showing an optimal EGF loading concentration of 75 ng/mL. c) FEA results showing the temporal evolution of the EGF concentration in the cell plating area for loading concentrations of 25, 50, 75, and 100 ng/mL. d) Migration velocity relative to a negative control in which BK was absent for loading concentrations of 50, 100, 200, and 400 µM showing an optimal BK loading concentration of 100 µM. e) FEA results showing the temporal evolution of the BK concentration in the cell plating area for loading concentrations of 50, 100, 200, and 400 µM (n = 3, Student’s t-test, P-value notation: * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001).

Figure 7.5 Competitive migration assay results associated with glioblastoma migratory response to spatially-opposing gradients of EGF and BK. a) Photograph and schematic of a 3D printed migration assay with spatially-opposing chemoattractant sources. b) Rose plot showing the direction and magnitude of cell migration (the EGF and BK sources located at 0 and 180°, respectively). Highlighted regions indicate octants around each source location. c) FEA results showing the temporal evolution of the spatial concentration profile at t = 4, 8, 24, and 48 hr. d) Analysis of the rose plot shown in panel b in terms of the percentage of cells heading toward the EGF and BK octants. e) Relative migration velocity of cells heading
toward the EGF and BK sources compared to that measured in the absence of EGF or BK, which served as the negative control. 
f) Relative migration velocity towards the EGF and BK sources in an experiment with competing chemoattractant sources compared to those measured with a single chemoattractant source (n = 3, z-test (d), Student’s t-test (e and f), P-value notation: * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001). .................................................................240

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**Figure S7. 8** Chemotactic response of BK (\( i.e. \), migratory response toward BK gradients) compared with that observed in the presence of uniformly-distributed BK. a) Spatiotemporal chemoattractant concentration profiles corresponding to an ‘optimal’ EGF gradient established by an EGF source initially loaded with 75 ng/mL EGF in cell culture medium that contains 0.7 \( \mu \)M BK. b) Relative migration velocity of human glioblastoma cells toward an ‘optimal’ EGF source in: 1) a single-source assay configuration (\( i.e. \), containing no competing chemoattractant), 2) the presence of a competing BK gradient, and 3) in the presence of uniformly-distributed BK. P-value notation: \( * = p \leq 0.05, ** = p \leq 0.01, *** = p \leq 0.001 \).

**Figure S7. 9** Demonstration of rapid prototyping capability regarding the spatial distribution of competitive chemoattractants shown using a four-channel model (a) and ability to integrate a 3D bioprinted tissue construct (1% alginate crosslinked using a saturated calcium chloride solution) using a one-pot fabrication approach (b; red dye added to enhance contrast of the bioprinted tissue construct).
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**Figure 8.5** Impedance magnitude (a) and phase angle (b) of stem cells (+ LIF) and spontaneously differentiated cells (– LIF), averages of n = 3 samples shown. Merged fluorescent micrographs (200x) associated with expression of SSEA-1, a mESC specific marker, in mESC cultures stabilized in a stem cell state (+ LIF) (c) and allowed to spontaneously differentiate (– LIF) (d), which served the basis of the stem cell-laden bioinks. e) Percentage of stem cells present in a stem cell state based on SSEA-1 expression data following 14 days of culture in +LIF and – LIF media (i.e., panels c and d) (** indicates p < 0.001, n = 3). Fluorescent micrographs (100x) associated with live/dead assays of stem cells in a stem cell state (+ LIF) (f and g) and in a spontaneously differentiated state (– LIF) (h and i) in both bioprinted and molded constructs using calcein AM, EthD-1, and Hoechst 33342 (nuclei). j) Summary of stem cell viability after following processing (* indicates p < 0.05, n = 3).
**Figure S8. 1** Schematic illustrating the wire bonding process that enables interface between the electroded multifunctional fiber and the potentiostat. Step 1: Electrodes at the distal end of the fiber near the 3D bioprinting nozzle were blocked with medical epoxy. Cladding material was subsequently removed from the outer walls of the fibers to expose the embedded copper wire. Step 2: Copper leads were subsequently bonded to the exposed copper wires using a silver epoxy. Step 3: The fiber was inserted into tubing. Step 4: 5-minute epoxy was then used to seal and reinforce the tubing-fiber connection and the bonded copper leads. The bioprinting nozzle was subsequently inserted in the tubing forming a tight seal that prevented the backflow of bioink.

**Figure S8. 2** a) Photograph of nozzle-fiber sensor assembly. b) Photograph illustrating 3D bioprinting through the hollow multifunctional fiber impedimetric sensor.

**Figure S8. 3** Micrographs of fiber cross sections demonstrating the ability to fabricate a range of inner channel diameters commonly used for 3D bioprinting: 100 (a), 200 (b), 300 (c), 400 (d), and 500 µm (e).

**Figure S8. 4** Fluorescence images of 5 x 10^5 cell/mL cultures of PC-12 cells in a cured alginate-NFC hydrogel on day 1 (a) and 7 (b) with a live/dead stain. c) Live/dead assay results show no significant difference between days 1 and 7.

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Both the negative control (a and b) and experimental (d and e) groups are shown. c) Live/dead results showed no significant difference between groups.

**Figure 9. 1 a)** Schematic of piezoelectric-excited millimeter cantilever (PEMC) sensor self-sensing and self-exciting design for sensor-based characterization of hydrogel rheological properties and real-time monitoring of sol-gel phase transitions. Photographs of a PEMC sensor from top-down (b) and side-view (c) perspectives. d) Sensor frequency response acquired via electrical impedance analysis shown in terms of the phase angle response (inset shows photograph of the PEMC sensor submerged in a concentrated solution of gel-forming polymer; spectra in air and vacuum correspond to 1 and 0.3 atm (vacuum), respectively).

**Figure 9. 2 a)** Schematic depicting the sensor-based sol-gel rheological characterization study and associated measurement principle (i.e., real-time monitoring of gelation processes via sensor signal tracking). Observed cantilever impedance phase angle over a 25 – 50 kHz sweep in air, solution (sol), and gel phases of 6 wt% gelatin (b), 0.25 wt% alginate (c), and 10 wt% PEGDA (d).

**Figure 9. 3** Limits of resonance persistence in increasingly concentrated hydrogels. a) 6, 8, 10, and 12 wt% gelatin. b) 0.5, 1, 1.5, and 2 wt% alginate. c) 5, 10, 15, and 20 wt% PEGDA.

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**Figure 9.** Real-time monitoring of high-frequency shear moduli at the resonant frequency ($\sim 35$ kHz) based on sensor resonant frequency and quality factor responses using the cantilever viscoelastic material-structure interaction model throughout gelation of 8 wt% gelatin (a) and 0.5 wt% alginate (b) solutions (green and blue lines show 25-point moving averages associated with the storage and loss moduli response, respectively). High-frequency shear moduli obtained at the resonant frequency of hydrogels formed from 6, 8, and 10 wt% gelatin (c) and 0.25, 0.5, and 0.75 wt% alginate (d) solutions ($n = 3$ experiments for each concentration). Sensor transfer functions associated with quality factor ($Q$) change vs. $G'$ and $E'$ with linear regressions shown (panels e and f, respectively).

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Figure S9. 2 Real-time monitoring of sensor signals during UV curing of a 10 wt% PEGDA shown in terms of the resonant frequency (a), phase angle (b), and quality factor (c) responses. Exposure to a negative control (light on with UV blocked) at 200 s and exposure to UV light begins at 600 s.

Figure S9. 3 Predicted gelation kinetic for chemical gelation of 0.25, 0.5, and 0.75 wt% alginate solutions based on the modified Hill modeling of associated sensor responses ($n = 3$ experiments for each concentration).
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Table 2.1 Summary of neural systems on a chip. Abbreviations: Hippocampal Neuron (HN), Cortical Neuron (CN), Motoneuron (MN), Neural Progenitor (NP), Schwann Cells (SC), Epithelial Cells (EC), Thalamic Neuron (TN), Peripheral Neuron (PN), Myoblast (MB), Astrocyte (AC), Oligodendrocyte (OC), Striatal Neuron (SN), Spinal Neuron (SPN), Myocyte (MC), Peripheral nervous system (PNS), Central Nervous System (CNS), External Tissue (EXT), Midbrain Dopaminergic Neuron (mDAN), Microglia (MG), Microelectrode Array (MEA), Polydimethylsiloxane (PDMS), Polycaprolactone (PCL), Polycarbonate (PC), Poly(ether imide) (PEI), Polytetrafluoroethylene (PTFE), Poly(ethylene glycol) diacrylate (PEGDA). Asterisk (*) indicates photolithography was also used. ........................................ 21

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Chapter One: Introduction

1.1 Background and Research Goal

Modern medicine depends on in-vitro cell culture models, small and large animal models, multi-phase clinical trials, and large-scale surveys to both understand the underlying pathology of diseases or injuries and to develop successful treatment options. However, even with these robust and well-designed approaches there remain significant knowledge gaps into disease pathology, particularly at the cellular and molecular levels, and many diseases have eluded successful treatment. In particular, neural diseases and injuries are some of the most difficult to understand and treat. One of the greatest challenges in addressing these knowledge gaps is poor translation from in-vitro cell culture to animal models to human clinical trials, which is largely attributed to over simplification of in-vitro models which do not represent in-vivo conditions and to differences in cell biology and biochemistry between animals and humans. A new tool which holds promise to improve these translational challenges is the microphysiological system, also referred to as an organ-on-a-chip, which is an in-vitro platform incorporating anatomical features such as three dimensionality and heterogeneity (both molecular and cellular), anatomical organization such as multiple cell type or tissue type ensembles, functional physical features such as flow, electrical or mechanical stimuli, and controlled release of biochemical cues or drugs. In addition to the greater biomimicry of these systems, another advantage of this approach is
the ability to incorporate sensors to collect real-time information related to dynamics of disease progression or response to drugs or other stimuli.

Broadly, microphysiological systems can be grouped into three general categories: microfluidic systems, chamber-based systems, and hydrogel-based systems. Microfluidic systems are based around cell culture in microchannels under flow conditions, where the flow is used to deliver nutrients or chemical stimulation in complex concentration distributions. The major advantage of microfluidic systems is the ability to carefully control the concentration distribution of dilute species. These systems are typically fabricated using poly(dimethyl siloxane) (PDMS) using soft lithography techniques. Chamber-based systems are also most often fabricated from PDMS using soft lithography. Chamber-based systems use multiple cell culture areas (chambers) separated by physical barriers permeable to chemical species or cell-components such as axons, but not cell bodies. The major advantage of chamber-based systems is the ability to study the interaction between different groups of cells or between a group of cells and a chemical stimulus in a controlled environment. In particular, chamber-based assays are commonly used for investigating axonal outgrowth and neural-network formation. Hydrogel-based systems implement 3D cell culture techniques to study cell behavior within a hydrogel matrix, mimicking the extra-cellular matrix of native tissue. The primary advantage of hydrogel-based systems is the cell-cell and cell-matrix interactions most accurately represent in-vivo conditions. These systems are typically manufactured using a casting process or through 3D bioprinting. 3D bioprinting a more advantageous manufacturing method as it allows for precise control of the distribution of cells and chemical species for heterogeneous tissue culture, as well as the ability to create
complex geometries with features such as vasculature or heterogeneous mechanical properties. While early microphysiological systems could be neatly placed into a single category, more contemporary systems blur the lines between categories often implementing aspects of multiple categories.

One of the advantages all microphysiological systems have over *in-vivo* studies is the ability for continuous measurement and more aggressive probing. While studies conducted in animal models or human clinical trials can only collect data at specific time-points, microphysiological systems allow sensor integration for real-time monitoring providing access to dynamics of disease progression or response to treatment. Additionally, through the use of different transducers or augmentation with selective reception sites, sensors can probe a wide range of physical or biological properties. While this is an important aspect of fully realizing the capabilities of microphysiological systems, sensor integration is only recently becoming commonly adopted in the research community.

This thesis describes improvements in manufacturing capabilities, available materials, physical modeling, and sensing capabilities for 3D printed neural microphysiological systems. The present research goal is to develop the capabilities for the production of heterogeneous miniaturized neural tissue constructs with controlled distributions of cells, dilute chemical species, and extra-cellular matrix components as well as sensing capabilities for both assuring quality during manufacturing and conducting long-term studies in the printed system. The research described herein can be generally categorized into three broad and interrelated categories: 1) manufacturing process
development and modeling, 2) materials formulation and synthesis, and 3) sensor characterization and implementation.

1.2 Objectives

The specific objectives of my thesis are as follows:

1. Improve 3D printing capabilities for manufacturing of hydrogel-based neural microphysiological systems through finite element analysis (FEA) of process physics and augmentation of 3D printing systems.

2. Expand the palette of available materials for 3D printing neural microphysiological systems by developing formulations with suitable rheological properties for microextrusion printing and mechanical and biochemical properties mimicking native neural tissue.

3. Develop and validate sensing capabilities for measuring cell quality (viability, type, and stemness) and hydrogel viscoelasticity both during and following the biomanufacturing process.

1.3 Contributions

Chapters 2 – 7 contain peer-reviewed contributions and chapters 8 and 9 contain contributions currently being prepared for, or undergoing, peer-review. The main contributions of this thesis are:

1. Found plasmonic properties could be achieved at macroscale in controlled distributions through integration of nanoparticles into 3D printed hydrogels using a new extrusion manifold. Manifold designs, experiments demonstrating plasmonic
distribution capabilities, and applications were reported in *Advanced Optical Materials* 5 (2017) 1700367. Details are in chapter 3.

2. Showed that microextrusion 3D printing could be used to fabricate pharmaceutical tablets with programmable distributions of active ingredients which corresponded to controlled release patterns. Modeling methods to predict changes in active ingredient concentration during manufacturing, demonstration of concentration distribution control, and release studies were reported in *Advanced Healthcare Materials* 7 (2018) 1800213. Details in Chapter 4.

3. Found that pick-and-place capabilities could be integrated into a printing system to allow incorporation of pre-fabricated electromechanical components during 3D printing. This approach was used to manufacture acoustofluidic devices for manipulation of microparticles. Design, experimental procedures, and demonstration of particle manipulation was previously reported in *Lab on a Chip* 18 (2018) 2087-2098. Details are in chapter 5.

4. Developed a hydrogel system using synthetic and biological macromolecules which exhibited Herschel-Bulkley fluid properties ideal for microextrusion 3D printing and could be cured into a gel with similar mechanical properties compared to natural neural extra-cellular matrix and supported high viability cell culture of multiple neural cell types. Synthesis, characterization, demonstration of 3D printing, and successful cell culture previously reported in *Biofabrication* 11 (2019) 025009. Details are provided in chapter 6.
5. Found that glioblastoma migration response towards a chemoattractant changes as a result of additional competing chemoattractants present, and that glioblastoma preferentially migrate towards epidermal growth factor (EGF) compared to bradykinin (BK) using a 3D printed migration assay. Design, modeling, and migration experiments were previously reported in *Advanced Biosystems* (2020) 1900225. Details are provided in chapter 7.

6. Found that electrode-functionalized hollow fibers could be used as extrusion tips for 3D bioprinting and extrusion of cell-laden hydrogel suspensions and provided an online impedance sensor to characterize cell quality such as cell type, viability, and stemness as described in a manuscript currently under preparation for submission to *Biofabrication*. Details are provided in chapter 8.

7. Showed that millimeter scale dynamic-mode piezoelectric cantilever resonance persists in polymer solutions and gels which allowed sensing of hydrogel viscoelastic properties through empirical and first principles approaches across the sol-gel transition providing valuable information into gelation dynamics and a potential high throughput approach for characterizing gel viscoelasticity as described in a manuscript currently under review at *Journal of Rheology*. Details are provided in chapter 9.

systems currently published in Biosensors and Biodetection (Springer, 2017) and Organ-on-a-Chip (Elsevier, 2020), respectively.

2.1. Abstract

Translational challenges associated with reductionist modeling approaches, as well as ethical concerns and economic implications associated with small animal testing, drive the need for developing microphysiological neural systems for modeling human neurological diseases, disorders and injuries. Here we provide a comprehensive review of microphysiological neural systems on a chip (NSCs) for modeling higher order trajectories in the human nervous system. Societal, economic, and national security impacts of neurological diseases, disorders and injuries are highlighted to identify critical NSC application spaces. Hierarchical design and manufacturing of NSCs are discussed with distinction of surface- and bulk-based systems. Three broad NSC classes are identified and reviewed: microfluidic NSCs, compartmentalized NSCs, and hydrogel NSCs. Emerging areas and future directions are highlighted, including the application of 3D printing to design and manufacturing of next-generation NSCs, the use of stem cells for constructing patient-specific NSCs, and the application of human NSCs to ‘personalized neurology’. Technical hurdles and remaining challenges are discussed. This review identifies the state-of-the-art design methodologies, manufacturing approaches, and performance capabilities of NSCs.
This work suggests NSCs appear poised to revolutionize the modeling of human neurological diseases, disorders and injuries.

2.2. Introduction

2.2.1. Societal Impacts of Neurological Diseases and Disorders: Neurological diseases, disorders and injuries (NDDIs) cause significant mortality rates and quality of life losses worldwide.\textsuperscript{[1]} For example, the World Health Organization has recently determined that 8 of 10 disorders in the highest disability class are neurological disorders.\textsuperscript{[2]} According to the American Academy of Neurology, genetic and infectious diseases and disorders of the nervous system currently affect over 6.4 million people in the United States (US) alone. In fact, stroke and Alzheimer’s disease are currently the third and sixth leading cause of death in the US, respectively.\textsuperscript{[3, 4]} Furthermore, the number of Americans diagnosed with Alzheimer’s disease is rising and expected to reach 16 million by 2050.\textsuperscript{[5]} Neurological disorders resulting from injury also pose critical problems. For example, according to the Centers for Disease Control and Prevention each year over 1.7 million people are currently living with a TBI.\textsuperscript{[6]} Several recent studies have also estimated that \textit{ca.} 20,000 new cases of spinal cord injury occur annually in the US with \textit{ca.} 270,000 currently living survivors.\textsuperscript{[6]} In addition to TBIs and spinal cord injuries, it has been estimated that over 200,000 peripheral nerve repair procedures are performed annually in the US to treat NDDIs that affect the peripheral nervous system.\textsuperscript{[7]}

Importantly, NDDIs also have significant impact on the economy and national security. For example, the direct healthcare costs associated with stroke and TBI alone in the US have been estimated to be greater than $46 billion US dollars (USD) per year with
indirect costs associated with work and productivity loss estimated near $91 billion USD per year.[6] Additionally, the high cost and time associated with drug development for NDDIs also contributes to both direct and indirect healthcare costs.[8] High incidence of neurological disorders and injuries among the warfighter and veterans is also of great concern and has far reaching implications toward national security. For example, TBI and posttraumatic stress disorder (PTSD) have been recognized as the most common problem requiring medical intervention.[9] Ultimately, considering the likelihood of continued civil conflict worldwide[10] and trend toward an aging population,[11] the prevalence of NDDIs is projected to further increase. Thus, there is a critical need for novel cost-effective molecular, cellular, and device-based medicines and therapies for the prevention and treatment of NDDIs.[12]

2.2.2. Limitations of State of the Art Models for Neurological Diseases, Disorders and Injuries: Historically, medicines and therapies for NDDIs have been developed by extending discoveries based on tissue culture and small animal models to clinical application. For example, two-dimensional (2D) monolayer cultures remain a standard model for many applications, such as high throughput drug screening. However, such ‘reductionist approaches’ often fail to replicate higher order features and trajectories of the in vivo nervous system, and although incorporating more cell types or use of human cells can somewhat improve the realism of such models, this approach sacrifices robustness.[13, 14] This tradeoff between robustness and realism as well as overall limitations in achievable complexity currently impede the use of reductionist approaches for modeling higher order trajectories of the nervous system.
Presently, three-dimensional (3D) histotypic and organotypic slice cultures, such as brain slice cultures, and small animal models are gold standards for modeling higher order features and trajectories of the *in vivo* nervous system.\(^{[13, 14]}\) However, such models are typically not compatible with human cells, which can challenge the translational impact of associated results due to cross-species differences. Furthermore, ethical concerns and high cost associated with animal testing also drive the need for alternative approaches. Driven by these limitations, ‘microphysiological neural systems’ (MPNS) appear poised to advance our fundamental understanding of higher order structure, function, and disease of the human nervous system, therein providing novel platforms for developing next-generation medicines and therapies for NDDIs.

2.2.3. Microphysiological Neural Systems-on-a-Chip: MPNS are defined as 3D biological constructs that reproduce higher order features, parameters and trajectories of the *in vivo* nervous system. Importantly, MPNS design and manufacturing processes are compatible with human cells endowing them with unparalleled translational value. In general, microphysiological systems (MPS) can be broadly categorized as: 1) scaffold-free or 2) scaffold-based.\(^{[15]}\) A discussion of scaffold-free MPS is beyond the scope of this review; various excellent reviews can be found elsewhere.\(^{[16, 17]}\) Alternatively, scaffold-based MPS are versatile platforms based on a tissue chip concept. Tissue chips are micro-fabricated devices that mimic human physiological responses. Tissue chips typically integrate scaffolding, mechanical cues, biochemical cues, and topographical cues to recreate physiological conditions. Recently, chip-based MPS have been applied to a number of tissue
and organ systems and now show significant promise in modeling higher order trajectories of the brain and nervous system.

Chip-based MPNS, hereinafter referred to as neural systems on a chip (NSCs), are scaffold-based 2D or 3D culture systems that possess higher order structure or functionality of the nervous system. In addition to compatibility with human cells, the concept of constructing biological architecture on a versatile functional substrate (i.e. a chip) provides flexibility, robustness, and efficiency in controlling system parameters, monitoring and stimulation.[18-20] Furthermore, NSCs are also highly attractive from a design and manufacturing perspective. For example, NSCs can be constructed using state of the art computer-aided design and robotic-assisted biofabrication approaches. Researchers have recently demonstrated that novel NSCs can be created using 3D printing techniques.[21] Given such desirable characteristics, highly biomimetic NSCs are now emerging to model higher order features and trajectories of the human nervous system. Here we provide a critical review of the state of the art of NSCs. Neural system on a chip (NSC) designs, manufacturing approaches, and applications to pathophysiological modeling of NDDIs are comprehensively reviewed. We also highlight emerging trends and techniques, technical challenges, and future directions.

2.3. Bio-inspired Design of Neural Systems on a Chip for Structure, Function and Disease

Design and engineering of NSCs is non-trivial as reproducing higher order neurophysiology or -pathophysiology requires realistic modeling of human neural anatomy, circuitry and microenvironmental parameters. Furthermore, the hierarchal structure and
function of the nervous system imposes additional design challenges. As a result, NSCs are designed using a ‘structure-function-disease’ heuristic. The typical bio-inspired design approach is multi-step process consisting of identifying the required anatomical and functional features needed for controlling system parameters toward a desired higher order trajectory.

2.3.1. Hierarchical Design toward Higher Order Neural Trajectories: NSCs typically possess one or more higher order anatomical features. As shown in Figure 2.1.A, such features include: 1) cellular heterogeneity; 2) clustering of multiple cell types; 3) spatial alignment of cell bodies; 4) spatial alignment of neurites; 5) controlled distribution of extracellular matrix (ECM); and 6) three-dimensionality. These features form the basis of complex anatomical systems found in NSCs. As shown in Figure 2.1.B, such systems include: 1) circuits of neuronal cells (e.g. used in Parkinson’s or Alzheimer’s disease modeling); 2) ensembles of neuronal and glial cells (e.g. used in brain tumor modeling); 3) ensembles of neuronal and other non-neuronal cells (e.g. used in neuromuscular junction modeling); and 4) ensembles of glial and other non-neuronal cells (e.g. used in models of the blood-brain barrier (BBB)).
Figure 2.1 Hierarchical design of neural systems on a chip toward assembly of higher order functional neural anatomical systems. Anatomical features (a) are assembled into anatomical systems (b) which ultimately contain functional features (c) that control microenvironmental parameters.
In addition to higher order anatomical features and systems, NSCs typically possess one or more higher order functional or augmented features. As shown in Figure 2.1.C, such features include: 1) fluidic channels, 2) controlled drug release systems, and 3) electroactive components. These features serve to program and control higher order microenvironmental parameters including: 1) mass transport of solutes; 2) static and dynamic mechanical stresses; and 3) spatiotemporal distributions of biochemical cues. Such features also enable the stimulation and monitoring of biology. Ultimately, as shown in Figure 2.2, the microenvironmental parameters established by functional or augmented features govern the higher order trajectories of realistic anatomical systems. Typical trajectories include: 1) cell and neurite outgrowth; 2) cell migration; 3) cell signaling; 4) circuit mapping; 5) phenotypic outcomes; and 6) gene expression. Thus, NSCs are a disruptive cell culture platform for the study of human NDDIs. However, the extent and flexibility by which the structure-function-disease design heuristic can be implemented depends on both the scaffold design and the manufacturing approach.
Figure 2. Hierarchical design of neural systems on a chip toward control of neural microenvironmental parameters and modeling of higher order trajectories. Control over microenvironmental parameters, such as transport of diluted species, mechanical stimulation, electrical stimulation, and spatiotemporal distribution of biochemical cues, through functional and augmented features, allows NSCs to model higher order trajectories of the human nervous system.

2.4. Manufacturing Approaches for Neural Systems on a Chip

Although design widely varies, NSCs are derived from the following components: 1) microchannels, 2) microchambers, 3) functionalized microdomains, 4) ECM, and 5) cells (see Figure 2.3). Microchannels have diverse application-dependent function. For example, microchannels are most commonly used to guide neurite outgrowth.\textsuperscript{[22, 23]} In some cases,
they also serve as scaffolds for assembling non-neuronal cells.\textsuperscript{[24]} In addition, microchannels are commonly used for fluid handling to provide perfusion\textsuperscript{[25]}, gradients of biochemical cues\textsuperscript{[26]}, and mechanical actuation\textsuperscript{[27]}. Microchambers are commonly used to spatially-isolate different cell types. Thus, they are useful components for guiding the formation of heterogeneous tissues.\textsuperscript{[28]} Functionalized microdomains are commonly used to spatially-control biochemical cue distribution, such as the patterning of ECM or growth factors. As a result, they are effective for controlling cell seeding locations and neurite outgrowth directions.\textsuperscript{[29]}
Figure 2.3 The fundamental building blocks of neural systems on a chip include microchannels (a), microchambers (b), functionalized microdomains (c), extracellular matrix (d), and cells (e).

Importantly, the ability to both make and assemble these building blocks is highly dependent on the manufacturing approach used. Thus, since these building blocks serve as the basis for constructing higher order anatomical and functional features, the manufacturing approach has a direct impact on the type of microenvironmental parameters that can be controlled and the resultant trajectories that can be modeled. Overall, NSC manufacturing consists of three steps: design, fabrication, and integration with biology (i.e. surface functionalization and cell seeding). However, these steps widely differ depending on the manufacturing process used and may include: 1) manual techniques, 2) computer-aided design (CAD), 3) medical imaging, and 4) computer-aided manufacturing (CAM). As a result,
the manufacturing process influences the robustness of the resultant NSC platform as discussed in greater detail in the following sections.

2.4.1. Manufacturing Processes: As shown in both Figure 2.4 and Table 2.1, five primary manufacturing processes are used to construct NSCs: 1) photolithography, 2) soft lithography, 3) contact printing, 4) laser patterning, and 5) 3D printing. The following techniques are briefly reviewed below.
a. Photolithography
   i. Oxidize top layer of silicon
   ii. Spin-cast negative photoresist
   iii. Selectively cure photoresist
   iv. Remove uncured photoresist
   v. Etch SiO₂
   vi. Remove cured photoresist

b. Soft lithography
   i. Creation of master
   ii. Pour on PDMS and cure
   iii. Peel off PDMS

c. Contact Printing
   i. Expose stamp to solution
   ii. Remove stamp from solution
   iii. Apply stamp to substrate
   iv. Remove stamp

d. Laser patterning
   i. Cure the bulk gel
   ii. Apply reactive biomolecules
   iii. Selectively react with laser
   iv. Wash excess biomolecules

e. 3D printing
   i. Create 3D CAD model
   ii. Prepare printing inks
      ECM
      Growth factors
      Cells
      Support material
   iii. 3D print
Figure 2. 4 Schematics of the five commonly used neural system on a chip manufacturing techniques: photolithography (a), soft lithography (b), contact printing (c), laser patterning (d), and 3D printing (e).

Table 2. 1 Summary of neural systems on a chip. Abbreviations: Hippocampal Neuron (HN), Cortical Neuron (CN), Motoneuron (MN), Neural Progenitor (NP), Schwann Cells (SC), Epithelial Cells (EC), Thalamic Neuron (TN), Peripheral Neuron (PN), Myoblast (MB), Astrocyte (AC), Oligodendrocyte (OC), Striatal Neuron (SN), Spinal Neuron (SPN), Myocyte (MC), Peripheral nervous system (PNS), Central Nervous System (CNS), External Tissue (EXT), Midbrain Dopaminergic Neuron (mDAN), Microglia (MG), Microelectrode Array (MEA), Polydimethylsiloxane (PDMS), Polycaprolactone (PCL), Polycarbonate (PC), Poly(ether imide) (PEI), Polytetrafluoroethylene (PTFE), Poly(ethylene glycol) diacrylate (PEGDA). Asterisk (*) indicates photolithography was also used.

<table>
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<th>Tissue</th>
<th>Design</th>
<th>Manufacturing</th>
<th>Materials</th>
<th>Length</th>
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<td>Brain</td>
<td>Compartmentalized</td>
<td>Soft lithography</td>
<td>PDMS</td>
<td>cm</td>
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<td>cm</td>
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<td>Isolation Method</td>
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<td>Soft lithography*</td>
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<td>Brain</td>
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<td>MEA</td>
<td>Microfabrication</td>
<td>Silicon</td>
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<td>Laser patterning</td>
<td>PDMS, PC</td>
<td>mm</td>
<td></td>
<td>Disease</td>
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</table>
2.4.1.1. Photolithography: Photolithography is a core process of microfabrication. The base material for photolithography is typically silicon. As shown in Figure 2.4.A, the first step involves oxidizing the wafer’s top surface followed by coating with a thin photoresist layer. A laser or photomask is subsequently used to selectively expose specific locations of the wafer to UV light, which initiates a photochemical reaction in the photoresist. The photoresist can then be selectively removed to expose specific locations of the wafer to subsequent chemical etchants. For example, hydrofluoric acid is often used to remove the exposed silicon oxide. The final step involves removing, also referred to as ‘stripping’, the remainder of photoresist from the wafer by exposure to chemical solutions, such as sulfuric acid. We note that many variations to the process have been developed and reviewed.
Advantages: High precision lasers and photomasks designed with CAD software allow highly precise designs. Subsequent etching or material deposition steps yield complex electronic systems. Disadvantages: Photoresist curing and material removal steps typically require high temperature, extreme pH, and exposure to radiation. As a result, the manufacturing process does not support simultaneous integration with biology during platform fabrication.

2.4.1.2. Soft Lithography: Soft lithography is a type of polymer casting process and has been extensively used for the fabrication of microfluidic devices. The process begins with creation of a rigid mold, commonly referred to as a ‘master’, using photolithography. This process transfers the geometric pattern of the photomask to the master. As shown in Figure 2.4.B, an elastomeric polymer, commonly polydimethylsiloxane (PDMS), is then cast onto the master and solidified via crosslinking. The solidified elastomeric material, which contains an imprint of the master, is then released from the mold. Typical features include open microchannels, chambers, pads and pillars. To form microfluidic devices, the solid elastomeric molds are bonded to rigid substrates, such as glass or plastic, thereby forming sealed channels. Advantages: Soft lithography is a high precision manufacturing process due to the use of photolithography for creating the master. Thus, it enables precision manufacturing of NSC building blocks, such as microchannels. The photomask creation step is also compatible with CAD resources. Disadvantages: Given soft lithography requires utilizes photolithography for master creation, the disadvantages of photolithography apply to soft lithography. Elastomer crosslinking also typically requires elevated temperatures...
and is impeded by moisture. As a result, similar to photolithography, the manufacturing process does not support simultaneous integration with biology during platform fabrication.

2.4.1.3. Contact Printing: Contact printing is a material deposition process and has been used extensively for functionalizing substrates for cell culture applications. The process begins with creation of an elastomeric stamp using soft lithography. As shown in Figure 2.4.C, the stamp is then coated with the desired adsorbate through exposure to an analyte-containing solution. Subsequently, the stamp is brought into mechanical contact with the substrate, thereby transferring the adsorbate from the stamp to the substrate. After transfer, the stamp is removed, resulting in a substrate functionalization pattern that matches the geometric pattern of the stamp. Advantages: Contact printing has similar advantages to soft lithography (e.g. precision and compatibility with CAD). Contact printing is also compatible with a wide range of analytes as the transfer mechanism is based on adsorption.\textsuperscript{[29, 58]} Contact printing can also create periodic functionalized microdomains across macroscopic length scales.\textsuperscript{[58]} Disadvantages: Repeated contact printing on the same substrate is challenging as the mechanical contact step is typically done manually. Contact printing can only be used to deposit small molecules and biomacromolecules, but faces challenges with depositing larger biologics, such as animal cells. Contact printing can only facilitate deposition of thin material layers.

2.4.1.4. Laser Patterning: Laser patterning in the scope of this review is a technique used to selectively bind biomolecules to a hydrogel in 3D.\textsuperscript{[61, 103]} Although varying mechanisms exist, all processes involve the laser-triggered reaction of photolabile groups within a hydrogel (see Figure 2.4.D). The most common technique is to bind mono- or di-
acrylated peptides to polyethylene glycol diacrylate (PEGDA) hydrogels given PEGDA hydrogels inherently contain unreacted acrylate groups from the curing step.\textsuperscript{[103, 104]} Another approach involves the laser-triggered cleavage of photolabile bonds, which exposes new reactive groups for selectively bonding biomolecules to the hydrogel.\textsuperscript{[61]} \textbf{Advantages:} The ability to spatially control 3D hydrogel chemistry offers unique advantages for directing cell growth in hydrogel NSCs.\textsuperscript{[61]} \textbf{Disadvantages:} Only hydrogels and biomolecules functionalized with photoreactive groups can be used, which significantly limits material availability.

2.4.1.5. \textit{3D Printing:} 3D printing is a biomanufacturing process and has been used extensively in tissue engineering applications. Various types of 3D printing processes exist, including stereolithography, inkjet printing, micro-extrusion printing and laser-assisted bioprinting. A detailed description of the individual processes can be found elsewhere.\textsuperscript{[105, 106]} While multiple types of 3D printing processes exist, they differ in terms of material deposition mechanism, process physics, material compatibility, multi-material printing capability, manufacturing speed, and precision. As shown in Figure 2.4.E, the first step to 3D printing is constructing the path information that describes the motion and triggering of the printing tool (\textit{e.g.} a laser or extruder) from a 3D digital model. The next step in 3D printing is the formulation of a printable material, commonly referred to as an “ink”. Subsequently, the ink is loaded into a dispensing tool or holding reservoir and the printing process is initiated. This enables the conversion of the 3D digital model to a physical object. \textbf{Advantages:} Digital models, and thus printer path information, can be derived from medical imaging data. Unlike contact printing, 3D printing is a CAM process. This aspect affords repeatability and robustness in multi-layer and -material assembly. Certain types of 3D
printing, such as micro-extrusion printing, are compatible with a diverse materials palette including thermosets, thermoplastics, composites, hydrogels, and solutions. 3D printing offers a one-pot biomanufacturing approach for directly interweaving biology with scaffold and functional materials. Disadvantages: 3D printing is currently a serial processing technique. Thus, throughput can be limited for large parts that contain intricate path geometries. However, we note that the development of advanced dies that accommodate the simultaneous printing of multiple parts can address this limitation similar to prior advancements in injection molding processes.

2.5. Classes of Neural Systems on a Chip

NSCs are scaffold-based architectures. Thus, they require cell seeding either on the surface or within the bulk of an exogenous material. As a result, NSCs differ with respect to the degrees of freedom for resultant trajectories, such as growth and migration. Importantly, the desired features and the manufacturing approach influence the scaffold design. In general, NSCs fall into one of two categories: 1) surface-based designs and 2) bulk-based designs. Surface-based designs are those that seed cells on the surface of an exogenous material. Thus, mono- or multi-layer cell growth, cell migration, cell-cell interactions and cell-matrix interactions occurs in 2D at the solid-liquid interface between the exogenous material and growth medium. Microfluidic and compartmentalized NSCs are the most common type of surface-based designs. In contrast, bulk-based designs seed cells within the bulk of an exogenous material. Thus, cell growth, cell migration, cell-cell interactions and cell-matrix interactions occurs in 3D within the bulk of the growth medium-infused exogenous material. Hydrogel NSCs are the most common type of bulk-based designs.
2.5.1. Microfluidic Neural Systems on a Chip: Microfluidic NSCs are surface-based designs based on isolated or interacting microfluidic channels (see Figure 2.5). The microfluidic channels are typically rectangular and range 1 - 500 µm in width and 2 - 25 mm in length. The microfluidic channels typically have three walls that arise from the bulk polymer material (e.g. PDMS) and one wall (the bottom wall) that arises from the substrate (e.g. glass or tissue culture plastic). In some cases, the bottom wall consists of a porous membrane that enables the cells in two adjacent microchannels to chemically interact. For example, one common design involves two co-directional microchannels separated by a polycarbonate transwell membrane.[82, 86, 91] **Advantages:** The ability to use transparent materials for microfluidic construction provides ease in optical characterization and stimulation techniques. Microfluidic NSCs also support fluid handling, which has various advantages including establishing: 1) convective flow of nutrients and biochemical cues[92], 2) diffusive profiles of biochemical cues[26], and 3) mechanical cues[90], such as shear stress or dynamic scaffold deformation. Additionally, the ability to utilize a wide range of substrates, such as conductive materials[97], offers the ability to establish unique functional and augmented features for stimulation and monitoring. **Limitations:** Given microfluidic NSCs are surface-based designs, the degrees of freedom for cell growth and migration are limited relative to bulk-based designs (i.e. to 2D instead of 3D). High order trajectories related to or derived from 3D cell growth, cell migration, cell-cell interactions and cell-matrix interactions are difficult to model using microfluidic NSCs. Further, microfluidic NSCs typically utilize simplistic surface functionalization approaches, such as surface coating with
adsorbed ECM components. Thus, it is relatively challenging to replicate native cell-matrix interactions using microfluidic NSCs.

Figure 2.5 Highlights of microfluidic neural systems on a chip (NSCs). a) Microfluidic NSC for studying differentiation of neural progenitor cells under the influence of chemical gradients. i) Schematic of a Shh/FGF8 or Shh/BMP4 gradient microfluidic device; ii) visualization of gradient; and iii) immunoassay of TuJ1 to quantify cell clusters and neurite
b) Microfluidic NSC for Alzheimer's disease studies. i) Schematic of β-amyloid gradient device; ii) live/dead assays of sections 1-5 with intensity plots and slopes shown on the right; and iii) imaging of synapsin-II distribution. Reprinted with permission.[89] Copyright John Wiley & Sons 2009.

Microfluidic NSC for modeling the blood-brain barrier (BBB). i) Schematic of the BBB; ii) live/dead stain of endothelial cells; iii) immunoassay of tight junction ZO-1 in endothelial cells; iv) immunoassay of GFAP in astrocytes; and v) environmental scanning electron micrograph of astrocytes. Reprinted with permission.[82] Copyright Royal Society of Chemistry 2012.

2.5.1.1. Manufacturing of Microfluidic Neural Systems on a Chip: Microfluidic NSCs consist of a microchannel-containing elastomeric cast bonded to a rigid substrate. Microfluidic NSCs are typically manufactured using a combination of photolithography and soft lithography (see Sections 2.4.1.1 and 2.4.1.2, respectively).[108,109] However, we also note that 3D printing has recently emerged as a technique for manufacturing microfluidic devices and NSCs (see Section 2.4.1.5).[21,60,110-112] In cases of microfluidic NSCs that contain convective fluid flow, microneedles are inserted to the inlet and outlet ports to interface with fluid handling components and instrumentation (e.g. tubing and pumps).

Microfluidic NSCs are typically functionalized by first exposing the fluidic channels to ECM-containing solutions in a static or flow mode. Subsequently, the coated fluidic channels are exposed to cell suspensions in a static or flow mode to seed cells on the coated surfaces, thereby resulting in a cell mono-layer. Cellular growth strongly follows topographical cues. If required, the process may be repeated to form multi-layers in the same microchannel (e.g. to establish a feeder layer).

2.5.1.2. Hierarchical Design of Microfluidic Neural Systems on a Chip: Given the ease of designing and fabricating microchannels via soft lithography, active fluid handling capability,
and flexibility with using alternative substrate materials, various higher order anatomical features of native neural systems can be reproduced using microfluidic NSCs (see Table 2). For example, microfluidic NSCs have been used to affect the: 1) clustering of different cell types; 2) spatial alignment of cell bodies; and 3) spatial alignment of neurites. Microfluidic NSCs have also been designed to reproduce higher order functional and augmented features given the ability to integrate: 1) fluidic channels, and 2) electroactive components.[25, 51, 52, 96-98] For example, Bianco et al. developed a microfluidic system in which neuroinflammation could be simultaneously monitored by microscopy and electrophysiological recordings.[79] As a result, microfluidic NSCs offer programing and control of various higher order microenvironmental parameters such as: 1) convective mass transport of solutes; 2) actuation of static and dynamic mechanical stresses; 3) spatiotemporal distributions of biochemical cues; and 4) stimulation and monitoring of cells. For example, Griep et al. have demonstrated that shear stress (5.8×10⁻¹ Pa) is an important parameter for improving endothelial cell function through tight junction formation in BBB models.[86] As shown in Figure 2.5.A, Park et al. used a microfluidic device to control spatiotemporal distributions of three different cytokines (Shh, FGF8, and BMP4) ranging from 0 - 500 ng/ml to affect the differentiation of neural progenitor cells.[89] Various studies have also used microfluidics to support the perfusion of tissues for long term studies.[81, 92, 113] Thus, microfluidic NSCs are useful platforms for modeling higher order trajectories of native neural systems such as: 1) neurite outgrowth; 2) cell migration; 3) cell signaling; and 4) gene expression. Chung et al. have used a microfluidic device to direct the proliferation and differentiation of human neural stem cells via gradients of epidermal growth factor, fibroblast growth factor 2, and
platelet-derived growth factor established by laminar flow and diffusive mixing.\textsuperscript{[26]} As shown in Figure 2.5.B, Booth \textit{et al.} developed a BBB model that showed endothelial cells began to express tight junction in flowing media at 2.6 µL/min after three days via zonula occludens-1 (ZO-1) imaging.\textsuperscript{[82]} Deosarkar \textit{et al.} also showed that endothelial cells exhibited tight junction formation, as measured by the expression of ZO-1 in microfluidic BBB models, and allowed endfeet-like neonatal astrocyte-endothelial cell interactions through a porous interface.\textsuperscript{[99]} Although beyond the scope of this review, we note that microfluidic tissue chips have been applied toward a number of organ systems.\textsuperscript{[18, 19, 114]} As a result, the microfluidic-based tissue chip design is commonly referred to as an ‘organ-on-a-chip’; however, we caution the reader that the organ-on-a-chip concept does not strictly apply to microfluidic designs as alternative design and manufacturing approaches now exist for constructing tissue chips (\textit{e.g.} 3D printing\textsuperscript{[24]} and molding\textsuperscript{[115]}).
Table 2.2 Summary of microfluidic neural systems on a chip. Abbreviations: Endothelial Cell (EC), Neural Progenitor Cell (NP), Extracellular Matrix (ECM).

<table>
<thead>
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<th>Study</th>
<th>Motivation</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
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<td>Neural differentiation in a co-culture with ECs</td>
<td>Developing a modular blood brain barrier model</td>
<td>[78]</td>
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<tr>
<td>Neuron viability following beta-amyloid insult</td>
<td>Neuroinflammation in different brain regions</td>
<td>[79]</td>
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<td>Perfusion optimization for brain slices</td>
<td>Improving brain-on-chip environment</td>
<td>[81]</td>
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<td>TEER response to histamine exposure</td>
<td>Blood brain barrier model</td>
<td>[82]</td>
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<tr>
<td>Application of oligomeric amyloid beta to neurons</td>
<td>Alzheimer’s disease</td>
<td>[83]</td>
</tr>
<tr>
<td>Neuronal response to behavior and olfactory stimulation</td>
<td>Developing a device for monitoring neural activity</td>
<td>[84]</td>
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<td>Application of growth factor gradients to NPs</td>
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<td>[26]</td>
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<td>Monitoring of axonal response to neural injury</td>
<td><em>In vivo</em> monitoring of neural injury</td>
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<td>Monitoring TEER response to shear</td>
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<tr>
<td>Brain slice survival with local microperfusion</td>
<td>Long-term brain slice studies</td>
<td>[92]</td>
</tr>
<tr>
<td>Application of potassium to induce cortical spreading depression</td>
<td>Brain injury and migraines</td>
<td>[93]</td>
</tr>
<tr>
<td>EC drug permeability in astrocyte-conditioned medium</td>
<td>Developing a platform for modeling drug delivery</td>
<td>[94]</td>
</tr>
<tr>
<td>Applying tacrolimus to regenerating nerves</td>
<td>Nerve regeneration</td>
<td>[95]</td>
</tr>
<tr>
<td>Monitoring glioma ECM remodeling</td>
<td>Brain tumors</td>
<td>[25]</td>
</tr>
</tbody>
</table>
2.5.2. Compartmentalized Neural Systems on a Chip: Compartmentalized NSCs are surface-based designs based on interconnected compartmentalized culture chambers (see Figure 6). The design supports the manual addition of media to individual chambers for studies in static fluid or under gravity-perfusion. Compartmentalized NSCs typically have channels ranging from 1 - 5 mm in width and 5 - 20 mm in length for compartmentalizing cell bodies, and microchannels ranging from 1 - 5 µm in width and 100 - 1,000 µm in length for directing axonal growth or creating diffusion gradients.\[22, 23\] For example, the most commonly used design consists of two compartmentalization channels separated by microchannels, as shown in Figure 2.6.A.\[22, 33, 37, 40, 44, 46\] In contrast to microchannels found in microfluidic NSCs, microchannels in compartmentalized NSCs may not contain a top wall, such as in Campenot chamber designs. Similar to microfluidic NSCs the bottom wall is formed by the underlying substrate (e.g. a 35 mm dish). Importantly, the primary distinction between microfluidic and compartmentalized NSCs is the absence of active fluid handling in the latter design. Compartmentalized NSCs also offer the ability to incorporate controlled release systems within the cell compartments, such as loaded hydrogels or microparticles, as the millimeter-sized chamber accommodate the manual incorporation of controlled release systems and passive release mechanisms.\[30, 40, 45\] Compartmentalized NSCs are
maintained using conventional manual cell culture techniques. **Advantages:** The separation of cell chambers via microchannels enables the co-culture of multiple cell types as well as the use of multiple media types and biochemical cues in the same platform. This affords the ability to study the interaction between cells that require significantly different biochemical cues as well as to restrict cellular interaction to neurite-based signaling and transport.\(^{29, 35, 41}\) Further, this feature enables the selective stimulation and inoculation of cells in a single chamber, which has importance for fundamental circuit mapping studies and modeling a wide range of NDDIs. **Limitations:** The type of cell-cell interactions are relatively restricted to those that occur through neurites. Similar to microfluidic NSCs, the fact that compartmentalized NSCs are surface-based designs makes high order trajectories related to or derived from 3D cell growth, cell migration, cell-cell interactions and cell-matrix interactions difficult to model.
Figure 2. 6 Highlights of compartmentalized neural systems on a chip (NSCs). a) Compartmentalized NSC for central nervous system axonal injury, regeneration and transport. i) Prismatic and cross-sectional views of a somal–axonal compartmentalized NSC; ii) demonstration of fluidic isolation with Texas Red dye, scale bar is 100 µm; and iii) application of Green Cell Tracker to the axonal side with backtracked identification of neurons in the somal chamber. Reprinted with permission[22] Copyright Nature Publishing Group 2005. b) Compartmentalized NSC for the neuromuscular junction. i) Schematic of a compartmentalized NSC for development of NMJs; ii) spinal cord motoneurons plated in the proximal channel extend axons into the distal channel to contact myotubes. Reprinted with permission[34] Copyright Elsevier 2016.
2.5.2.1. **Manufacturing of Compartmentalized Neural Systems on a Chip:**

Compartmentalized NSCs consist of a multi-chamber bonded to a rigid substrate. Depending on the design, the multi-chamber is typically composed of either PDMS or Teflon (e.g. Campenot chamber designs), but can also be made of soft materials, such as hydrogels. Thus, the microchannels and microchambers are typically fabricated via molding processes, as PDMS and Teflon can be molded at relatively low temperatures using soft lithography (see Section 2.4.1.2), extrusion, pressing or injection molding. It was also recently demonstrated that compartmentalized NSCs can be fabricated using 3D printing, which enables the rapid prototyping of microchannel and microchamber design and geometry.[24] Although chamber-substrate bonding process is typically done manually, 3D printing approaches have recently emerged as a one-pot bottom-up biomanufacturing process for compartmentalized NSCs.[24]

Compartmentalized NSCs are typically functionalized prior to chamber-substrate bonding using standard manual surface coating approaches (i.e. exposure to solutions containing soluble ECM components). Thus, contact printing can also be used to create patterned functionalized microdomains prior to chamber-substrate bonding (see Section 2.4.1.3). Cell seeding is accomplished by conventional manual plating techniques (i.e. pipetting of cell suspensions into the cell compartments). Similar to microfluidic NSCs, cellular growth strongly follows topographical cues.

2.5.2.2. **Hierarchical Design of Compartmentalized Neural Systems on a Chip:** Given the ability to co-culture multiple cell types and program cell-cell interactions via guided neurite outgrowth, various higher order anatomical features of native neural systems can be
produced using compartmentalized NSCs (see Table 2.3). For example, compartmentalized NSCs have been used to affect the: 1) formation of heterogeneous tissues; 2) clustering of multiple cell types; 3) spatial alignment of cell bodies; and 4) spatial alignment of neurites. For example, as shown in Figure 2.6.B, Ionescu et al. developed a compartmentalized NSC for study of the neuromuscular junction, which enabled the monitoring of muscle-neuron communication.[34] Berdichevsky et al. used a compartmentalized NSC to co-culture cortical and hippocampal neurons separated by microgrooves to monitor the development of neurite pathways.[49] A compartmentalized NSC containing a cell-laden hydrogel component was used by Shin et al. to co-culture endothelial and neural progenitor cells in a 3D environment allowing them to study the effect of vasculature on neural progenitor cell differentiation.[42] Compartmentalized NSCs have also been designed to reproduce higher order functional and augmented features given the ability to integrate: 1) controlled drug release systems, and 2) electroactive components. For example, although not a controlled drug release system, Millet et al. demonstrated a microfluidic-based surface functionalization approach for compartmentalized NSCs to enable the study of laminin and poly-L-lysine gradients on neurite outgrowth.[29] Johnson et al. demonstrated that 3D printing could be used to guide neurite outgrowth in compartmentalized NSCs onto conductive grids to support transmission electron microscopy measurements of single axons.[21] As a result, compartmentalized NSCs offer programming and control of various higher order microenvironmental parameters such as: 1) spatiotemporal distributions of biochemical cues; and 2) stimulation and monitoring of cells. For example, Johnson et al. developed a 3D printed compartmentalized NSC with spatially
segregated distributions of nerve growth factor and Schwann cell media to study Schwann cell-axon self-assembly and axon-to-cell viral spread.[24] Ch’ng et al. used a compartmentalized NSC to study the spread of viral infection between neuronal and epithelial cells via axonal transport.[116] Thus, compartmentalized NSCs are useful platforms for modeling higher order trajectories of native neural systems such as: 1) neurite outgrowth and tissue self-assembly; 2) cell migration; 3) cell signaling; 4) circuit mapping, and 5) gene expression profile. For example, one of the most common uses of compartmentalized NSCs is to establish aligned neurite outgrowth. This is a useful technique for both studying neurite physiology and pathophysiology, such as structure, transport, degradation, regeneration, depolarization, as well as establishing neural circuits.[39] Southam et al. directed neurite outgrowth from a neuronal and glial compartment into a chamber containing skeletal muscle cells to form neuromuscular junctions.[28] Liu et al. used a compartmentalized NSC to track pseudorabies virus transport between cell bodies and axons.[35] A similar compartmentalized NSC was used by Taylor et al. to track viral spread from infected neurons along neurites through size exclusive barriers into a separate epithelial cell-containing compartment using fluorescence microscopy.[117] Bérangère et al. have shown that compartmentalized NSCs can also be used to study the effect of biochemical cues on neurite pathophysiology, such as the dying back of axons exposed to β-amyloid (Aβ).[32] Compartmentalized NSCs have also provided useful platforms for investigating the effect of mechanical injury on neural system components. Siddique et al. developed a compartmentalized NSC that supports co-culture of spinal cord and peripheral nerves for studying the effect of growth factors on axonal regeneration following mechanical injury.[43]
Koyuncu et al. showed although pseudorabies virus induces protein synthesis to enable retrograde virus transport, damaging axons prior to infection decreases virion transport, suggesting that virus particles and damage signals compete for retrograde transport.[118]

Shin et al. used gene expression analysis to study the effect of neural progenitor-endothelial cell interaction on neural progenitor cell morphology and differentiation.[42]

**Table 2.3** Summary of compartmentalized neural systems on a chip. Abbreviations: Central Nervous System (CNS), Peripheral Nervous System (PNS), Neuromuscular Junction (NMJ), Glial-derived Neurotrophic Factor (GDNF).

<table>
<thead>
<tr>
<th>Study</th>
<th>Motivation</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variation of matrix crosslink density and orientation</td>
<td>A new platform for drug discovery</td>
<td>[30]</td>
</tr>
<tr>
<td>Application muscimol to a neural network</td>
<td>Synaptic competition</td>
<td>[31]</td>
</tr>
<tr>
<td>Application of beta-amyloid to a cortico-hippocampal network</td>
<td>Synapse die-back, Alzheimer’s disease</td>
<td>[32]</td>
</tr>
<tr>
<td>Measurement of excitotoxin induced degradation of axons</td>
<td>Alzheimer’s disease and brain injuries</td>
<td>[33]</td>
</tr>
<tr>
<td>Observation neuromuscular junction formation and activity</td>
<td>Development of a NMJ model on a chip</td>
<td>[34]</td>
</tr>
<tr>
<td>Observation of viral transport and gene expression</td>
<td>Demonstrating the application of 3D printing for NSCs</td>
<td>[24]</td>
</tr>
<tr>
<td>N/A</td>
<td>CNS/PNS regeneration</td>
<td>[23]</td>
</tr>
<tr>
<td>Observation of neural infection and neuron to cell infection</td>
<td>Understanding the mechanism neural infection</td>
<td>[35]</td>
</tr>
<tr>
<td>Monitoring movement of labeled mitochondria in axons</td>
<td>Axon degeneration, neurodegenerative diseases</td>
<td>[36]</td>
</tr>
<tr>
<td>Studying neural development in response to gradient cues</td>
<td>Guiding neuron development</td>
<td>[29]</td>
</tr>
<tr>
<td>N/A</td>
<td>Improving brain on chip capabilities</td>
<td>[37]</td>
</tr>
<tr>
<td>Investigation of myelination of axons by isolated oligodendrocytes</td>
<td><em>In vitro</em> modeling of glia/axon interaction</td>
<td>[38]</td>
</tr>
<tr>
<td>Addition of astrocytes to established networks</td>
<td>A new platform for neural co-cultures</td>
<td>[39]</td>
</tr>
<tr>
<td>Local exposure of biomolecular cues to neurons</td>
<td>Understanding axonal growth</td>
<td></td>
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<tr>
<td>-----------------------------------------------</td>
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<td></td>
</tr>
<tr>
<td>Stimulation of neural network formation</td>
<td>Neural network construction</td>
<td></td>
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<tr>
<td>Differentiation in a vascular microenvironment</td>
<td>Improving brain microenvironment</td>
<td></td>
</tr>
<tr>
<td>Monitoring nerve regeneration</td>
<td>Nerve regeneration</td>
<td></td>
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<tr>
<td>Monitoring amyloid beta transmission in neural networks</td>
<td>Alzheimer’s disease</td>
<td></td>
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<tr>
<td>Observation of the formation of neuromuscular junctions</td>
<td>Drug screening and motor neuron pathophysiology</td>
<td></td>
</tr>
<tr>
<td>Application of 3- and 4-repeat tau protein to neural cultures</td>
<td>Alzheimer’s disease</td>
<td></td>
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<tr>
<td>Isolation of axonal mRNA</td>
<td>Axonal injury and regeneration</td>
<td></td>
</tr>
<tr>
<td>Imaging of compartmentalized neurons</td>
<td>Synapse visualization and manipulation</td>
<td></td>
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<tr>
<td>Observation of the formation of a NMJ</td>
<td>A new NMJ model on a chip</td>
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<tr>
<td>Application of GDNF to a neuromuscular co-culture</td>
<td>Development of neuromuscular junctions</td>
<td></td>
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<tr>
<td>Recording activity between cortical and thalamic neurons</td>
<td>Cortical and thalamic connectivity</td>
<td></td>
</tr>
<tr>
<td>Recording activity between cortical and thalamic neurons</td>
<td>Isolating networks in a controlled environment</td>
<td></td>
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<tr>
<td>Development and activity of connections between brain slices</td>
<td>Understanding neural pathways</td>
<td></td>
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<tr>
<td>Measuring muscular contraction in response to neural stimulation</td>
<td>Drug screening</td>
<td></td>
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<tr>
<td>3D neural network patterning control through thermal etching</td>
<td>Studying neuron-glial signaling, drug screening</td>
<td></td>
</tr>
<tr>
<td>Manipulating neural network connections through thermal etching</td>
<td>Individual-cell electrophysiological monitoring</td>
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</tr>
<tr>
<td>Individual-cell measurements of a controlled neural network</td>
<td>A new platform for neural network research</td>
<td></td>
</tr>
<tr>
<td>Monitoring spontaneous firing among spatially controlled networks</td>
<td>Investigating neural network function</td>
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</table>
2.5.3. Hydrogel Neural Systems on a Chip: Hydrogel NSCs are bulk-based designs involving the growth of suspended cells within a 3D hydrogel scaffold (see Figure 2.7). Hydrogel NSCs are typically a few hundred micrometers in thickness and may extend millimeters to centimeters in width. As shown in Table 1, hydrogel based NSCs currently make up less than 10% of research efforts, however, this is likely to increase with the emergence of biomanufacturing approaches, such as 3D printing. Hydrogels are formed from either synthetic or natural polymers. This feature enables the selection and design of hydrogels to mimic the native human ECM of neural tissue. For example, many naturally derived hydrogels used for NSCs are based on collagen, hyaluronic acid, or matrigel.[57, 59, 60] Primary cells and cell lines are used to construct hydrogel NSCs. Hydrogel NSCs are also compatible with spheroids, which enables further design of biomimetic cell-cell interactions. 

Advantages: Hydrogel NSCs are 3D architectures. Thus, they enable the study of high order trajectories related to or derived from 3D cell growth, cell migration, cell-cell interactions and cell-matrix interactions.[61] An important distinguishing feature of hydrogel NSCs is the flexibility to design heterogeneous tissues through: 1) the suspension of multiple cell types within a single hydrogel phase, 2) the adjacent crosslinking of cell-laden hydrogels that contain different cell types, or 3) the adjacent crosslinking of hydrogels that contain different ECM compositions. Such features enable hydrogel NSCs to examine the effect of multiple higher order parameters, such as ECM composition and biochemical cue distributions, on the growth of heterogeneous neural tissues. Given hydrogel NSCs consist of 3D architectures and are derived from extrudable cell- and biochemical cue-laden hydrogels, 3D printing approaches can be leveraged to model novel higher order trajectories of the human nervous
**Limitations:** The physical dimensions of hydrogel NSCs are constrained by the ratio of the bioconversion rate to the diffusion rate, which can be described by the Damköhler number. For hydrogel NSCs, the effective diffusivity of the rate-limiting substrate in the cell-laden hydrogel limits the maximal thickness to a couple hundred micrometers. Thus, hydrogel NSCs that exceed this value will develop an internal necrotic zone extending from the center of the hydrogel to a certain critical distance over which the concentration of the limiting substrate is below the threshold to sustain cell viability. To overcome this barrier, efforts are now ongoing to vascularize cell-laden hydrogel matrices.\[^{119}\]
Figure 2. Examples of hydrogel neural systems on a chip (NSCs). a) Hydrogel NSC containing photolabile properties for study of 3D cellular migration. Top down (i) and prismatic (ii) images of fluorescently labeled oligopeptide channels within a 3D hydrogel – scale bars are 200 µm; and (iii) primary rat dorsal ganglia growing exclusively within a GRGDS peptide modified column – scale bar is 100 µm. Reprinted with permission.[61] Copyright Nature Publishing Group 2004. b) Hydrogel NSC for modeling cortical neuron outgrowth in brain-like environments. i) 3D printed layered brain-like structure; ii) confocal image of neurons after 5 days – scale bar is 100 µm; and iii) magnified image of area inside square showing axonal projection into the cell-free gel – scale bar is 100 µm. Reprinted with permission.[60] Copyright Elsevier 2015. c) Hydrogel NSC for study of neural progenitor cell differentiation. i) Neural progenitor cell-laden 3D printed porous hydrogel structure; ii) live/dead assay of hydrogel construct; and iii) scanning electron micrograph of a neuron in the 3D structure with the arrows indicating the soma and axon, respectively. Reprinted with permission.[57] Copyright John Wiley & Sons 2016.

2.5.3.1. Manufacturing of Hydrogel Neural Systems on a Chip: Hydrogel NSCs are composed one or more compositionally-unique 3D hydrogel domains. Cells are first propagated to reach high cell density and subsequently suspended in a nutrient-rich uncrosslinked hydrogel. Given uncrosslinked hydrogels are typically incapable of maintaining free-standing 3D structures, molding approaches are commonly used. For example, an uncrosslinked cell-laden hydrogel is first added to the mold cavity, subsequently crosslinked, and then removed. In addition to molding, 3D printing can also be used to additively assemble 3D hydrogel structures (see Section 2.4.1.5). 3D printed hydrogel NSCs are first constructed by developing printable hydrogel ‘bio-inks’ that contain the desired cell type and biochemical cues. Subsequently, the bio-inks are loaded into dispensing tools, such as cartridges syringes, or cast on energy absorbing plates for 3D printing. 3D printing provides a one-pot manufacturing process for hydrogel NSCs as it is possible to print both support materials and bio-inks using the same printing system.[107]
In contrast to microfluidic and compartmentalized NSCs, which require post-processing steps to integrate biology through functionalization and cell seeding steps, hydrogel NSCs do not require such post-processing. This unique aspect of hydrogel NSCs arises due to bio-compatible manufacturing (i.e. biomanufacturing) processes, such as 3D printing.

2.5.3.2. Hierarchical Design of Hydrogel Neural Systems on a Chip: Given the ability to additively assemble hydrogels via 3D printing, use hydrogels as carriers for multiple components (e.g. cells and biochemical cues), and assemble adjacent hydrogel systems of unique composition, various higher order anatomical features of native neural systems can be reproduced using hydrogel NSCs (see Table 2.4). For example, hydrogel NSCs have been used to affect the: 1) formation of heterogeneous tissues; 2) clustering of multiple cell types; 3) controlled distribution of extracellular matrix; and 4) construction of three-dimensional systems. Hydrogel NSCs also contain higher order functional and augmented features given the ability to integrate: 1) fluidic channels, and 2) controlled drug release systems. For example, Lee et al. combined a microfluidic flow chamber with a hydrogel NSC to model gliomas.[25] These functional features enable the programing and control of various higher order microenvironmental parameters such as: 1) mass transport of solutes (e.g. gases and biomolecules) and 2) spatiotemporal distributions of biochemical cues (e.g. rate and profile). For example, in that same study, Lee et al. leveraged fluidic flow through hydrogels to control the mass transport of fresh media and growth factors to glioma cells.[25] As shown in Figure 7.A, Luo et al. have shown that hydrogel NSCs can be created with controlled distributions of biochemical cues for directing cell growth.[61] Thus, hydrogel NSCs are useful platforms for
modeling higher order *trajectories* of native neural systems, such as: 1) tissue self-assembly; 2) neurite outgrowth, 3) cell migration; 4) phenotypic outcomes, and 5) gene expression. For example, as shown in Figure 2.7.B, Lozano et al. demonstrated hydrogel NSCs composed of biomimetic layered brain-like structures could be used to examine the neurite outgrowth from cortical neurons between adjacent hydrogels.\cite{60} Gu et al. demonstrated 3D printed hydrogel NSCs could be used to examine the differentiation of neural progenitor cells into neurons and glia using gene expression analysis as shown in Figure 2.7.C.\cite{57}

**Table 2.4** Summary of hydrogel neural systems on a chip. Abbreviations: Traumatic Brain Injury (TBI).

<table>
<thead>
<tr>
<th>Study</th>
<th>Motivation</th>
<th>Ref.</th>
</tr>
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<tbody>
<tr>
<td>Characterization of 3D printed neural structures</td>
<td><em>In vitro</em> drug screening and disease modeling</td>
<td>57</td>
</tr>
<tr>
<td>Characterizing the properties of neurons in 3D culture</td>
<td>Developing biomimetic and relevant tissue models</td>
<td>59</td>
</tr>
<tr>
<td>Printing and imaging of a layered brain like structure</td>
<td>Traumatic brain injury and disease modeling</td>
<td>60</td>
</tr>
<tr>
<td>Growing neurons in a peptide patterned medium</td>
<td>Directing cell growth in three dimensions</td>
<td>61</td>
</tr>
<tr>
<td>Monitoring neurite outgrowth in response to guidance cues</td>
<td>Understanding nerve regeneration processes</td>
<td>63</td>
</tr>
<tr>
<td>Development of <em>in vivo</em> 3D neurite outgrowth assay</td>
<td>Drug screening</td>
<td>64</td>
</tr>
<tr>
<td>Effect of TBI on a modular 3D brain model</td>
<td>Response to TBI</td>
<td>65</td>
</tr>
<tr>
<td>Dual-hydrogel system for cell culture with protein gradients</td>
<td>Developing a 3D microenvironment with molecular patterns</td>
<td>62</td>
</tr>
</tbody>
</table>

2.6. Applications of Neural Systems on a Chip

NSCs have various applications given their ability to model a variety of higher order physiological and pathophysiological phenotypes and trajectories of the human nervous
system. The applications of NSCs can be broadly classified as: fundamental research (e.g. modeling of complex neural systems or disease phenotypes), drug discovery (e.g. creating biomimetic models of NDDIs derived from human cells as high throughput drug screening platforms), or personalized medicine (e.g. creating patient-specific disease models as personalized drug screening platforms). As a result, NSCs have a significant impact on basic, translational (i.e. applied), and clinical research in neuroscience, neurology, and neural engineering. Below we highlight the application of NSCs to various disease, disorder, and injury models.

2.6.1. Alzheimer’s Disease: Alzheimer’s disease (AD) is a prevalent and serious neurological disorder and is currently the sixth leading cause of death in the US. Our current understanding of AD suggests it is driven by extracellular deposition of Aβ and intracellular accumulation of tau proteins. As a result, the presence of Aβ plaques, tau tangles, oxidative stress, and brain inflammation are hallmark characteristics of AD pathology. Researchers are now using NSCs to create platforms to study higher order pathophysiological trajectories associated with AD. As shown in Figure 8.A for example, Song et al. used a compartmentalized NSC to show that Aβ is transmitted through neural connections toward an attempt to improve our understanding of the mechanism by which Aβ plaques lead to loss of synapsis. Stoothoff et al. used a compartmentalized NSC to show that differences in tau levels change the mitochondrial distribution within a cell and affect axon transport dynamics. Kunze et al. developed a microfluidic NSC that allowed them to spatially control the concentration of hyperphosphorylated tau proteins throughout a cell population, thereby creating an AD model with co-cultured “healthy” and “diseased”
tissues. Choi et al. used a microfluidic NSC to determine that Aβ fibrils had little neurotoxic effect, but oligomeric Aβ assemblies resulted in atrophy. A microfluidic NSC designed by Park et al. based on applying interstitial flow to 3D neurospheroids showed that Aβ was significantly more destructive under flowing conditions than static conditions.

**Figure 2.** Applications highlights of neural systems on a chip (NSCs) for modeling neurological diseases, disorders and injuries. a) A compartmentalized NSC for studying long-distance transport of β-amyloid for better understanding Alzheimer’s disease, with a schematic showing three compartments and fluorescent images the transport of fluorescein isothiocyanate – tagged β-amyloid monomer across all three compartments. Reprinted with permission. Copyright John Wiley & Sons 2014. b) A compartmentalized NSC for studying peripheral nerve repair. i) Schematic of a compartmentalized NSC for manipulating, injuring, or treating isolated neurites; ii) regeneration of an untreated axonal injury; and iii) degeneration following the same axonal injury with the application of Nocodazole. Scale bar is 0.2 mm. Reprinted with permission. Copyright Elsevier 2014.
2.6.2. Parkinson’s Disease: Parkinson’s disease (PD) affects over 10 million people worldwide. Our current understanding of PD suggests it is driven by the progressive impairment and deterioration of dopaminergic neurons in the substantia nigra. As a result, the presence abnormal protein aggregates, known as Lewy bodies, are hallmark characteristics of PD pathology. Although still emerging, researchers are now using NSCs to create platforms to study higher order pathophysiological trajectories associated with PD. For example, Lu et al. fabricated a compartmentalized NSC to study 6-hydroxydopamine-mediated axonal degradation, which proceeds the degeneration of dopaminergic neurons in Parkinson’s disease. This NSC also allowed researchers to study mitochondrial transport dynamics in conditions replicating Parkinson’s disease.

2.6.3. Traumatic Brain or Nerve Injury: TBI, spinal cord injuries, and peripheral nerve injuries affect millions of people annually in the US. Further, nerve injuries are especially challenging to model and treat given the wide variations in anatomy, injury mechanism, and regenerative pathways among the brain, spinal cord and peripheral nerves. Importantly, NSCs are useful platforms for studying nerve injuries because they allow researchers to induce injuries to higher order neural anatomies and directly monitor trajectories associated with both injury and regeneration. For example, Hosie et al. used a compartmentalized NSC to isolate soma and axons toward the study of site-directed glutamate excitotoxicity in TBIs. As shown in Figure 2.8.B, Siddique et al. have developed a compartmentalized NSC that allows researchers to manually induce nerve injuries and administer isolated treatments to axonal components in a biomimetic 3D environment. Ghannad-Rezaie et al. created a microfluidic NSC that enabled live imaging of the neural response to peripheral
axonally injury in *Drosophila* larvae.[85] Yin *et al.* developed a microfluidic NSC to study and optimize drug candidate dosages for nerve regeneration toward the goal of minimizing harmful side effects, such as tumors.[95]

### 2.7. Emerging Areas and Future Directions

**2.7.1. 3D Printing:** 3D printing has enabled developments across a wide range of disciplines, including electronics, materials science, and tissue engineering, and is now poised to reconceptualize the design and engineering of NSCs. For example, although soft lithography is the gold standard for creating microfluidics and microchannels, and 3D printing now offers the ability to construct microfluidic networks of complex 3D geometry within a wide range of materials.[110, 111] As shown in Figure 2.7.C, Gu *et al.* demonstrated that 3D printed neural progenitor cells in hydrogel bio-inks can be differentiated *in situ* to synapse-forming predominantly gamma-aminobutyric acid (GABA)-expressing neurons.[57] Johnson *et al.* used micro-extrusion 3D printing to construct multi-material compartmentalized NSCs containing microfluidic channels of complex geometry and embedded electroactive components, see Figure 2.9.A.[21] In that study, they also demonstrated viable printing of a wide range of cell types including primary embryonic neurons.[21] Another advantage of 3D printing is the ability to use medical imaging techniques, such as computed tomography (CT),[124] magnetic resonance imaging (MRI),[124] or structured-light scanning (SLS),[125] to reverse engineer anatomical geometry that would be otherwise be difficult to design and manufacture using traditional approaches. Of all 3D printing techniques, micro-extrusion 3D printing in particular lends itself to developing NSCs, due to its compatibility with processing the most expansive materials set, including
solutions, cell suspensions, cell-laden hydrogels, thermoplastics, thermosets, elastomers, and composites.\textsuperscript{[107]} 3D printing is also able to create 3D heterogeneous biomimetic neural tissues containing distributed ECM proteins and growth factors through the ability to control the composition of individually printed bio-inks.\textsuperscript{[107]} For example, the ability to spatially distribute bio-inks containing different formulations of biochemical cues in 3D scaffolds via 3D printing was recently shown to selectively direct the growth of sensory and motor nerves.\textsuperscript{[125]}
Figure 2. Highlights of emerging areas and future directions for neural systems on a chip (NSCs). a) 3D printed NSCs. i) Schematic of 3D printing process for a compartmentalized NSC; ii) three parallel microchannels with neurons and axons shown in the first chamber; iii) axons from the first chamber associated with self-assembled Schwann cells within the
second chamber; and \textit{iv}) axon termini from the first and second chamber interacting with epithelial cells in the third chamber. Reprinted with permission.\cite{21} Copyright Royal Society of Chemistry 2015. \textit{b}) Hydrogel NSC for modeling brain tumors. \textit{i}) Prismatic and side view schematics of a microfluidic chip coupled with a glioma-laden hydrogel; \textit{ii}) structure of the biomimetic hydrogel; and \textit{iii}) a SEM image of the electrospun fiber separating the microfluidic channel from the hydrogel. Reprinted with permission.\cite{25} Copyright John Wiley & Sons 2014.

2.7.2. Electronic Augmentation: Creating next-generation NSCs will require the seamless integration of electroactive components for enhanced stimulation and monitoring functionality with neural anatomy. To date, efforts toward this goal have been achieved by integrating neural tissue with microelectrode arrays (MEAs). For example, Kanagasabapathi \textit{et al.} have created MEA-coupled compartmentalized NSCs for monitoring cortical and thalamic cell connectivity.\cite{51, 52} Various studies have also used MEA-coupled microfluidic NSCs to study the effect of biochemical cues on neural networks.\cite{96-98} Smith \textit{et al.} have created a cantilever-based NSC that measures muscle contraction following the stimulation of a motoneuron.\cite{50}

2.7.3. Human Cells: A major advantage of NSCs is their compatibility with human cells. Although the majority of NSCs to date have been constructed using non-human cells, human NSCs (\textit{i.e.} NSCs constructed with human cells) are beginning to be explored. For example, Griep \textit{et al.} used a human brain endothelial cell line (hCMEC/D3) for BBB applications.\cite{86} Lee \textit{et al.} implemented a human glioma cell line (A-172) into their microfluidic/hydrogel NSC to study the migration of glioma cells in 3D.\cite{25} Stoothoff \textit{et al.} used H4 human neuroglioma cells to study the mitochondrial axonal transport for AD applications.\cite{45} Yeon \textit{et al.} cultured primary human umbilical vein endothelial cells (HUVEC) and human astrocytes for BBB drug permeability studies.\cite{94} The ability to use human cells provides unique opportunities for
preclinical drug testing, such as target identification, target validation, target-based screening, phenotypic screening, pharmacodynamics, pharmacokinetics, absorption-distribution-metabolism-excretion (ADME) studies, and toxicology testing. Thus, human NSCs provide novel platforms that could reduce the cost and time associated with drug discovery for NDDIs. For a detailed discussion, we refer the reader elsewhere to comprehensive reviews on the application of tissue chips to drug discovery. The ability to utilize human stem cells is also an emerging area, which we discuss in greater detail in the following sections.

2.7.4. Personalized Medicine: Tissue chips have been suggested to enable future paradigms of personalized medicine and pharmacology. Likewise, the ability to construct NSCs from patient-derived cells now potentially enables the ‘personalized’ treatment of NDDIs via target-based or phenotypic screening conducted using patient-specific NSC disease models. For example, the ability to model higher order pathophysiological phenotypes and trajectories of the human nervous system could establish highly effective treatments. The continued evolution of computer-aided biomanufacturing processes, such as 3D printing, also provides novel opportunities for customization and prototyping of patient-specific NSC disease models. Ultimately, given the sustained demand for personalized medicine, NSCs are expected to play an integral role in the future personalized treatment of NDDIs. The ability to utilize human stem cells is also critical for developing patient-specific NSCs, which we discuss in greater detail in the following sections.

2.7.5. Biomimicry: In vivo human neural systems consist of cells growing in soft 3D ECM in the presence of both immobilized and diffusive spatiotemporal distributions of
biochemical cues. Cells also interact with 3D multi-scale topographical cues. Native neural systems are also influenced by mechanical factors, such as pulsatile fluid flow as well as vasodilation and vasoconstriction effects. Further, native neural tissue has highly controlled mechanical property matching. For example, with the exception of the meninges, neural tissue does not grow in direct contact with highly rigid materials \textit{in vivo}. Lee \textit{et al.} have approached this by coupling a glioma laden hydrogel with a microfluidic device, as shown in Figure 2.9.B.\textsuperscript{[25]} Biomimetic NSCs should strive to possess each of the above features. Unfortunately, the vast majority of NSCs developed to date contain only one, or at most a few, of the aforementioned features. Thus, achieving realistic and balanced biomimicry of the \textit{in vivo} nervous system in NSCs must be improved to achieve the most successful translational and clinical outcomes.

2.7.6. Stem Cells: The ability to construct NSCs from stem cells offers unique opportunities for studying the development and regeneration of neural systems, developing biomimetic models of human NDDIs, and creating personalized NSCs. Specifically, induced pluripotent stem cells (iPSCs)\textsuperscript{[127]} provide useful tools for such applications. For example, the ‘holy grail’ in biomedical research is to generate a transgenic mouse model of the human illness. The MECP2 mutation in Rett syndrome or the mutant gene causing Huntington’s disease are excellent examples where animal models reproduce salient features of the disease. Unfortunately, the majority of human neurological and neuropsychiatric illnesses are believed to be poly-genetic, consisting of multiple, and often unknown, gene alterations, making the generation of transgenic animals highly challenging. However, iPSCs are beginning to come to the rescue. For example, fibroblasts can be readily harvested from
patients and induced to form neurons or glial cells in the dish to study their molecular changes. This approach has recently been used to reveal unexpected changes in neuron complexity in Costello syndrome,[128] a rare developmental disorder with autism traits, suggesting that NSCs derived from human iPSCs provide novel opportunities for modeling rare and complex human NDDIs. Given iPSCs are also patient-derived, NSCs constructed using iPSCs may also serve as personalized drug screening platforms where libraries of drugs can be examined regarding their ability to correct a protein or signaling pathway deranged by disease. Finally, NSCs constructed from iPSCs may eventually serve as platforms for differentiating and programming cells for neural regeneration and other cell-based therapies. For example, such cells could be differentiated into the cell of interest and corrected to express the right complement of genes in NSCs, and subsequently be collected for implantation. Since the iPSCs were initially harvested from the patient, the resultant autologous graft or cell-therapy will not elicit an immune response (e.g. immune rejection).

In addition, the ability to construct NSCs from stem cells offers unique opportunities for understanding the development and regeneration of the nervous system. For example, NSCs constructed using neural progenitor cells derived from human embryonic stem cells have been used to study the effect of biochemical cues on the differentiation and formation of complex neurite networks.[89]

2.8. Technical Hurdles, Remaining Challenges and Opportunities

Although NSCs have progressed significantly over the past decade, there are still major technical hurdles and remaining challenges to overcome. It is established that cells exhibit different trajectories in 2D vs. 3D environments.[129-131] However, we still face
significant manufacturing challenges associated with embedding functional and augmented
features, such as fluidic channels and electroactive components in 3D. Another challenging
technical hurdle is to simultaneously program and control multiple microenvironmental
parameters toward mimicking or reproducing signaling cascades. This is a critical
requirement for modeling higher order trajectories associated with developing nervous
systems and NDDIs. The use of NSCs for drug discovery and personalized medicine
applications also involves regulatory considerations. For example, the future use of NSCs as
alternatives to small animal models for preclinical drug testing may require steps equivalent
to Animal Model Qualification, which is required by the FDA to rely on the evidence from
animal studies regarding drug effectiveness. Alternatively, use of NSCs as patient-specific
disease models intended for the diagnosis or treatment of NDDIs would subject NSCs to the
regulatory requirements of biomedical devices.\textsuperscript{[132]} Ultimately, both the translational impact
and the regulatory barriers of NSCs are tied to the challenge of creating highly robust and
reproducible NSCs. However, as noted in Sections 3 and 4, the vast majority of NSCs, with
the exception of 3D printed NSCs, involve manual assembly, functionalization or seeding
steps. Thus, the creation of robust NSCs hinges on eliminating manual processing steps
toward fully automated biomanufacturing processes. As discussed in the Section 2.6,
realistic biomimicry is a major driving force for NSC design. Specifically, mimicry of
vascularized brain tissue remains a critical challenge. Additionally, the coupling of NSCs to
hemodynamic processes, such as hemoglobin-based oxygen transport or lipoprotein-based
lipid uptake, is required to advance NSCs.
2.9. Conclusions

NSCs appear poised to shift the paradigm for modeling human NDDIs. The ability to model higher order anatomical features, functional and augmented features, microenvironmental parameters, and ultimately, trajectories of the human nervous system is highly dependent on the NSC design (e.g. microfluidic, compartmentalized, or hydrogel NSCs). Emerging biomanufacturing processes, such as 3D printing, are now enabling the design and manufacturing of robust novel NSCs. The field of NSCs is currently in a developmental stage heading toward increased biomimicry, functional-augmentation, and personalized medicine and pharmacology. Opportunities exist in terms of addressing various technical and regulatory hurdles that remain toward NSC application to drug discovery and personalized medicine, including achieving more realistic biomimicry of the human nervous system and robustness in NSC manufacturing approaches.

2.10. References


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Chapter Three: 3D Printed Functionally Graded Plasmonic Constructs

3.1. Abstract

A new class of 3D functionally graded plasmonic materials and devices manufactured through 3D printing is presented. Up to eight different plasmonic inks are interwoven into a single functionally graded construct. Both continuous and discrete 3D gradients in plasmonic properties are realized. The approach is applied toward engineering of next-generation plasmonic devices. Specifically, the manufacturing of a novel functionally graded plasmonic night-vision contact lens is demonstrated.

3.2. Introduction

The ability to assemble and interweave multiple plasmonic nanomaterials from the bottom-up into macroscopic 3D structures would both shift the paradigm for plasmonic materials processing and bring about new classes of plasmonic materials and devices. Presently, state of the art plasmonic materials and devices can be classified as plasmonic arrays,[1, 2] discrete plasmonic structures,[3, 4] and plasmonic nanocomposites[5, 6]. Plasmonic arrays consist of patterned 2D arrays of micro- and nano-scale plasmonic features or constructs on rigid or flexible substrates and are typically fabricated using nanomanufacturing or directed assembly approaches.[5] Discrete plasmonic structures consist of micro- or nano-scale 3D constructs and are typically fabricated using microfabrication and nanomanufacturing processes.[3] Plasmonic nanocomposites consist
of dispersed plasmonic nanoparticles within a matrix phase and are typically fabricated using casting or directed assembly approaches.[5] Although each class provides unique opportunities, nanocomposite-based approaches appear poised to transform next-generation plasmonic materials and devices as they offer the potential to integrate and interweave multiple nanomaterials into complex multi-component 3D systems.

To date, 3D printing has led to the development of novel multi-functional materials and devices through the additive assembly of nanomaterials into 3D structures.[7] For example, quantum dot inks were recently used to create novel 3D printed quantum dot light-emitting diodes.[8] Inks containing gold nanorods were recently used to create 3D printed stimuli-responsive plasmonic controlled release capsules.[9] Inks containing silver nanoparticles have also been extensively used. For example, silver inks were used to create 3D printed conformal antennas.[10] An ink based on graphene oxide nanosheets was also recently printed to create freestanding reduced graphene oxide nanowires.[11]

In addition to novel nanomaterial processing opportunities, 3D printing also offers unique opportunities in multi-material manufacturing.[7, 12-15] Specifically, the capacity for 3D printing to interweave multiple materials from the bottom-up has led to the creation of next-generation functionally graded materials. Two different approaches have been used to create materials with graded properties via 3D printing, which can be classified by spatial variations in: 1) geometric patterning, or 2) material composition. These approaches have enabled the processing of materials and manufacturing of devices with graded mechanical, electrical, and optical properties. For example, it was recently demonstrated that materials with graded dielectric permittivity could be created by spatially varying the geometric
pattern of the 3D printed material throughout the construct. A similar technique was also used to create novel graded index lenses and electromagnetic field devices. Alternatively, a biomimetic soft robot with a graded modulus was created by spatially varying the composition of the 3D printed material throughout the construct. Thus, 3D printing appears poised to revolutionize the creation of next-generation plasmonic materials and devices based on 3D functionally graded plasmonic nanocomposites.

Here we show multi-material 3D printing can be leveraged to create novel plasmonic materials and devices endowed with three-dimensionally graded plasmonic properties. We demonstrate that up to eight different plasmonic inks can be interwoven into a single 3D printed functionally graded plasmonic construct. We also report flexible utility of the concept for diverse nanocomposite systems using both crosslinking and non-crosslinking polymer matrices. A novel multi-material microextrusion 3D printing technique that contains dynamic multi-component mixing capability is presented. We show that the versatile technique enables the printing of functionally graded plasmonic constructs with continuously graded 3D properties. Ultimately, we highlight the potential of the concept to catalyze the development of next-generation plasmonic materials and devices by 3D printing a functionally graded night vision contact lens.

3.3. Results and Discussion

As shown in Figure 3.1a, our concept for creating next-generation functionally graded plasmonic materials and devices is founded on the additive assembly of multiple inks with varying plasmonic properties into multi-component 3D nanocomposite constructs. In order to demonstrate the promise of this concept, we used a class of plasmonic nanoparticles
with highly tunable plasmonic properties. Silver nanoprisms (AgNPs) are an emerging class of plasmonic nanoparticles that have highly tunable plasmonic properties.\cite{21} AgNPs also exhibit higher in-plane dipole resonance and plasmonic sensitivity relative to spherical silver nanoparticles offering unique opportunities for sensing and filtering applications.\cite{21} Thus, we selected AgNPs as the functional nanomaterial for preparing the plasmonic inks. The ability to control the size of AgNPs is representatively shown in Figure 3.1b (see Supporting Information Figure S3.1 for TEM images of all AgNPs). Our TEM data show individual batches of AgNPs could be synthesized over the range of 15 - 79 nm in size. As shown in Figures 1c and d, the absorbance peak of AgNPs in aqueous solution red-shifted with increasing nanoparticle size and ranged from ~460 - 790 nm.
Figure 3.1 a) Concept for creating next-generation functionally graded plasmonic materials and devices using 3D printing. Multiple inks with varying plasmonic properties are additively assembled and interwoven by 3D printing to form novel multi-component 3D nanocomposite constructs. b) Representative TEM images of plasmonic silver nanoprisms across the scale of nanoparticle sizes used in this study. c) Absorbance spectra of aqueous solutions containing different size silver nanoprisms. d) Relationship between silver nanoprism size and wavelength of the absorbance peak (standard deviation based on n = 50 - 100 nanoparticles in the same batch). e) Photograph of Pluronic F-127 plasmonic inks and corresponding absorbance spectra of the gelled non-printed material (inset shows the printed inks; scale bar is 5 mm). f) Photograph of PEGDA plasmonic inks and corresponding absorbance spectra of the gelled non-printed material (inset example shows printed inks; scale bar is 5 mm).

The data in Figures 3.1b - d show AgNPs represent highly tunable nanomaterials for preparing the plasmonic inks. Thus, we next examined the potential to create inks with varying plasmonic properties across the visible spectrum. Given the versatility of 3D printing is highly dependent on ink chemistry, we demonstrated that our concept for constructing next-generation 3D printed plasmonic materials and devices is compatible with multiple ink formulations. Specifically, we chose to examine two matrices for plasmonic ink formulation: Pluronic F-127 and poly(ethylene glycol) diacrylate (PEGDA). Pluronic F-127 was selected due to its advantageous rheological properties (i.e. viscoplasticity and high yield stress) that enable the printing of free-standing 3D structures.[22] PEGDA was selected due to its ability to form mechanically robust structures for tissue engineering and biomedical device applications.[23, 24] Additionally, the two ink formulations demonstrated the utility of the proposed concept given they have disparate rheological properties, mechanical properties, and gelation chemistry (i.e. uncrosslinked vs. crosslinked). As shown in Figures 3.1e and f, plasmonic inks with absorbance bands across the UV-Vis spectrum could be prepared using both matrices (see Supporting Information Figure S3.2 for
additional data over the full UV-Vis spectrum). Importantly, the data in the insets of Figures 1e and f also show that the AgNPs remained functionally active after gelation (see Supporting Information Figure S3.3 for UV-Vis data of uncrosslinked PEGDA inks). The absorbance of both plasmonic ink systems exhibited a linear dependence on nanoparticle concentration (see Supporting Information Figure S3.4).

Having demonstrated ink plasmonic properties can be controlled over the UV-Vis spectrum by varying the type of AgNPs that are dispersed in the polymer matrix phase, we next examined the ability to grade the plasmonic properties of 2D plasmonic constructs using a novel multi-material 3D printing approach that contains multi-component dynamic mixing capability. Figure 3.2a shows a schematic of our novel multi-material 3D printing approach for printing functionally graded plasmonic materials. The process is based on simultaneous multi-component microextrusion and dynamic mixing. This approach enables us to control the composition of the printed plasmonic material in real-time by dynamically regulating the volumetric flow rates of the individual components. As a result, the printed material contains a mixture of the plasmonic properties found in each component (i.e. exhibits an absorbance spectrum that contains multiple absorbance bands). This approach allows us to realize two distinct regimes for grading the functional properties of materials and devices: discrete vs. continuous. Analogous to the behavior of mathematical functions, which can be either discontinuous or continuous with respect to changes in the independent variable, the properties of functionally graded materials may exhibit either smooth or sharp changes with respect to the material’s spatial dimensions. Thus, hereinafter we define constructs that exhibit sharp discontinuous changes in spatial properties as ‘discretely
graded’ and constructs that exhibit smooth continuous changes in spatial properties as ‘continuously graded’. We note that due to the layer-by-layer nature of additive manufacturing, continuously graded constructs may exhibit localized discontinuity in spatial properties at the interface between adjacent layers; however, continuously graded constructs composed of a high number of layers would exhibit relatively smooth changes in spatial properties across the global domain. We first demonstrated the ability to 3D print discretely and continuously graded 2D plasmonic constructs. As shown in Figure 3.2b, the flow rates of the individual ink components are modulated by dynamically controlling the extrusion pressure for each component across the spatial printing dimensions (see Supporting Information Figure S3.5 for a description of the individual ink formulations). We note that a non-zero pressure for each component was required at all times (even for pure component printing) to prevent backflow. Importantly, as shown in Figures 3.2c and d, this approach enabled us to print novel 2D plasmonic materials with discretely and continuously graded plasmonic properties across macroscopic length scales. In addition to the visually observable grading profiles, the heat maps shown in Figures 3.2c and d quantitatively validate the grading outcomes (see Supporting Information Figure S3.6 for a schematic of the measurement approach). For example, the heat map for the continuously graded plasmonic material shows the fraction of component A decreases linearly from 1 to 0.48 to 0 at x = 0, 12.5 and 25 mm (i.e. L = 0, 0.5L, and L), respectively. In contrast, the fraction of component A in the discretely graded plasmonic material was either 1 or 0 at all positions.
Figure 3.2 a) Schematic of the simultaneous multi-component microextrusion and dynamic mixing 3D printing technique using multiple plasmonic inks. b) Spatially-dependent printing pressure profiles used to achieve continuous or discrete gradients. Photograph of a printed continuously and discretely functionally graded 2D plasmonic construct (c and d, respectively) and corresponding heat map showing the grading of plasmonic properties in terms of the spatial distribution of ink A (scale bar is 5 mm). Constructs were printed with Pluronic F-127 ink.
Having established the proof of concept for creating 3D printed functionally graded plasmonic constructs, we next examined the ability to create plasmonic materials with functionally graded plasmonic properties in 3D. Thus, we next printed discretely and continuously graded 3D constructs. As shown in Figure 3.3, we were able to create novel 3D printed discretely graded plasmonic materials of diverse geometry, including spheres, cylinders, pyramids, and cubes using the Pluronic F-127 ink formulation (see Supporting Information Video S3.1). As shown in Figure 3a, the sphere was graded vertically (z-direction) using two plasmonic inks containing AgNPs of either 39 or 71 nm in size. The cylinder was graded radially using two plasmonic inks containing AgNPs of either 39 or 71 nm in size (see Figure 3.3b). The pyramid was graded in the x-y plane using four plasmonic inks containing AgNPs of 15, 49, 71 or 78 nm in size (see Figure 3.3c). The cube was graded in three dimensions using eight plasmonic inks containing AgNPs of 15, 28, 39, 49, 53, 58, 71 or 79 nm in size (see Figure 3.3d). Importantly, as shown in Figure 3, the presence of graded plasmonic properties throughout the 3D printed constructs was both visually observable and quantifiable via 3D transmittance measurements. The 3D transmittance measurements were acquired by subjecting different locations of the construct to incident light and subsequently recording the resulting optical properties. The incident light was initially directed top-down through the construct at low values of the sample rotational angle ($\theta$) and incrementally moved toward the side of the construct with increasing values of $\theta$ (see Supporting Information Figure S3.7). Given $\theta$ determines the location at which the plasmonic properties are measured throughout the 3D construct, and therefore, the number
of ink components that the incident light encounters, variation in the UV-Vis spectrum at different values of $\theta$ indicates the plasmonic properties are graded throughout the 3D construct. Importantly, given the absorption band location of the plasmonic ink depends on the nanoparticle size (see Figure 3.1d), light passing through a graded plasmonic nanocomposite that contains either multiple single-component inks with different size nanoparticles or a single multi-component ink that contains multiple different size nanoparticles will exhibit bands of high transmittance across the UV-Vis spectrum, which we refer to as transmittance bands. For example, the cubic construct exhibits multiple transmittance bands across the UV-Vis spectrum over $\theta = 10 - 20^\circ$ due to the interaction of incident light with multiple discrete internal cubic cells that contain different nanoparticles. In contrast, the cylindrical construct has a filled inner cylinder that contains nanoparticles of 39 nm in size that exhibit an absorbance band near 520 nm, and an outer cylindrical domain that contains nanoparticles of 71 nm in size that exhibit an absorbance band near 730 nm. As shown in Figure 3.3b, the graded coaxial feature can be identified in the transmittance plot by the presence of two notable transmittance bands. For example, the first band is present near 750 nm at low angles, where incident light passes along the longitudinal axis of the cylinder. The second band emerges near 450 nm at 20$^\circ$, where the rotation of the sample causes incident light to pass through the annular domain before entering the internal cylinder. We note that uniform changes in transmittance across the full UV-Vis spectrum arise from a varying path length effect as the sample rotates throughout the measurement. For example, this effect is observable in the transmittance plot of the spherical construct (see Figure 3.3a).
As shown in Figure 3.4, we next created complementary 3D printed continuously functionally graded plasmonic materials of identical geometries using the Pluronic F-127 ink formulation (see Supporting Information Video S3.2). The sphere, pyramid, and cube were graded vertically, while the cylinder was graded radially. Grading was achieved using two
plasmonic inks containing AgNPs of either 39 or 71 nm in size. As shown in Figure 3.4, in contrast to the discretely graded 3D constructs shown in Figure 3, the continuously graded 3D constructs did not exhibit sharp changes in spatial plasmonic properties. For example, as shown in Figure 3.4b, the continuously graded cylinder did not exhibit a sharp increase in transmittance value at 450 nm and 20° as observed in the discretely graded cylinder (see Figure 3.3b). In contrast, the transmittance plot of the continuously graded cylinder exhibits a gradual increase in transmittance at 450 nm with increasing $\theta$ due to the smooth decrease in the concentration of the 39 nm size nanoparticles in the positive radial direction. Similar to the differences in grading regime that can be identified by comparing transmittance plots from the same construct geometry (e.g. the aforementioned differences in transmittance plots of the discretely vs. the continuously graded cylinders), one can also identify differences in geometry when comparing the transmittance plots of constructs fabricated with the same grading regime. Such geometric differences are observable when comparing the transmittance plots of the continuously graded pyramid and cube shown in Figures 3.4c and d, respectively. For example, although both constructs have the same vertically graded composition (continuous bottom-up grading from 39 to 71 nm nanoparticles), the transmittance plots exhibit different dependence on $\theta$ due to the geometry-dependent cross-sectional pathways of incident light (see Supporting Information Figure S3.7).
Figure 3.4 Highlight of 3D printed continuously functionally graded 3D plasmonic constructs (sphere (a), cylinder (b), pyramid (c), and cube (d)). Each column shows the rendering, prismatic view, front view, top view, and 3D transmittance (%T) plot for a given construct in vertically descending order (maximum transmittance ($T_m$) = 31 (a), 31 (b), 6 (c), 31 (d)). Constructs were printed with Pluronic F-127 ink. All scale bars are 5 mm.

The functionally graded 3D constructs shown in Figures 3.3 and 3.4 demonstrate the promise to create next-generation plasmonic devices using 3D printing. Given hydrogels serve as materials for state-of-the-art contact lens applications, we next sought apply our approach toward the engineering of a next-generation contact lens. It is well established...
that red light, centered at ~650 nm, provides night vision by achieving and preserving dark adaptation.[25] Additionally, blue light, centered at ~475 nm, is known to pass through the eye to the retina, causing eye fatigue and potentially macular degeneration.[26, 27] This information suggests it may be possible to develop a novel ‘night vision’ plasmonic contact lens technology that spatially and selectively filters incident light on the eye. As shown in Figure 3.5a, we designed a novel functionally graded plasmonic night vision contact lens that filters all visible wavelengths other than red light (650 nm) from entering the pupil as well as all blue light (475 nm) from entering the surrounding regions of the eye. We selected the PEGDA plasmonic ink formulation given the use of PEGDA in commercially available contact lens.[23] Thus, we next designed blue filter and red band-pass filter PEGDA plasmonic inks. The blue filter ink contained 15 nm AgNPs and had an absorbance peak at 463 nm. The red band-pass filter ink contained all AgNPs except for the 58 nm AgNPs, which had an absorbance peak at 600 nm. The absorbance spectra for the two ink filters are available in Supporting Information Figure S3.8. Photographs of the 3D printed functionally graded plasmonic night vision contact lens are shown in Figures 3.5b and c. We note that although we selected a low molecular weight PEGDA formulation as a proof of concept to achieve a mechanically robust graded lens, the transparency of the lens can be tuned using higher molecular weight formulations.[28, 29] Importantly, the 3D transmittance plot shown in Figure 3.5d confirms the desired spatial filtering characteristics of the lens achieved by the functionally graded plasmonic properties. For example, Figure 3.5d shows high transmittance of red light and low transmittance of other wavelengths at the center of the lens (low θ values), which would provide night vision by achieving and preserving dark
adaptation. The data also show the transmittance of blue light decreases to zero toward the outer lens regions, which would protect the eye from fatigue and macular degeneration.
Figure 3. a) Schematic of a novel functionally graded plasmonic night vision contact lens. The functionally graded night vision contact lens is designed to filter all visible wavelengths other than red light (650 nm) from entering the pupil as well as all blue light (475 nm) from entering the surrounding regions of the eye. Photographs of a 3D printed PEGDA night vision contact lens on a glass slide (b) and finger (c) (scale bars are 10 mm). d) Representative 3D transmittance plot of the night vision contact lens verifying red band-pass filtering at the center of the contact lens and blue filtering toward the outer contact lens regions.

3.4. Conclusions

This communication demonstrates a paradigm shifting approach for creating next-generation plasmonic materials and devices. Specifically, we showed 3D printing enables the fabrication of novel functionally graded 3D plasmonic constructs with distinct classes of graded material properties (i.e. discretely vs. continuously graded) through the additive assembly and dynamic mixing of multiple plasmonic inks. Flexible utility with both crosslinking and non-crosslinking systems was successfully realized. The impact on development of next-generation plasmonic devices was demonstrated through the design and fabrication of a novel 3D printed functionally graded plasmonic night vision contact lens. Ultimately, this work opens the door for creating novel plasmonic materials and 3D printed
functional materials as well as paves the way for new applications of plasmonic materials and devices.

3.5. Experimental Section

3.5.1. Silver Nanoprism Synthesis and Characterization: Silver nanoprism (AgNPs) were synthesized following the seed-mediated method in the previous reports.[21, 30] First, small silver seeds were prepared by sequentially adding 0.75 mL of 0.5 mg mL\(^{-1}\) poly(sodium 4-styrenesulfonate) (PSSS, average \(M_w\sim 1,000\) kDa) and 0.9 mL of ice-cold 10 mM sodium borohydride (NaBH\(_4\)) to a beaker containing 15 mL of 2.5 mM sodium citrate solution. Afterwards, 15 mL of 0.5 mM silver nitrate solution was added at a constant rate of 6 mL min\(^{-1}\) using a syringe pump. The seeds were aged for 30 min and then used for synthesizing silver nanoprisms. To synthesize AgNPs, 1.35 mL of 10 mM ascorbic acid solution was added to 230 mL of ultrapure deionized water followed by various amount of seeds (260 µL - 8 mL). The amount of Ag seeds determines the size of the final AgNPs. Afterwards, 5.4 mL of 5 mM AgNO\(_3\) was added to the mixture at a rate of 1.8 mL min\(^{-1}\) using a syringe pump. Once the synthesis was complete, 9 mL of 25 mM of sodium citrate solution was added to stabilize the AgNPs. The resultant solutions ranged in concentration from 82 pM for the largest particles to 2 nM for the smallest particles. The solutions were stirred during all procedures. To characterize the AgNP size, 5 - 10 µL of the nanoparticle solution was drop-casted onto carbon-coated copper grids for TEM imaging. The samples were dried overnight and then imaged using a Philips EM 420 electron microscope. The extinction spectra of the AgNP solutions were obtained using an Agilent Cary 5000 UV-Vis-NIR spectrophotometer.
3.5.2. Plasmonic Ink Preparation: Two plasmonic ink formulations were prepared: crosslinking and non-crosslinking inks. To prepare the crosslinking ink, a 10 wt% 750 Da PEGDA (Sigma Aldrich) solution containing 1 wt% 2,2-dimethoxy-2-phenylacetophenone (Sigma Aldrich) photoinitiator was prepared in aqueous solutions of AgNPs. The solution was first heated to 40°C and mechanically stirred for one hour. Subsequently, the solution was cooled to room temperature prior to printing. To prepare the non-crosslinking ink, a 30 wt% Pluronic F-127 (Sigma Aldrich) solution was prepared in aqueous solutions of AgNPs at 4°C. The solution was mechanically stirred for 20 minutes, and then placed in a refrigerator overnight. Subsequently, the solution was warmed to room temperature prior to printing.

3.5.3. 3D Printing of Functionally Graded Plasmonic Constructs: A custom 3D printing system was used for this work. The system was composed of a customized assembly of an industrial dispensing robot (F5200N; Fisnar) and a block of high precision digital dispensers (Ultimus V; Nordson EFD). A custom mixing manifold was created by linking the multiple syringe barrels with independent pressure control via Luer lock fittings (Component Supply Company) to a single 27 GA tapered tip (Nordson). The ratio of the ink components exiting the extruder (i.e. the compositional blend of the printed inks) was controlled by the applied pressure to the individual syringe barrels. The tool paths were programmed using vendor-provided software (RobotEdit; Fisnar). Constructs composed of Pluronic F-127 inks were printed using linear print speeds ranging from 0.5 - 4 mm s\(^{-1}\) and pressures ranging from 14 - 26 psi. Constructs of PEGDA inks were printed in Pluronic F-127 scaffolds. A sacrificial Pluronic F-127 scaffold was first printed at 15 psi with a print speed of 4 mm s\(^{-1}\).
Subsequently, the PEGDA ink was printed with a stationary deposit and pressures ranging from 0.1 - 1 psi. We note that a non-zero pressure for each component was required at all times (even for pure component printing) to prevent backflow. The PEGDA ink was crosslinked by a two minute exposure to 365 nm UV radiation from a 6 W lamp (UVP UVGL-55). The Pluronic F-127 scaffold was then removed by flushing with cold water.

3.5.4. UV-Vis Characterization of Plasmonic Inks and 2D Constructs: The UV-Vis absorbance data for the individual plasmonic inks were collected over 300 - 999 nm with a 1 nm step size (Synergy Hybrid H1m; BioTek). 200 µL of each ink was deposited into a 96 well plate for testing. All measurements were done at room temperature. The PEGDA inks were characterized both before and after crosslinking. The 2D heat maps shown in Figure 3.3c and d were acquired by printing constructs directly into a 6 well plate. Subsequently, the plate reader was used to measure the UV-Vis spectrum of the functionally graded 2D construct at 10 equally distributed points across the construct. A schematic depicting the measurement approach is shown in Supporting Information Figure S3.6. The resulting spectra were then used to calculate the position-dependent fraction of each ink component in the functionally graded 2D construct as:

$$F_i = \frac{A_i}{\sum A_i} = \frac{A_i}{A_{tot}}$$  \hspace{1cm} (1)

where $F_i$ is the fraction of the $i^{th}$ component in the functionally graded material, $n$ is the number of components, $A_i$ is the area under the $i^{th}$ peak, and $A_{tot}$ is the total area under all peaks.

3.5.5. UV-Vis Characterization of 3D Plasmonic Constructs: White light absolute transmittance and specular reflectance of all functionally graded 3D plasmonic constructs
were obtained on a Cary 5000 UV-Vis-NIR spectrophotometer with universal measurement accessory (UMA). The angle resolved measurements were performed by controlling the angle of incidence light, the angle of collection, and the angle of the stage rotation. The light incidence angle was systematically varied from 6 - 65° with a 1° increment by rotating the sample stage. Initially all the measurements were started by focusing the light beam on the center of the 3D construct from a top down perspective (the construct’s z-direction) and then rotating the sample from 6 - 65° with respect to the sample’s y-axis (see Supporting Information Figure S3.7). For all measurements, the incident beam collimation aperture and the detector aperture were set to maximize the signal.
3.6. References


3.7. Supporting Information

These supporting information include: TEM images of silver nanoprisms (Figure S3.1), absorbance spectra of both F-127 and PEGDA plasmonic inks (Figure S3.2), absorbance spectra of uncrosslinked PEGDA inks (Figure S3.3), measurements of hydrogel absorbance properties over a range of nanoparticle concentrations (Figure S3.4), absorbance spectra of pure and blended F-127 ink components used in Figure 3.2 (Figure S3.5), a schematic of the UV-Vis measurement for generating the 2D absorbance heat map shown in Figure 3.2 (Figure S3.6), a schematic of the 3D UV-Vis measurement approach (Figure S3.7), absorbance spectra of the two PEGDA inks used for constructing the 3D printed functionally graded plasmonic night vision contact lens (Figure S3.8), and videos of graded plasmonic construct 3D printing shown in Figures 3.3 and 3.4.
Figure S3.1 Highlight of the range of silver nanoprism sizes used in this study for plasmonic ink formulations. TEM images of silver nanoprisms with diameters of 15 ± 4 nm (a), 28 ± 6 nm (b), 39 ± 7 nm (c), 49 ± 3 nm (d), 53 ± 5 nm (e), 58 ± 4 nm (f), 71 ± 13 nm (g), and 79 ± 11 nm (h).
Figure S3. 2 UV-Vis spectra of F-127 (a) and PEGDA (b) inks used in Figures 3.2e and f.
Figure S3. 3 UV-Vis spectra of uncrosslinked PEGDA inks.
Figure S3.4 Peak absorbance intensity for 15 and 79 nm nanoparticles in PEGDA and Pluronic F-127 inks as a function of nanoparticle concentration. The concentration at dilution factor = 1.0 corresponds to the concentration used for graded plasmonic constructs shown in the main text.
Figure S3. 5 UV-Vis spectra of the pure and blended F-127 inks used in Figure 3.2.
Figure S3. 6 Schematic of the UV-Vis measurement used to generate the 2D absorbance heat map shown in Figure 3.2. Absorbance spectra were acquired across the 3D printed sheet at 10 equally distributed points (represented schematically by crosses).
**Figure S3.** 7 *a*) Schematic of the 3D UV-Vis measurement principle corresponding to data shown in Figures 3.3 and 3.4. *b*) Schematic of the path of incident light along the 3D constructs (solid line) that occurs by rotating the sample through the angle $\theta$ during the measurement (start of the path corresponds to low angle values; dashed line shows scan direction).
Figure S3.8 Absorbance spectra of the blue filter (A) and red band-pass filter (B) inks used for the functionally graded plasmonic night vision contact lens shown in Figure 3.5.
Chapter Four: Programming of Multicomponent Temporal Release Profiles in 3D Printed Polypills via Core-shell, Multilayer, and Gradient Concentration Profiles

4.1. Abstract

Additive manufacturing (AM) appears poised to provide novel pharmaceutical technology and controlled release systems, yet understanding the effects of processing and post-processing operations on pill design, quality, and performance remains a significant barrier. Here, we report a systematic study of the relationship between programmed concentration profile and resultant temporal release profile using a 3D printed polypill system consisting of an FDA approved excipient (Pluronic F-127) and therapeutically relevant dosages of three commonly used oral agents for treatment of type 2 diabetes (300 - 500 mg/pill). A dual-extrusion hydrogel microextrusion process enabled the programming of three unique concentration profiles, including core-shell, multilayer, and gradient structures. Experimental and computational studies of diffusive mass transfer processes revealed that programmed concentration profiles are dynamic throughout both pill 3D printing and solidification. Spectrophotometric assays showed that the temporal release profiles could be selectively programmed to exhibit delayed, pulsed, or constant profiles over a 5-hour release period by utilizing the core-shell, multilayer, and gradient distributions, respectively. Ultimately, this work provides new insights into the mass transfer processes that affect design, quality, and performance of spatially graded controlled
release systems, as well as demonstrates the potential to create disease-specific polypill technology with programmable temporal release profiles.

4.2. Introduction

Although traditional processing operations, such as compaction and wet granulation, currently enable high-throughput production of quality pharmaceuticals, they place various constraints on achievable therapeutic value.[1, 2] For example, traditional large-scale pharmaceutical manufacturing processes restrict the flexibility to incorporate multiple active ingredients, and thereby, limit the ability to produce patient-specific pill technologies.[2] However, treatment plans associated with common diseases and disorders, such as diabetes, are often complex, involving multiple drugs, patient-specific dosages, and carefully tuned temporal release profiles.[3] For example, treatment plans for type 2 diabetes commonly involve prescriptions for multiple oral agents, including metformin, acarbose, and glyburide, among others, often administered in the form of tablets that contain dosages ranging from 1 - 1,000 mg per pill.[4-8] New pharmaceutical manufacturing processes that offer the ability to produce pills capable of delivering multiple actives in a controlled and patient-specific fashion could lead to optimized treatments for a variety of diseases and disorders.

Additive manufacturing (AM), also commonly referred to as 3D printing, is a computer-aided manufacturing process, that assembles materials into parts via layer-by-layer deposition or solidification processes.[9] AM offers various advantages, including rapid prototyping, multi-material integration, fabrication of complex geometries, and importantly, fabrication of biological constructs.[10, 11] For example, AM has enabled the production of
artificial tissues and organs,\textsuperscript{[9]} \textit{in vitro} platforms,\textsuperscript{[12, 13]} biomedical devices,\textsuperscript{[14-17]} and bioelectronics,\textsuperscript{[18, 19]} and now appears poised to transform pharmaceutical manufacturing and pill technology\textsuperscript{[20-29]}. Among the various ways that AM can be leveraged toward improving pharmaceutical manufacturing capabilities, pill technology, healthcare logistics, and ultimately patient health, the ability to fabricate novel pill technologies composed of multiple actives, commonly referred to as ‘polypills,’ has received considerable focus.\textsuperscript{[10, 20-22, 26-28]} To date, fused filament fabrication,\textsuperscript{[25]} microextrusion printing,\textsuperscript{[22, 28]} and inkjet printing\textsuperscript{[23, 24]} AM processes have been leveraged to fabricate compartmentalized polypills,\textsuperscript{[28]} oral drug delivery films,\textsuperscript{[30]} patient-specific topical drug delivery systems,\textsuperscript{[31]} stimuli-responsive pills,\textsuperscript{[32]} nanomedicine containing pills,\textsuperscript{[33]} and polypills with orthogonal release profiles,\textsuperscript{[28, 34]} demonstrating their promise for achieving increased tablet complexity, personalized care, and on-demand manufacturing.\textsuperscript{[20, 21, 26, 27, 35]} For example, microextrusion printing was used to manufacture bilayer tablets that used diffusion and osmotic pressure to achieve zero- and first-order release kinetics.\textsuperscript{[22]} Microextrusion printing was also used to fabricate polypills containing up to five actives.\textsuperscript{[28]} A low temperature fused filament fabrication method that reduced the risk of thermal degradation to the active ingredients was used for production of patient-specific rapid release pills.\textsuperscript{[25]} In another example, a combination of 3D printing and casting was implemented to create pills with customizable release profiles.\textsuperscript{[36]} However, although the aforementioned studies have established the initial body of knowledge in the area of 3D printed pharmaceuticals, various technical challenges and knowledge gaps exist, including online blending of actives and understanding
the effects of mass transfer throughout processing and post-processing intervals on polypill design, performance, and quality.

Here, we provide a systematic study of the relationship between programmed concentration profile and temporal release profile in 3D printed polypills using a combination of experimental and computational techniques. We show microextrusion 3D printing processes enable the fabrication of polypills containing up to three oral agents commonly used for treatment of type 2 diabetes (metformin hydrochloride, glyburide, and acarbose). Experimental measurements and finite element analysis (FEA) of diffusive mass transfer processes during both the processing and post-processing intervals revealed that the programmed concentration profiles can be highly dynamic, and fabrication speed and solidification time are important process parameters that govern the difference between final and programmed concentration profiles. Drug release studies showed that the temporal release profile of individual actives from solid Pluronic F-127 polypills, an FDA approved excipient, could be programmed \textit{ab initio} to exhibit delayed, pulsed, and constant temporal profiles by changing the concentration profiles among core-shell, multilayer, and gradient distributions, respectively. Ultimately, this work suggests that AM offers expansive opportunities for designing patient- and disease-specific polypills with programmable temporal release profiles, and suggests that internal mass transfer processes throughout processing and post-processing intervals are important considerations for polypill design, quality, and performance.
4.3. Materials and Methods

4.3.1. Materials

All materials were purchased from Sigma-Aldrich.

4.3.2. Preparation of Hydrogel Inks for Microextrusion 3D Printing

Inks were prepared in 30 wt% aqueous solutions of Pluronic F-127. In addition to a pure ink void of actives and a pure ink containing red dye, three active-containing inks were prepared and contained one of the following: 12.5 mg mL\(^{-1}\) metformin hydrochloride (molecular weight (MW) = 165.6 g mol\(^{-1}\)); 100 mg mL\(^{-1}\) glyburide (MW = 494.0 g mol\(^{-1}\)), or 1 mg mL\(^{-1}\) acarbose (MW = 645.6 g mol\(^{-1}\)). The concentrations of actives were selected based on the dynamic range of spectroscopic assays, but we found that metformin hydrochloride and acarbose were also printable under similar conditions at higher therapeutically relevant concentrations of 300 and 100 mg mL\(^{-1}\), respectively. The actives were first dissolved in de-ionized water (Direct-Q 3UV; EMD Millipore) at 4 °C, then mixed with Pluronic F-127, and subsequently stored overnight at 4 °C to dissolve the Pluronic F-127. The cold active-containing polymer solutions were subsequently mixed using a centrifugal mixer (ARE-310; Thinky) for 30 s at 2000 rpm to ensure homogenization and uniformly suspend any undissolved solids, such as solid active particles in the case of super-saturated inks (i.e., those containing the active above its solubility limit as in the case of glyburide). This was followed by exposure to light vacuum for 10 min to remove air bubbles. The solutions were then loaded into different 55 mL syringe barrels and brought to room temperature to induce gelation.
4.3.3. Pill Design and 3D Printing

Cylindrical pills of radius \( R \) and height \( H \) equal to 6.5 and 10 mm, respectively, were fabricated using a custom microextrusion 3D printing system\(^{[15,37]}\). The system was composed of a gantry robot (F5200N.1; Fisnar), multiple dispensing systems (Ultimus V; Nordson), and a desktop computer. Hydrogel inks were printed from 55 mL syringe barrels using 27 GA tapered tips (Nordson), except for glyburide inks, which were printed using 22 GA tapered tips (Nordson) to accommodate its relatively higher viscosity. A custom static mixing nozzle as reported earlier\(^{[37]}\) was used for printing the gradient distributions. This nozzle enabled real-time mixing of two separate components, here one of the three drug-containing inks with the drug-free ink, such that the composition of the drug in the extruded ink could be varied between zero and the concentration of the drug-containing ink by controlling the pressure applied to each syringe in real-time. Pills were printed at a linear velocity of 10 mm s\(^{-1}\). A printing pressure of 12 psi was used for all pills except glyburide pills, which used a pressure of 8 psi, and gradient pills, which used pressures ranging from 38 - 46 psi. The build time (\textit{i.e.}, time to print one pill) was approximately 90 min. Pills were solidified by drying under light vacuum for 12 hr resulting in solid Pluronic F-127 pills. All pills contained 2 mm thick top and bottom drug-free Pluronic F-127 caps to reduce the effect of axial losses during dissolution on the measured temporal release profile, thereby leaving a remaining 6 mm of the pill’s center for active-containing material (schematically shown in Figure 1). This space contained one to three of the oral agents for type 2 diabetes (\textit{i.e.}, metformin hydrochloride, acarbose, and glyburide). We examined three experimental groups and one control group that consisted of the following programmed concentration
profiles (i.e., spatial distributions of actives): core-shell, multilayer, gradient, and uniform distributions, respectively. The pills with core-shell distributions contained a 3 mm radius active-containing core surrounded by a drug-free shell from a radius of 3 mm to the pill boundary located at 6.5 mm. The pills with multilayer distributions contained a 2 mm radius active-containing core, surrounded by a drug-free band from a radius of 2 to 4.5 mm, and an active-containing shell from a radius of 4.5 mm to the pill boundary. The pills with gradient distributions contained a linearly decreasing concentration profile that changes from the maximum concentration of the ink at the center of the pill \((r = 0)\) to a value of \(c = 0\) at the pill boundary. The polypills contained a combination of the aforementioned spatial distributions with 2 mm sections along the axial direction dedicated to a different distribution. The pills with uniform distributions contained the same concentration of active at all positions.

4.3.4. Characterization of Concentration Profiles in 3D Printed Pills during Processing and Post-Processing Intervals via Image Processing Techniques

To both assess the time-varying concentration profiles of actives in printed pills and characterize the diffusion of active species within the 3D printing and solidification intervals (i.e., processing and post-processing intervals, respectively), pills described in Section 2.3 were printed using red dye in place of the active. The drug-free caps were excluded to improve contrast for the active-containing region of the pill. Top-down photographs were then obtained (D7200; Nikon) yielding a two-dimensional concentration profile. The concentration profiles were then analyzed using image processing software (ImageJ; National Institutes of Health). The images were first reduced to 8-bit grayscale, and the grayscale value \((x)\) was then measured along the pill’s radius. The grayscale value indicates
the intensity of the dye at a given location, and therefore, the relative concentration of the dye. The normalized concentration profile \( \frac{c}{c_{\text{max}}} \) was then calculated as \( \frac{x - x_{\text{min}}}{(x_{\text{max}} - x_{\text{min}})} \), where \( x_{\text{max}} \) and \( x_{\text{min}} \) are the maximum and minimum greyscale values.

### 4.3.5. Pill Dissolution Studies

Measurements began by submerging solid pills in 30 mL of de-ionized (DI) water. Top-down brightfield micrographs were then taken at 10 min intervals for a duration of 300 min using a fluorescence stereoscope (AxioZoom; Zeiss). The pill radius was calculated as the average of the radius measured at the pill’s top and bottom focal planes. Error bars correspond to the standard deviation of six repeated dissolution studies \( (n = 6) \).

### 4.3.6. Finite Element Modeling of Diffusive Mass Transfer Processes during Processing and Post-Processing Intervals

Given that the polypills contain adjacent internal domains of differing concentration, it is of interest to consider the diffusion of drugs within the pill during both the 3D printing process and the post-fabrication solidification process. Modeling of the diffusive mass transfer processes during both the processing and post-processing intervals (i.e., 3D printing and solidification) were modeled using commercially available finite element analysis (FEA) software (v5.3; COMSOL Multiphysics). Mass transfer of actives within the pills was modeled using the Transport of Diluted Species module. **Mass Transfer during the Processing Interval:** Modeling of active species transport within the 3D printed pill during fabrication requires solution of the diffusion equation across a computational domain that increases in size with time. The simulation began with the first printed layer. The initial concentration profile corresponded to one of the aforementioned spatial distributions of actives, which included
core-shell, multilayer, or gradient structures. No flux boundary conditions were
implemented at the borders of the pill and flux continuity boundary conditions were
implemented at the interfaces between adjacent sub-domains of different concentration.
Calculations were done assuming Fickian diffusion and the diffusion constant \( D_o \) of the
active in the printed Pluronic-127 gel inks was 5\( \times \)10\(^{-11}\) m\(^2\) s\(^{-1}\).[38] The computational domain
was discretized using a physics-controlled mesh. The concentration profile was obtained by
solving the governing equations at various mesh densities ranging from 937 to 1,293,947
elements over a time interval equal to the time to print a single layer \( (t_{p,1}; \text{here 1.5 min}) \) using
a time step of 0.01 min. The resulting concentration profile was then stored. Convergence
was obtained at a mesh density of 238,832 elements within 1% of the previous mesh.
Subsequently, the computational domain was extended vertically by the addition of another
sub-domain to represent the addition of another printed layer of the pill. The governing
equations were then resolved across the new computational domain for a duration of \( t_p \)
subject to previously described boundary conditions but a modified initial condition that
included both the previously stored solution as well as the programmed concentration
profiles corresponding to one of the aforementioned spatial distributions of actives. This
process was repeated \( n-2 \) times, where \( n \) is the number of the printed layers (here \( n = 40 \)).
Upon addition of the final layer, the time interval of the simulation was then extended into a
post-processing interval of duration greater than \( t_p \). Mass Transfer during the Post-Processing
Interval: Modeling of active species transport within the 3D printed pill during solidification
was done using the final computational domain and concentration profile obtained from the
aforementioned simulation. The concentration profile within the pill during solidification
was then calculated by solving the diffusion equation for an extended time period, $t_{pp} = 12$ hr, but with a time-dependent diffusion constant ($D(t) = D_0 [1 - t / t_{pp}]$, where $t$ is the time and $t_{pp}$ is the post-processing time, here a drying-based solidification process). This relation assumes the diffusion coefficient of a small molecule within a hydrogel decreases with decreasing hydration\[39\] and diffusion within the solid phase is comparatively negligible\[40\].

4.3.7. Characterization of Temporal Release Profiles from 3D Printed Polypills

To begin experiments the solidified pills were immersed in 20 mL of DI water. Small aliquots (volumes described below) were subsequently removed at 25 minute intervals for a duration of 300 min. Briefly, to detect metformin hydrochloride, 50 µL of solution was removed at each time point and transferred to a well plate. To each well, 30 µL of 30% hydrogen peroxide and 140 µL of 5M sodium hydroxide were added and the well plate was heated to 70 °C for 1 hr. The absorbance peak at 400 nm was then measured.\[41\] To detect acarbose, 100 µL of solution was removed at each time point and transferred to a well plate. To each well, 100 µL of 0.5M sodium hydroxide and 100 µL of 0.01M potassium permanganate were added and allowed to react at room temperature for 30 min. The absorbance peak at 610 nm was then measured.\[42\] To detect glyburide, 30 µL of solution was removed at each time point and transferred to a well plate. To each well, 270 µL of dimethyl sulfoxide was added to dissolve the glyburide. The absorbance peak at 307 nm was then measured.\[43\] In the polypill experiments, separate aliquots for assay of each drug were removed at each time point to be tested. All measurements were conducted against a drug-free blank using a fluorescence microplate reader (Synergy H1M; BioTek). The fraction of active released was calculated as $A(t)/A_f$ where $A(t)$ is the absorbance value measured at the
wavelength of interest at time $t$ and $A_f$ is the final absorbance measured after the pill dissolved. All assays were run in triplicate.

4.3.8. Statistical Analysis

Statistical analysis was conducted in OriginPro. All data are presented showing the mean value with error bars to one standard deviation. Sample size for pill dissolution was $n = 6$ (pH = 7) and $n = 3$ (pH = 3). Sample size for drug release profiles was $n = 3$ for each pill design. All normalized data were calculated using feature scaling.

4.4. Results and Discussion

4.4.1. Dynamics of Programmed Concentration Profiles in 3D Printed Pills throughout Processing and Post-Processing Intervals

As shown schematically in Figure 4.1, microextrusion 3D printing processes offer a unique opportunity to both fabricate polypills and systematically explore the effect of programmed concentration profiles (i.e., spatial distributions of actives) on temporal release profiles, as such is critical toward the manufacturing of quality ‘smart’ and personalized pharmaceuticals. The premise for this study was that the temporal release profile of an active is governed by its programmed concentration profile, and that these concentration profiles are dynamic throughout both the processing and post-processing intervals. Pluronic F-127 was selected as an excipient, also referred to as bulking agent or diluent, based on its high yield stress at room temperature, which enables formation of free-standing hydrogel structures,[44] and given it is FDA approved for use in food additives and pharmaceuticals.[45, 46] As shown schematically in the middle panel of Figure 1, we examined three programmed spatial distributions of actives in the Pluronic F-127 pills, including core-shell, multilayer,
and gradient structures. A dye-containing ink was used as a model active-containing ink given its relatively similar MW to active species and ability to facilitate real-time measurement of the concentration profiles.

**Figure 4.1** Schematic showing the concept of programming temporal release profiles of individual actives from a single polypill by controlling their spatial distributions (i.e., concentration profiles). Microextrusion 3D printing processes enable the fabrication of polypills containing multiple actives distributed with varying concentration profiles, and was leveraged here to provide a systematic study of the relationship between programmed spatial distribution and temporal release profile.
Photographs of the printed pills after the 90 min processing and 12 hr post-processing intervals (i.e., the 3D printing and solidification intervals, respectively) are shown in Figures 4.2.A and B, respectively. Pills were printed at relatively low speeds to conduct real-time process monitoring for quality assurance. However, we found the print time could be reduced to approximately 20 minutes using the same 3D printing system by increasing the feed rate. The process is also scalable via the use of multiple extruders to enable multiplexing, and thus, improve throughput. Water loss during the solidification process resulted in a 13% and 30% decrease in pill’s diameter and height, respectively (see Figure S1 of Supplementary Materials). As shown in Figure 4.2.A, the visible spatial distribution present immediately after 3D printing qualitatively matched the programmed distributions (i.e., the tool path described by G-code). Furthermore, the photographs shown in Figure 4.2.B suggest that the programmed distributions persisted in the presence of pill shrinkage and solidification. We found that the solidified pills could be released from the glass substrates and withstand typical handling forces without fracture or disassembly (see Figure S4.2 of Supplementary Materials). However, although the final distributions obtained after post-processing resembled the programmed concentration profiles, the data suggest that the programmed concentration profiles are dynamic throughout the processing and post-processing intervals. In other words, the final concentration profiles do not exactly match the programmed structures. To better understand the potential mechanisms that drive the dynamic behavior of programmed concentration profiles throughout both the processing and post-processing intervals, we next quantified the differences between the programmed distributions and those obtained following processing and post-processing.
Figure 4.2.C provides a quantitative measure of the radial concentration profile in the pills shown in Figures 4.2.A and B. As shown in Figure 4.2.C, the final concentration profiles qualitatively matched the programmed distributions (i.e., tool path, here described by G-code), but were dynamic throughout the processing and post-processing intervals. As shown by comparing the final and programmed concentration profiles among each of the different spatial distributions, the core-shell and multilayer structures exhibited relatively larger differences relative to the gradient structure. For example, the difference between the final and programmed normalized concentration at $r = 0$ mm was 0.4, 0.2, and 0.1 for the core-shell, multilayer, and gradient structures, respectively. The correlation between shape of the programmed profile and magnitude of change observed suggests diffusion processes may drive programmed concentration profile dynamics (i.e., large differences in concentration over short distances present in core-shell and multilayer structures establish relatively larger driving forces for diffusive mass transfer when compared with gradient structures). Thus, to better understand such potential mechanisms that drive the dynamics of programmed concentration profiles, we next examined how diffusive mass transfer processes affect the programmed (i.e., tool path-defined) concentration profiles throughout the processing interval.
Figure 4. 2 Microextrusion printing of polypills with varying concentration profiles and analysis of concentration profile dynamics throughout processing and post-processing intervals. A) Top-down photographs of the 3D printed hydrogel Pluronic F-127 pills containing core-shell, multilayer, and gradient concentration profiles acquired after the 90-min processing interval (i.e., microextrusion 3D printing process). A red dye facilitates the measurement of radial concentration profile using image processing techniques. B) Top-down photographs of the same 3D printed Pluronic F-127 pills shown in (A) acquired after the 12-hr post-processing interval (i.e., drying-based solidification process). C) Dimensionless radial concentration profile corresponding to the dashed lines in (A) and (B) showing the dynamics of programmed concentration profiles (G-code) throughout pill processing (hydrogel) and post-processing (solid).
4.4.2. Modeling of Mass Transfer Processes in 3D Printed Pharmaceuticals throughout the Processing Interval

To understand the mechanism driving the dynamics of programmed concentration profiles in the 3D printed pills, we next modeled the temporal evolution of the various spatial distributions in the pill throughout both the 3D printing (processing) and solidification (post-processing) intervals using a novel finite element model. Similar to modeling of heat transfer processes in fused filament fabrication AM processes,\textsuperscript{[47]} modeling of mass transfer processes in microextrusion printing throughout the manufacturing interval is non-trivial due to the fact that the computational domain (\textit{i.e.,} size of the part) increases with time. We present here for the first time a model capable of calculating concentration profile dynamics in 3D printed parts across a computational domain that changes in size throughout the simulation. As shown schematically in Figure 4.3.A, the computational domain extends in the print direction throughout the simulation interval based on the known speed of the 3D printing process (\textit{e.g.,} the linear speed or the time to print a single layer ($t_{p,l}$)), here 10 mm s\textsuperscript{-1} and 90 s. Prior to examining the simulation results, it is useful to consider the potential role diffusive mass transfer processes may have considering both the pill design and the processing parameters. For example, it is possible to estimate the time scale for diffusion ($t_d$) as $L_c^2/D$, where $L_c$ is the characteristic length and $D$ is the diffusion constant of an active species in a hydrogel.\textsuperscript{[48]} Considering AM is a layer-by-layer fabrication process, $L_c$ may be approximated as the thickness of a single layer (250 µm). Thus, given $D = 5 \times 10^{-11}$ m\textsuperscript{2} s\textsuperscript{-1},\textsuperscript{[38]} the time scale for diffusion is 21 min (\textit{i.e.,} $t_d = 21$ min). We remind the reader that the build time ($t_p$) was 90 min. Considering the ratio of the build time to the diffusive time scale ($t_p/t_d$)
is on the order of one \( \frac{t_p}{t_d} = 90 \text{ min}/21 \text{ min} \sim 4.3 \), diffusive mass transfer processes are likely to drive changes in programmed concentration profiles throughout the processing interval, such as those shown in Figures 4.2.A and C.

Figures 4.3.B and C show the final concentration profile in the 3D printed pills after the processing interval for each of the three spatial distributions: core-shell, multilayer, and gradient. The data show that diffusive mass transfer throughout the processing interval causes changes to each of the programmed concentration profiles. As shown in Figures 4.3.B and C, the largest differences between the final and programmed concentration profiles occurred in the core-shell and multilayer structures. The maximum differences occurred at the interface between domains of different concentration (denoted by positions \( h_1 \) and \( h_3 \)). We remind the reader that such was also observed in the 3D printed pills from a top-down perspective shown in Figure 4.2. Alternatively, as shown in the right panel of Figures 4.3.B and C, the pills with gradient structures exhibited relatively smaller deviations between final and programmed concentration profiles. For example, the maximum difference between the final and programmed concentration profile for the gradient structure was 4.3 mg mL\(^{-1}\) and was located at \( r = 0 \text{ mm} \) near the bottom of the pill. The difference between the final and programmed concentration profiles was also a function of height, with relatively larger changes occurring toward the pill’s base for all structures examined. This observation can be described by the longer time for diffusive mass transfer processes to affect initially printed layers.
Figure 4. 3 Finite element modeling of mass transfer in 3D printed pills during the processing (i.e., 3D printing) interval. A) Schematic of the computational approach for modeling mass transfer in 3D printed parts, such as polypills, throughout the processing interval as a series of transient simulations. B) Results from the finite element model described in (A) showing the predicted two-dimensional concentration profiles of active in the radius ($r$)-height ($h$) plane after the processing interval for the core-shell, multilayer, and gradient structures ($c_{\text{max}} = 12.5 \text{ mg/mL}$). C) Radial concentration profile corresponding to the dashed lines in (B) showing both the dynamics of programmed concentration profiles throughout the pill processing interval and the effect of height and position on concentration profile dynamics ($h_1 = 2.25 \text{ mm}, h_2 = 4.25 \text{ mm}, h_3 = 6 \text{ mm}$).
4.4.3. Modeling of Mass Transfer Processes in 3D Printed Pharmaceuticals throughout the Post-Processing Interval

As previously shown in Figure 4.2, a comparison of the pills before and after post-processing revealed that the concentration profiles were also dynamic throughout the solidification process. Thus, we next examined the temporal evolution of the programmed core-shell, multilayer, and gradient concentration profiles throughout the post-processing interval using the finite element model. As shown in Figures 4.4.A and B, the experimentally observed changes in programmed concentration profile can also be described by diffusion of actives between 3D printed layers under the driving force imposed by the concentration difference between the adjacent sub-domains (see Figure S4.3 of Supplementary Materials for predicted concentration profiles in the $r$-$h$ plane). This observation supports the hypothesis that the concentration difference between adjacent filaments (i.e., sub-domains) is an important consideration in predicting the deviation between programmed distributions and those obtained following post-processing. The data in Figure 4.4.B show the largest differences between the final and programmed concentration profiles occurred at the center of the pill. To interpret the larger changes in concentration profile observed during the post-processing interval relative to the processing interval, it is useful to consider the ratios of the processing time ($t_p$) and post-processing time ($t_{pp}$) to the diffusive time scale ($t_d$) (i.e., $t_p/t_d$ and $t_{pp}/t_d$, respectively). For example, $t_p/t_d = 90 \text{ min}/21 \text{ min} = 4.3$ compared with $t_{pp}/t_d = 720 \text{ min}/21 \text{ min} = 34.3$, suggesting that diffusive mass transfer effects during the post-processing interval are larger than during the processing interval. We remind the reader that $t_d$ is actually time-dependent throughout the post-processing interval given the
diffusivity decreases with time due to pill solidification (see Section 4.3.6 of the Materials and Methods Section), yet the previous calculation provides a useful estimate for interpreting the observed concentration profile dynamics. Overall, the data shown in Figures 4.2 – 4.4 describe the mechanisms underlying changes in programmed concentration profiles throughout the processing and post-processing intervals, and demonstrate it is possible to achieve 3D printed pills with concentration profiles that exhibit core-shell, multilayer, and gradient structures. We next examined the temporal release profiles associated with the three spatial distributions using single-active pill systems.
4.4.4. Measurement of Dissolution Kinetics

Prior to investigating the temporal release profiles for the three different spatial distributions, we first examined the dissolution kinetics of the solid Pluronic F-127 pills.
given the ratio of the diffusive time scale \((t_d)\) to the dissolution time \((t_e)\) serves as a useful measure of the time scale for drug release \((i.e.,\) the temporal release period\). In this case, \(L_c\) can be approximated as the radius of the pill \((6.5\) mm\), since the case of drug release from the pill is now being considered as opposed to diffusion between adjacent layers as discussed in Figures 3 and 4. Thus, given \(D = 5 \times 10^{-11} \) m\(^2\) s\(^{-1}\), the diffusive time scale \((t_d) = 23.5\) hr. As shown in Figure 4.5.A, the pill radius decreased throughout the dissolution process, over which the pill exhibited an interior solid domain surrounded by a thin gel layer. As shown by the trend in Figure 4.5.B, the dissolution process at pH = 7 followed Higuchi-like kinetics, commonly associated with diffusion-driven release processes\.[49] A change in the trend was observed approximately halfway through the dissolution process \((t = 130\) min\) and corresponded to the onset of dissolution in the axial direction (see Figure 4.5.A), thereby potentially accelerating the dissolution rate. We also found that the dissolution process in a low pH solution (pH = 3) exhibited similar kinetics (see Figure 4.5.B). Overall, the dissolution studies shown in Figure 5 suggest two important considerations when interpreting temporal release profiles: 1) the time scale of the temporal release period for the 3D printed Pluronic F-127 pills will be on the order of the dissolution time \((t_e\), here 5 hr\), given it is smaller than the diffusive time scale \((t_d = 23.5\) hr\), and 2) payload release by pill erosion \((i.e.,\) polymer gelation followed by dissolution into the liquid phase\) drives the temporal release process. Having examined the pill dissolution kinetics, we next examined the temporal release profiles achieved by the three different spatial distributions.
Figure 4.5 Dissolution studies of 3D printed pills in aqueous solution. A) Top-down micrographs of dissolving 3D printed Pluronic F-127 pills at various time points throughout the dissolution process (t = 0, 100, 200, and 300 min). B) Dissolution profile of the 3D printed Pluronic F-127 pills in DI water (n = 6 for pH = 7; n = 3 for pH = 3).
4.4.5. Relationships between Programmed Concentration Profiles and Temporal Release Profiles in Single Active-Containing 3D Printed Pills

Although AM enables the integration of multiple actives within a single pill, also referred to as polypills, we first examined the temporal release profiles associated with pills that contain only a single active, here metformin hydrochloride, distributed in a core-shell, multilayer, or gradient structure (see Figure 4.6.A). The concentration of active in the metformin hydrochloride ink was selected based on the dynamic range of the spectroscopic assay used for quantification of drug release; however, we also found metformin hydrochloride was printable at higher therapeutically relevant concentrations up to 300 mg mL\(^{-1}\). As shown in Figure 4.6, the core-shell, multilayer, and gradient structures (i.e., programmed concentration profiles) exhibited unique temporal release profiles over the course of a 300-min release period. For example, as shown in Figure 4.6.B, core-shell spatial distributions resulted in a delayed temporal release profile. The delayed temporal profile exhibited no release until 125 min, after which the total payload was released at a constant rate. Alternatively, the temporal release profile of the control pills that contained a uniform spatial distribution of metformin hydrochloride exhibited the commonly observed exponential release characteristics. As shown in Figure 4.6.C, multilayer distributions resulted in a pulsed temporal release profile. The pulsed temporal release profile initially resembled that of control pill, but instead of continually increasing, the pulsed release profile plateaued at 65% payload release over the time range of 75 to 200 min. Following the plateau, a second exponential release phase was observed for the duration of the release period. As shown in Figure 4.6.D, gradient distributions (here linear profiles) resulted in
relatively constant temporal release profiles. Collectively, the results in Figure 6 show the ability to achieve programmable temporal release profiles of therapeutics from 3D printed pills containing a single active. Specifically, we found programmed concentration profiles with core-shell, multilayer, and gradient structures generate delayed, pulsed, and constant temporal release profiles, respectively.

Figure 4.6 Temporal release profiles of single active-containing pills with core-shell, multilayer, and gradient concentration profiles. A) Schematic of capped metformin hydrochloride pills with varying concentration profiles. Temporal release profiles of metformin hydrochloride pills containing core-shell (B), multilayer (C), and gradient (D) distributions (n = 3 for each pill type). Control pills contained a uniform distribution of
metformin hydrochloride. Core-shell, multilayer, and gradient concentration profiles enable programming of delayed, pulsed, and constant linear release profiles in 3D printed pills containing a single active (here metformin hydrochloride).

4.4.6. Relationships between Programmed Concentration Profiles and Temporal Release Profiles in 3D Printed Polypills

Having demonstrated a correlation between programmed concentration profile and the resultant temporal release profile using single active-containing pills, we next examined the temporal release profiles achieved in polypills containing three commonly used oral agents for treatment of type 2 diabetes: metformin hydrochloride, acarbose, and glyburide. Treatment plans for type 2 diabetes commonly involve combinations of the aforementioned drugs administered by tablets containing dosages ranging from 1 - 1,000 mg and release periods ranging from 1 - 8 hr.[50, 51] Furthermore, research is still ongoing regarding the use of drug combinations for treatment of type 2 diabetes, suggesting that polypill technology may play a role in future treatment plans.[52] The polypill design is shown in Figure 4.7.A. The stacked polypill contained one layer with a core-shell spatial distribution of glyburide, a second layer with a multilayer distribution of acarbose, and a third layer with a gradient distribution of metformin hydrochloride. The polypill also had two end caps that contained no drug to decrease payload loss in the pill’s axial direction. Similar to the studies using single active-containing metformin hydrochloride pills discussed in Figure 4.6, the concentration of actives in the printed acarbose and glyburide inks were selected based on the dynamic range of the spectroscopic assays. The glyburide inks exhibited a higher yield stress and viscosity relative to the metformin hydrochloride and acarbose inks due to a larger amount of suspended solids based on the relatively lower solubility limit of glyburide.
Such behavior is consistent with previous observations from inks for nanocomposite printing that also exhibit yield stress increase due to increase in solid filler content.\textsuperscript{[54]} The resultant temporal release profiles of the individual actives are shown in Figures 4.7.B - C. Comparison of the temporal release profiles from the pills with single actives with those from the polypills (see Figures 4.6.B - D and 4.7.B - D, respectively) reveals that the correlations between spatial distribution and temporal release profile observed for case of single active pills also hold for pills containing multiple actives. For example, as shown in Figures 4.7.B - D, glyburide, acarbose, and metformin hydrochloride exhibited delayed, pulsed, and constant temporal release profiles, respectively. Specifically, as shown in Figure 4.7.B, glyburide was not detected until 175 minutes, which compared reasonably to the value of 125 minutes observed for the metformin hydrochloride pills (see Figure 4.6.B). As shown in Figure 4.7.C, acarbose exhibited a pulsed temporal release profile characterized by a plateau near 50% payload release over the time range of 125 to 225 minutes. The acarbose response also contained a short delay period not observed in the metformin hydrochloride pills shown in Figure 4.6.C. Similar to the behavior observed in the single active pills, metformin hydrochloride was released from the polypills continuously over the 300-min release period; however, close inspection of the profile shows two separate intervals of differing release rates rather than a single interval as observed in the absence of other actives. We note that the polypills exhibited a higher relative standard deviation in temporal release profile compared with the pills that contained a single drug, but the origin cannot be elucidated at this time. Ultimately, these results suggest that it is possible to program the temporal release profiles of individual actives from polypills by controlling
their concentration profiles. Specifically, we showed that programmed concentration profiles with core-shell, multilayer, and gradient structures can be used to produce delayed, pulsed, and constant temporal profiles, respectively. Overall, these results suggest unique opportunities for the design and manufacturing of patient- and disease-specific polypills with programmable temporal release profiles.

Figure 4. 7 Temporal release profiles of polypills with core-shell, multilayer, and gradient concentration profiles. A) Schematic of a stacked polypill containing interior glyburide, acarbose, and metformin layers and drug-free caps. Temporal release profiles of glyburide
(B), acarbose (C), and metformin (D) from the polypills. Control pills contained a uniform distribution of the actives. Core-shell, multilayer, and gradient concentration profiles enable programming of delayed, pulsed, and constant linear release profiles in 3D printed polypills (here, three commonly used oral agents for treatment of type 2 diabetes - metformin hydrochloride, acarbose, and glyburide).

4.5. Conclusions

Microextrusion 3D printing is a relatively new manufacturing process that is poised to address several of the current challenges in pharmaceutical tablet manufacturing. Here, we reported a systematic study of the effect of programmed concentration profile on temporal release profile in Pluronic F-127 pills which contained up to three commonly prescribed oral agents used for treatment of type 2 diabetes. Programmed concentration profiles of varying spatial distribution were examined in this work, including core-shell, multilayer, and gradient structures. A combination of experimental studies and finite element models revealed programmed concentration profiles in 3D printed pills are dynamic throughout both the 3D printing (processing) and solidification (post-processing) intervals. A novel finite element model revealed that the experimentally observed dynamics can be described by the diffusion of active species within the pill under driving forces imposed by the concentration gradient associated with a given spatial distribution. Temporal release studies showed that programmed concentration profiles with core-shell, multilayer, and gradient structures can be used to produce delayed, pulsed, and constant temporal release profiles, respectively. Overall, this work suggests unique opportunities for approaching the design and manufacturing of quality ‘smart’ and personalized polypills with programmable temporal release profiles. In addition, this work suggests that the ability to
understand mass transfer processes within pills during both processing and post-processing intervals is critical toward design and quality control of future 3D printed pharmaceuticals.

4.6. References


4.7. Supporting Information

These supplementary materials contain: measurements of pill shrinkage during the solidification interval (Figure S4.1), photographs of 3D printed pills following the solidification interval withstanding typical handling (Figure S4.2), and changes in concentration profiles in pills calculated during the post-processing interval corresponding to data shown in Figure 4.4 of the main text presented from a side view (Figure S4.3).
Figure S4. Measurements of pill shrinkage during the solidification process. Measurement of pill shrinkage during solidification was measured by acquiring diameter and height measurements before and after drying with digital calipers. Pills shrank 13% and 30% in diameter and height, respectively. Measurements on pills before drying also confirmed that the AM process accurately produced the programmed pill dimensions, yielding pills within 100 µm of the programmed diameter and height (i.e., that defined by the tool path). Measurements correspond to $n = 3$ different pills.
Figure S4. 2 Photograph of two 3D printed solid Pluronic F-127 polypills released from the substrate following the post-processing (*i.e.*, drying) interval. The solid pills could withstand handling forces without fracture or crumbling.
Figure S4. 3 A) Schematic of the programmed concentration profiles (i.e., those described by the tool path) in 3D printed pills. B) Predicted two-dimensional concentration profile of metformin hydrochloride in the r-h plane for core-shell, multilayer, and gradient structures following the post-processing interval corresponding to data shown in Figure 4 of the main text ($c_{max} = 12.5$ mg mL$^{-1}$).
Chapter Five: Additive Manufacturing of Three-dimensional (3D) Microfluidic-based Microelectromechanical Systems (MEMS) for Acoustofluidic Applications

5.1. Abstract

Three-dimensional (3D) printing now enables the fabrication of novel 3D structural electronics and microfluidics. However, conventional subtractive manufacturing processes for MEMS fabrication relatively limit device structure to two dimensions and require post-processing steps for interface with microfluidics. Thus, the objective of this work is to create an additive manufacturing approach for fabrication of 3D microfluidic-based MEMS devices that enables 3D configurations of electromechanical systems and simultaneous integration of microfluidics in a one-pot manufacturing process. Here, we demonstrate the ability to fabricate microfluidic-based 3D microelectromechanical systems (MEMS) that contain orthogonal out-of-plane piezoelectric sensors and actuators using additive manufacturing. The devices were fabricated using a microextrusion 3D printing system that contained integrated pick-and-place functionality. Additively assembled materials and components included 3D printed epoxy, polydimethylsiloxane (PDMS), silver nanoparticles, and eutectic Gallium-Indium as well as robotically embedded orthogonal out-of-plane piezoelectric chips (lead zirconate titanate (PZT)). Electrical impedance spectroscopy and finite element modeling studies showed the embedded PZT chips exhibited multiple resonant modes of varying mode shape over the 0 – 20 MHz frequency range. Flow visualization studies using neutrally buoyant particles (diameter = 0.8 – 70 µm) confirmed the 3D printed MEMS devices
generate bulk acoustic waves (BAWs) capable of size-selective manipulation, trapping, and separation of suspended particles in droplets and microchannels. Flow visualization studies in continuous flow format showed suspended particles could be moved toward or away from the walls of microfluidic channels based on selective actuation of in-plane or out-of-plane PZT chips. This work suggests additive manufacturing potentially provides new opportunities for the design and fabrication of acoustofluidic and microfluidic devices.

5.2. Introduction

Additive manufacturing, also referred to as 3D printing, has emerged as a flexible multi-material processing technique and appears poised to enable the design and manufacture of novel functional materials and devices.[1-6] In particular, the ability to 3D print multiple materials, notably polymers and functional nanomaterials, has led to the fabrication of novel electronic[4, 6-9] and microfluidic devices.[1, 5, 10] The current paradigm for 3D printing functional multi-material and -component systems, such as 3D electronics, is based on formulating all materials and components into printable matrices. For example, structural and functional materials can be formulated as printable suspensions, foams, gels, resins, and melts.[2, 11, 12] However, in situ integration of non-printed components, such as microchips, remains an open challenge that currently limits the design space for 3D printed electronics. Thus, new approaches for robust integration of non-printed functional components with 3D printed architectures could expand our ability to create novel 3D printed electronics, and ultimately, functional multicomponent systems.[13]

Embedding of non-printed components within 3D printed architectures provides a means of enhancing the performance and functionality of 3D printed systems, and thus, has
been leveraged toward the fabrication of novel electronic devices.\cite{14, 15} For example, 3D printed magnetic flux sensors,\cite{16} electrode-integrated microfluidic devices,\cite{17} circuits,\cite{7, 16} and nuclear spin polarizers\cite{18} have been produced by embedding non-printed electronic components in 3D printed architectures. Embedding techniques are classified by the mechanism for grasping and placement of non-printed components, such as in pick-and-place techniques.\cite{19} Although various pick-and-place techniques exist (e.g. manually or robotically guided\cite{20} adhesion, suction,\cite{21, 22} or mechanical\cite{23} mechanisms),\cite{24} robotic suction-based techniques are especially promising given tolerance for surface characteristics of the embedded component, ability to control grip force, and fast cycle time.\cite{19} To date, the most common embedded components have included conductive materials,\cite{25, 26} discrete circuit components,\cite{15, 27} and integrated circuits.\cite{27, 28} Given ceramic materials serve as functional components for a variety of electronic devices due to their electromechanical coupling properties, the ability to 3D print ceramic-based multicomponent systems would be desirable. However, although 3D printing appears to be an emerging technique for shaping ceramic materials,\cite{29} processing and post-processing requirements\cite{30} (e.g. the need for support materials, sintering, and poling, respectively) impede the integration of ceramics into 3D printed polymeric architectures.\cite{31} Consequently, the ability to construct 3D printed ceramic-based electronic devices remains an open challenge. Thus, the ability to interweave non-printed technical ceramics, such as piezoelectric materials, with 3D printed materials via embedding could potentially enable the fabrication of novel multicomponent electronic devices,\cite{13} such as microelectromechanical systems (MEMS).
Acoustofluidic devices are a versatile class of analytical platforms for small-volume static liquid and flow-based biosensing and bio-separation applications. Flexibility in biological and biomedical applications arises from the various methods for actuating surface and bulk acoustic waves via electroded piezoelectric materials (e.g. via interdigitated or parallel plate electrodes, respectively). To date, transmitted surface acoustic waves (SAWs) actuated by interdigitated electrodes have been applied in both static and flow formats (e.g. droplet- or microfluidic-based, respectively). Although microfabrication approaches have enabled the creation of novel interdigitated electrode geometries for SAW-based acoustofluidic devices, the techniques for anchoring and embedding the piezoelectric components for bulk acoustic wave (BAW)-based acoustofluidic devices remain limited. Thus, the ability to embed piezoelectric chips in 3D printed architectures in situ during the 3D printing process could provide unique electroding, anchoring, and acoustic matching strategies, thereby generating new opportunities for the design and application of BAW-based acoustofluidic devices. Furthermore, the ability to 3D print acoustofluidic devices provides a potential pathway toward highly reconfigurable and integrated acoustofluidic platforms.

Here, we highlight a novel approach for integrating piezoelectric materials with multi-material 3D printed microfluidic architectures using a pick-and-place microextrusion 3D printing process. Specifically, we demonstrate the utility of the electronic integration technique via the printing of 3D microfluidic-based MEMS devices composed of thermosetting polymers for structural anchoring, elastomers for acoustic impedance matching, functional nanomaterials for conductive pathways, and embedded lead zirconate...
titanate (PZT) chips for sensing and actuation purposes. Electrical impedance spectroscopy and finite element modeling studies show the printed devices exhibit multiple resonance modes and corresponding mode shapes over the 0 – 20 MHz frequency range. We also show via flow visualization studies that the acoustic trapping patterns and streaming profiles correlate with the embedded PZT chip mode shapes. The printed devices exhibit frequency- and particle size-dependent regimes of acoustic particle trapping and streaming, with a transition zone occurring at ~15 MHz and ~1 µm, respectively. This work shows 3D printing provides new opportunities for the design and fabrication of 3D MEMS, acoustofluidic, and microfluidic devices.

5.3. Materials and Methods

5.3.1. Materials

Glass cover slips (22 × 22 mm²) were purchased from AmScope. Epoxy (Hysol E-90FL) was from Loctite. Lead zirconate titanate sheets (PZT-5A; 127 µm thick) with nickel (Ni) electrodes were from Piezo Systems, Inc. (Woburn, MA). Sylgard 184 silicone (PDMS) was from Dow Corning. Polyethylene particles (70 and 25 µm) were from Cospheric (Santa Barbara, CA). Polybead particles (6 and 0.8 µm) were from Polysciences, Inc. (Warminster, PA). Fluorescent Fluoro-Max particles (26, 6, and 0.8 µm) were from ThermoFisher Scientific. Ultrapure de-ionized water (DIW) was from a commercially available DIW system (Direct-Q 3UV; Millipore). Poly (ethylene glycol) dimethacrylate (PEG-DA) (Mw = 750 Da), silver nitrate, poly(acrylic acid) sodium salt (PAA) (Mw = ~15 kDa, 35% in water), diethanolamine, ethylene glycol, ethanol (200 proof), and eutectic Gallium-Indium (EGaIn) were from Sigma Aldrich. Hydrochloric acid (HCl) was from Fisher Scientific.
5.3.2. Preparation of 3D Printed and Embedded Materials

PZT sheets were diced into $5 \times 1 \times 0.127$ mm$^3$ chips for use as the embedded piezoelectric component in the 3D printed acoustofluidic devices (American Dicing; Liverpool, NY). The 3D printed materials included an elastomer (PDMS), a thermosetting polymer (epoxy), and a suspension of conductive (silver; Ag) nanoparticles. PDMS was prepared as a 10:1 ratio by weight of the base to curing agent. Epoxy was prepared as a vendor-specified mixture of the two-part system. The Ag nanoparticle suspension was synthesized as previously described.$^{[40]}$ Briefly, 40 g of diethanolamine and 3.6 g PAA were dissolved in 50 mL of DIW and stirred for 2 hours at room temperature. Separately, a solution of 20 g silver nitrate in 20 mL water was prepared. The silver nitrate solution was slowly added to the diethanolamine-PAA solution while stirring vigorously. The mixture was stirred gently for 24 hours until black. The resulting solution was then titrated with 120 mL ethanol to form a precipitate. The supernatant was decanted, and the Ag slurry was centrifuged at 5,000 rpm for 40 minutes (Storvall ST16; Thermo Scientific). The supernatant was again removed. Then a humectant solution (30 wt% ethylene glycol in water) was added to the nanoparticles at 10 wt% and homogenized. The resultant suspension was placed under light vacuum for 30 minutes prior to printing.

5.3.3. Fabrication of Acoustofluidic Devices via the Pick-and-Place 3D Printing Process

A custom pick-and-place microextrusion 3D printing system comprised of an industrial dispensing robot (F5200N; Fisnar; repeatability = 20 µm) and high precision digital dispensers (Ultimus V; Nordson EFD) was used for this work (see Figure S1 of
Supporting Information). Tool paths were written using vendor-provided software (RoboEdit; Fisnar). Printing of the device began by placing a glass substrate on the heated print stage \((T = 100^\circ C)\) using the suction-based pick-and-place tool (16 gauge nozzle). Ag was then printed from the edge to the center of the substrate using a 33 gauge tip at 4 mm/s with an applied pressure of 10 – 15 psi. Subsequently, the pick-and-place tool was used to grasp a PZT chip from the edge of the build area and place it on the end of the Ag pathway located in the center of the substrate by applying a vacuum of 0.65 psi to grasp the chip and a pressure of 0.1 psi to release it, respectively. Epoxy was then printed around the chip using a 27 gauge tapered tip and a high pressure adapter (HP7x; Nordson) at 2 – 4 mm/s with a pressure of 30 – 40 psi. The stage was then heated to 100\(^\circ\)C for 10 minutes using a Peltier to cure the epoxy. Next, Ag was printed from the top face of the PZT chip at the center of the device to the edge of the substrate extending in the opposite direction of the first Ag pathway. Subsequently, the printed device was heated for 10 minutes at 100\(^\circ\)C to evaporate excess liquid from the printed Ag suspension. An epoxy boundary was then printed at the edges of the substrate located 1 mm inside the ends of printed conductive pathways and allowed to solidify for 10 minutes at 100\(^\circ\)C. PDMS was then printed within the external epoxy boundary using a 20 gauge tip at 8 mm/s with an applied pressure of 4 psi, and the printed device was allowed to cure for two hours at 100\(^\circ\)C. This process resulted in a device that contained a single in-plane transducer. Subsequently, a second pick-and-place operation was used to integrate a second out-of-plane transducer on the chip that was orthogonal to the previously embedded in-plane transducer by the following steps. First, a single filament of silicone was printed on the chip in the direction of the printed Ag conductors displaced by a distance of 3
mm from the center of the in-plane transducer using a 27 gauge tip at 1 mm/s to provide a medium with high adhesion for the subsequently embedded piezoelectric chip. Subsequently, a second piezoelectric chip was grasped from the long edge (i.e., thickness dimension) using a second pick-and-place tool based on an adhesive grasping mechanism (here, a silicone-filled 27 gauge tip) and inserted 200 µm into the silicone filament for placement. The pick-and-place tool was subsequently retracted leaving the piezoelectric chip placed in an out-of-plane configuration with the normal vector of the chip’s face orthogonal to that of the previously integrated chip. The silicone was then allowed to cure. An epoxy anchor was printed around the base of the piezoelectric chip covering the silicone filament and making mechanical contact with the chip and PDMS substrate on all sides leaving 0.5 mm of the piezoelectric chip exposed to air. This process resulted in a device that contained a 3D configuration of orthogonal acoustic transducers (i.e., one in-plane and one out-of-plane transducer).

A Y-shaped microfluidic channel was then printed in between the two transducers according to a previously reported technique.\cite{41} Briefly, two EGaIn filaments were printed in a Y-shape on the PDMS substrate directly in between the two orthogonal transducers using a 20 gauge tip with a pressure of 0.1 – 0.3 kPa and print speed of 1.7 – 2.2 mm/s. Following printing, the EGaIn filament and out-of-plane acoustic transducer were insulated by application of a second PDMS layer on the device. After PDMS curing, the EGaIn was removed from the microchannel by continuous flow of a 1M HCl solution down the channel. The channel was then rinsed with DIW and the inlet was connected with a syringe pump via external tubing.
5.3.4. Profilometry Studies

The height and cross-sectional profile of each 3D printed and embedded component in the printed construct was characterized using a profilometer (Form Talysurf S3F; Taylor Hobson) throughout the 3D printing process. All profiles were acquired at a scan speed of 0.5 mm/s using a 2 µm diamond conispherical stylus tip.

5.3.5. Electrical Impedance Spectroscopy

The electrical impedance responses of the 3D printed devices were measured using an impedance analyzer (E5061B; Keysight). The electrical impedance response at 100 mV AC with zero DC offset was recorded as phase angle ($\phi$) and impedance ($Z$) versus frequency data over the 0 – 20 MHz frequency range.

5.3.6. Finite Element Analysis of Electromechanical Frequency Response and Modal Mechanics

Electromechanically-coupled eigenfrequency and frequency response simulations of the printed device were done as previously reported.$^{[42, 43]}$ All studies were performed using commercially available finite element analysis (FEA) software (COMSOL Multiphysics, Version 5.2a). A frequency domain analysis was conducted in 3D using the piezoelectric device interface, which enables the coupling of a solid mechanics problem using the Structural Mechanics module with an electrostatics problem using the AC/DC module. The domain was generated using the graphics-user interface according to the dimensions of the embedded PZT chip ($5 \times 1 \times 0.127$ mm$^3$). The material properties of PZT were selected from the software’s built-in library. Damping was modeled as an isotropic structure loss factor ($\eta = 0.01$).$^{[42]}$ The mechanical boundary conditions included a fixed constraint condition on all
chip faces except the top and bottom faces. No initial deformation, stress, or external forces were applied. The electrostatic boundary conditions included a zero-charge condition on all chip faces except the top and bottom faces. A 20 V electrical potential and a ground condition were applied on the top and bottom faces, respectively. The model was then discretized using a physics-controlled mesh. Subsequently, the mode shapes and electrical impedance spectrum were obtained over 0.1 – 20 MHz at a step size of 0.01 MHz using an eigenvalue solver. Proper density of the mesh was validated by examining the convergence of the maximum displacement ($D$), $Z$, and $\varphi$ by iterating from an extremely coarse to an extra fine mesh element size. The total current ($I$) was calculated by integrating the boundary current density over the electrode area at each frequency value. $Z$ and $\varphi$ were then calculated as $Z = \frac{V}{I}$ and $\varphi = \tan^{-1}\left[\frac{\text{Im}(I)}{\text{Re}(I)}\right]$, respectively.

5.3.7. Sensing of Acoustic Waves

Acoustic waves generated by the electrically excited embedded PZT chip were sensed by integrating a secondary PZT chip with the elastomeric layer above the epoxy-embedded chip. The separation distance between the epoxy-embedded and secondary chips was ~1 mm. Sensing of acoustic waves was done by recording the voltage signal ($V_s$) generated in the secondary chip using an oscilloscope (DS1050E; Rigol) while the epoxy-embedded chip was excited at 20 V<sub>p-p</sub> ($V_a$). Voltage responses were acquired while the device was driven at each resonant mode. The fast Fourier transform (FFT) of the acquired voltage time series data was calculated using MATLAB. We note that the secondary PZT sensing chip was not present for subsequent acoustofluidic flow visualization studies (e.g. particle manipulation studies), but served to characterize the acoustic waves generated by the device.
5.3.8. Droplet- and Continuous Flow-based Particle Separation Studies

All polymer microparticle suspensions, including neutrally buoyant polyethylene (70 µm and 25 µm), Polybead (6 and 0.8 µm), and Fluoro-Max (26, 6, and 0.8 µm) particles, were prepared in DIW at a concentration of 1 mg/mL. For droplet-based particle separation studies, 3 µL of a given particle suspension was deposited on the top surface of the printed device 1 mm from the long side of the in-plane PZT chip (located in between the two orthogonal transducers) in the absence of vibration, thus forming a droplet that contained suspended particles. After allowing five seconds for the particle distribution to reach a stable initial condition, the in-plane transducer was then excited at 20 V_{p-p} at a fixed frequency. Micrographs and videos of particle trajectories throughout the stimulation period were acquired using a stereoscope (OM4413; Omo). Fluorescence micrographs were acquired using a fluorescence microscope (Axio Zoom.V16; Zeiss). For continuous flow-based particle separation studies, a particle suspension containing 6 µm polybead particles was first flowed through the 3D printed microfluidic channel at 25 µL/min in the absence of vibration, thus allowing a steady state flow profile to develop. The in-plane, out-of-plane, or both in- and out-of-plane transducers were then excited at 20 V_{p-p} at a fixed frequency. Micrographs of the flow profile throughout the stimulation period were acquired using a stereoscope (OM4413; Omo).

5.3.9. Image Analysis of Particle Distributions

Light and fluorescence micrographs were analyzed in ImageJ to quantify the particle distributions for the droplet-based particle separation studies. Micrographs were first imported into ImageJ and grey scaled. Subsequently, the grey scale intensity across the
diameter of the droplet was plotted. Intensity profiles were then normalized by the maximum grey scale value.

5.4. Results and Discussion

5.4.1. 3D Printing of Acoustofluidic Devices via Robotic Embedding of Piezoelectric Components

Although 3D printed electronics widely vary regarding design, function, and utility, the incorporation of electromechanically coupled materials remains an open challenge due to limitations associated with ceramic integration. As a result, manufacturing processes that enable the assembly of ceramics, polymers, and nanomaterials could potentially provide a novel fabrication approach for ceramic-based electronics, such as MEMS. As shown schematically in Figure 5.1, a multi-material pick-and-place 3D printing process could potentially enable the in situ integration of electroded technical ceramics, such as piezoelectric materials, into 3D printed polymer architectures, thus providing a novel pathway toward creating novel MEMS, such as acoustofluidic devices. We next examined the ability to 3D print acoustofluidic devices based on robotically embedded PZT chips, given they serve as actuators for a variety of MEMS, including dynamic-mode biosensors and acoustofluidic devices.[44-46] As shown in Figure 5.2a, the multi-step printing process involved: 1) pick-and-place of a glass substrate (22 × 22 mm^2; 227 mg); 2) 3D printing of a conductive Ag pathway on the substrate; 3) pick-and-place of the PZT chip (5 × 1 mm^2; 2.5 mg) on the conductive pathway with an in-plane configuration, thereby bonding the printed Ag pathway to the PZT Ni electrode; 4) 3D printing of an epoxy anchor; 5) 3D printing of a conductive Ag pathway from the top of the chip onto the substrate, thereby forming a symmetrically electroded PZT chip; 6) 3D printing of an epoxy support wall; and 7) 3D
printing of a PDMS insulation layer. As shown in Step 3 of Figure 5.2a, the robotically guided suction-based pick-and-place tool enabled placement of the chip on the printed Ag pathway with high precision. As shown by comparison of Steps 3 and 4 in Figure 5.2a, the release pressure associated with chip placement did not change the chip’s position relative to the release location. In addition, the shear stress exerted by the 3D printed epoxy anchor on the PZT chip during printing did not change the position of the PZT chip relative to the placement location (see Steps 3 and 4). Given the printed and embedded materials exhibit differences in thickness, the device exhibits a non-uniform height profile throughout the printing process. For example, the profilometry data in Step 3 show that embedding of the in-plane PZT chip causes a 143 µm height difference between the substrate and the top of the embedded PZT chip, which requires the subsequent printing of a 3D conductive Ag pathway to the chip’s top electrode. As shown in Step 4, the height profile of the printed epoxy was conducive for printing a 3D conductive Ag pathway (17 µm high and 750 µm wide) that extended from the top electrode of the PZT chip to the substrate (see Figure S5.2 of Supporting Information for a magnified view of the profilometry data). Ultimately, as shown in Figure 5.2b, the printing process resulted in a 729 µm thick, five-layer device composed of an embedded and electroded PZT chip within a matrix of 3D printed polymers and functional nanomaterials. A video of the printing process is shown in Video S5.1 of Supporting Information.
Additive manufacturing concept for fabrication of 3D microfluidic MEMS devices. A combination of 3D printing and robotic embedding facilitates the integration of orthogonal in-plane and out-of-plane piezoelectric transducers, functional 3D printable materials, and microfluidic channels. In-plane and out-of-plane piezoelectric transducers facilitate the trapping of continuously flowing particles in microfluidic channels in transverse and lateral directions.

Figure 5.2 a) Highlight of the seven fabrication steps (I-VII) for the 3D printed acoustofluidic device including 3D printing and embedding processes. Each step shows in vertically
descending order: the assembly schematic, a photograph of the device during the fabrication step, and the height profile of the device after completion of the step. b) Cross-sectional schematic (top) and photograph (bottom) of the device.

5.4.2. Frequency Response and Modal Mechanics of the 3D Printed Acoustofluidic Devices

As a dynamic electromechanical component, the embedded PZT chip exhibits various natural modes of vibration (i.e. resonant modes). Thus, we next examined the electrical impedance response of the in-plane PZT transducer over the 0 – 20 MHz frequency range. As shown in Figure 5.3a, the in-plane transducer exhibited various resonant modes as identified by peaks or inflection points in phase angle and impedance data, respectively. For example, the in-plane transducer exhibited four distinct resonant modes at 1.9 ± 0.1, 4.7 ± 0.1, 7.4 ± 0.1 and 17.2 ± 0.6 MHz (n = 13 devices). The observed coupling of mechanical motion to impedance change in the PZT layer is consistent with millimeter-scale piezoelectric sensors and actuators.[42, 43] Having identified the frequencies of the in-plane transducer’s multiple resonant modes via electrical impedance measurements, we next conducted finite element simulations to examine the modal mechanics associated with each resonant mode. Given the PZT was embedded in a printed epoxy anchor (see Figure 5.3b), the transducer was modeled as an externally clamped vibrating structure. As shown by the first panel of Figure 5.3a, the simulated electrical impedance response of the in-plane transducer exhibited resonant modes with high impedance-coupling at 1.6, 4.8, 7.6, and 17.3 MHz, which agreed reasonably to the experimentally measured values. Figure 5.3c shows the 3D mode shape at each resonant frequency in terms of the transverse (out-of-plane) displacement (\(D(z)\)) (see Figure S3 of Supporting Information for corresponding displacement profiles in the width direction). As shown by the 3D mode shapes and
corresponding displacement profiles in Figure 5.3c, the in-plane transducer exhibited transverse modes at 1.9 and 4.7 MHz and combination modes at 7.4 and 17.2 MHz with maximum transverse displacements \( (D(z)_{\text{max}}) \) of \(~18, 14, 12, \) and \(3 \) nm, respectively.

![Image](image-url)

**Figure 5.3** a) Experimentally measured and simulated electrical impedance response of the 3D printed acoustofluidic devices over 0 – 20 MHz and highlight of experimentally measured impedance \( (Z) \) and phase angle \( (\phi) \) characteristics from four resonant modes that exhibit strong impedance-coupling.  b) Photograph of the embedded PZT chip highlighting the acoustic source. c) Calculated 3D mode shape and displacement profile (dashed line) for each of the resonant modes shown in terms of the transverse displacement. d) Secondary transducer configuration for sensing of acoustic waves generated by the embedded PZT chip showing the corresponding voltage signal generated in the secondary transducer \( (V_{s,p-p}) \) at each mode. e) Comparison of the voltage generated in the secondary acoustic transducer with the maximum total displacement \( (D_{\text{max}}) \) calculated using finite element simulations.
for each resonant frequency \((f_n)\). Also shown is the fast Fourier transform of the measured voltage signal shown in (d).

5.4.3. Sensing of Acoustic Radiation

Having shown that the in-plane transducers exhibit various resonant modes of different mode shape and displacement magnitude, we next examined the ability to sense acoustic waves generated by the transducer through embedding of a secondary piezoelectric transducer. As shown schematically in Figure 5.3d, excitation of the in-plane transducer with voltage \((V_a)\) produces an acoustic wave that can be measured in terms of a generated voltage \((V_s)\) in a secondary piezoelectric transducer. Figure 5.3d shows the measured voltage generated in the secondary piezoelectric transducer for each of the four modes in the presence of device excitation at \(V_a = 20\) V. As shown in Figure 5.3e, the magnitude of the generated voltage in the secondary piezoelectric transducer correlated with the calculated maximum total displacement \((D_{max})\) for each mode. For example, the generated voltage was 540, 170, 184, and 80 mV at 1.9, 4.7, 7.4, and 17.2 MHz, respectively, and \(D_{max}\) was 45, 20, 22, and 8 nm, respectively. As shown in Figure 5.3e, we also verified that the frequency of the generated voltage matched the excitation frequency. Ultimately, the data in Figure 5.3 show the in-plane transducer exhibits various resonant modes and generate acoustic waves that propagate through the top printed PDMS layer. Thus, the coupling of fluid systems, such as a droplets or microfluidic streams, with the 3D printed MEMS device could enable novel MEMS architectures.
Having shown that the in-plane transducers exhibit various resonant mode shapes across a wide frequency range and transmit acoustic waves through the printed PDMS layer, we next examined the acoustofluidic effects produced by each mode. Given the increasing demand for small volume analytical techniques, droplet-based acoustofluidic applications (e.g. for droplet-based mixing and particle trapping) have received significant attention. Thus, as shown in Figure 5.4a, we examined the potential to manipulate suspended particles in droplet-based systems using the in-plane transducer to verify the acoustic coupling of the robotically embedded PZT materials with surrounding fluids. It is well established that propagating acoustic waves subject suspended particles to acoustic radiation and streaming forces that can result in particle trapping or dynamic mixing. As shown in Figure 5.4b, each resonant mode of the in-plane transducer enabled the trapping and mixing of 0.8 – 70 µm suspended particles in microliter droplets (see Video S5.2 of Supporting Information for a representative real-time video of particle dynamics). Similar to the dependence of mode shape on frequency discussed in Figure 5.3, we also observed frequency-dependent acoustic trapping patterns for all particle sizes. For example, particle trapping patterns consisted of parallel and concentric zones of trapped particles at low and high frequency, respectively. We note that concentric trapping patterns in droplets have also been observed using SAW devices over kHz – MHz frequency ranges with particles ranging from ~1 – 10 µm in diameter. We found the number of nodes present in a given trapping pattern (i.e. the number of individual trapped features) increased with frequency for all particle systems (see Figure 5.4b). For example, the number of trapping zones
increased from ~5 at 1.9 MHz to ~50 at 7.4 MHz with corresponding feature sizes ranging from ~125 µm wide to the width of a single particle, respectively. The data in Figure 5.4b also show frequency- and size-dependent regimes of particle trapping or streaming behavior, with a transition zone occurring between 7.4 and 17.2 MHz and 800 nm and 6 µm, respectively. For example, as shown in Figure 4b, all modes exhibited trapping behavior, except the 17.2 MHz mode, which caused streaming behavior. Likewise, all particles exhibited trapping behavior, except the 800 nm particles, which exhibited streaming behavior. As shown in Figure 5.4b, all particle sizes exhibited streaming behavior at 17.2 MHz. We note that the 800 nm particles exhibited streaming behavior regardless of the frequency value. We also observed that the streaming velocity increased with increasing excitation frequency for the 800 nm particle system. These observations are consistent with the dependence of $F_{PR}$ and $F_D$ on particle diameter ($R$) and frequency ($f$). Overall, the results shown in Figures 5.3 and 5.4 show that the 3D printed devices are functional and provide acoustofluidic coupling.
Figure 5.4 a) Schematic of droplet-based flow visualization studies using the in-plane piezoelectric transducer. Acoustic waves generated by the robotically embedded in-plane transducer propagate into the droplet producing pressure oscillations (P) and streaming flow (v) that exert forces on suspended particles (diameter = D). b) Micrographs of the suspended particle systems under excitation at each mode of the 3D printed acoustofluidic device show distinct regimes of trapping and streaming behavior for particles ranging from 0.8 – 70 µm in size. c) Fluorescence micrographs and particle distribution plots of multi-particle systems under excitation at each mode of the 3D printed acoustofluidic device show size-selective separation of particles and mode-dependent separation profiles.

5.4.5. Droplet-based Acoustofluidic Particle Separation Studies

The dependence of trapping patterns on particle size at a fixed frequency suggests that the in-plane transducer may provide the ability to size-selectively separate suspended...
particles. As shown in Figure 5.4c, we examined the ability to acoustically separate particles of different size using a fluorescent particle system that contained a mixture of 26, 6, and 0.8 µm particles. As shown by the fluorescence micrographs and radial intensity profiles in Figure 5.4c, all modes provided size-selective particle separation, yet exhibited different final configurations. As shown by comparison of Figures 4b and c, the trapping zone geometries observed for a single particle size (see Figure 5.4b) were consistent with those observed when particles of a different size were also present (see Figure 5.4c). For example, at 1.9 MHz, the 6 and 26 µm particles separated into distributed linear trapping zones, while the 800 nm particles remained relatively dispersed throughout the droplet. Alternatively, at 17.2 MHz the 6 µm particles formed symmetric ellipsoidal patterns, while the 26 µm particles became concentrated toward the droplet’s outer edge. However, it is important to note that separated particles at 1.9, 4.7, and 7.4 MHz were static, while those at 17.2 MHz were dynamic. We note that frequency-dependent dynamic particle separation profiles in droplets have also been observed using SAW devices at frequencies ranging from 10 to 130 MHz.[38, 47]

5.4.6. Integration of an Orthogonal Out-of-Plane Acoustic Transducer

Having demonstrated the functionality of the 3D printed in-plane transducers using droplet-based flow visualization studies, we next leveraged the 3D printing and pick-and-place process to construct 3D MEMS devices that contain additional orthogonal out-of-plane transducers, a novel configuration not found in SAW devices (see Figures 5.5a and b). As shown in Figures 5.5c-e, in contrast to the vacuum-based mechanism used in for embedding of the in-plane transducer (see Figure 5c), the out-of-plane transducer was embedded using
an adhesion-based pick-and-place mechanism (see Figures 5.5d and e). As shown by the photographs in Figure 5.5f, the process resulted in an out-of-plane transducer that was displaced ~3 mm from the in-plane transducer. It is noteworthy that similar to a previous report using BAW transducers, the normal vectors of the embedded piezoelectric chip faces are orthogonal, as such configurations are difficult to achieve using SAW devices. SAW devices with multiple interdigitated electrodes that establish orthogonal pressure waves have been reported however, the multiple systems for actuators are typically in-plane (i.e., the normal vectors are parallel and not orthogonal) as the planar electrodes are deposited via conventional microfabrication approaches and not 3D printing as done here. We note that in addition to controlling the orientation of the integrated piezoelectric materials (e.g., in-plane vs. out-of-plane with respect to the substrate), the use of additive manufacturing offers a greater accuracy and precision with respect to integration of piezoelectric components relative to hand positioning approaches, specifically 5.7 ± 2.5 vs. 15.2 ± 3.9 % error with respect to a target position, respectively (see Figure S5.4 of Supporting Information). The bonding and anchoring of the piezoelectric chips to the substrate via 3D printing also improves the accuracy and precision of the fabrication process given the established control and resolution of direct-write 3D printing processes relative to manual material deposition approaches. Figure 5.5g shows the electrical impedance response of both the out-of-plane and in-plane transducers over the 0 – 20 MHz range. The orthogonal out-of-plane transducer exhibited resonant modes at 1.8 ± 0.1, 4.6 ± 0.3, 7.3 ± 0.1, and 17.2 ± 0.7 MHz (n = 5 devices), which were similar to locations of the in-plane transducer modes (see Figures 5.2 and 5.5g), suggesting that fluidic systems, such as droplets or
continuously flowing liquid, could be simultaneously actuated via orthogonal in-plane and out-of-plane transducers. A detailed discussion of the origins of impedance-coupled resonant modes in anchored piezoelectric-excited millimetre-sized transducers has been reported elsewhere.[43]

**Figure 5.** a) Schematic of a 3D microfluidic MEMS device containing two orthogonal piezoelectric chips (one in-plane and one out-of-plane). b) Concept of orienting piezoelectric transducers with both the in-plane and out-of-plane components of a microchannel using additive manufacturing to facilitate manipulation of continuously flowing particles (e.g., whole cells). c) Vacuum-based robotic embedding principle associated with integration of the in-plane piezoelectric transducer. d–e) Adhesion-based robotic embedding principle associated with integration of the out-of-plane piezoelectric transducer. f) Photographs of the device before and after printing of Ag interconnects to the out-of-plane piezoelectric transducer. g) Electrical impedance spectra of the in-plane and out-of-plane piezoelectric transducers over the 0 – 20 MHz frequency range.
5.4.7. Continuous Flow-based Acoustofluidic Particle Separation Studies

The data in Figure 5.5 suggest that 3D printing offers the ability to create 3D MEMS devices that contain multiple embedded BAW transducers with orthogonal out-of-plane configurations. Given the advantages of microfluidics regarding increased throughput and biosensing applications, we next examined the acoustofluidic effects of the 3D orthogonal transducer configuration on suspended particles in a continuous flow format. As shown in Figure 6a, a microfluidic channel printing approach based on EGaIn\[41\] was used to construct a Y-shaped microchannel on the device in between the in-plane and out-of-plane transducer. Figure 6b shows the process resulted in a PDMS encapsulated 281 ± 11 µm wide and 197 ± 9 µm high microfluidic channel (n = 3 devices). Although the microchannel dimensions used here demonstrate the ability to interweave microfluidics within 3D MEMS via 3D printing, selection of different nozzle diameters for EGaIn printing can potentially enable matching between the transducer and the channel half-wavelength mode resonant frequencies.\[55\] As shown schematically in Figure 5.6c, the fluid and suspended particles within the microchannel could be subjected to acoustic stimulation through both the in-plane and out-of-plane transducer either individually or simultaneously. Thus, we next examined the effect of these stimulation conditions on a continuously flowing suspension of 6 µm particles. Figures 5.6d–g show the flow profile for the following four stimulation conditions: only the in-plane transducer on at 1.9 MHz (Figure 5.6d), only the out-of-plane transducer on at 1.8 MHz (Figure 5.6e), both transducers on (Figure 5.6f), and both transducers off (Figure 5.6g), which served as the negative control. As shown in Figure 5.6d, stimulation using only the in-plane transducer resulted in particle concentration near the walls of the microfluidic
channel. The suspended particles also exhibited a longitudinal trapping characteristic characterized by ‘strings’ of trapped particles one particle wide that extended across the width of the microchannel. In contrast as shown in Figure 5.6e, stimulation using only the out-of-plane transducer resulted in transverse concentration of the flowing particles toward the top of the channel causing them to leave the focal plane of the microscope. The micrograph also shows concentration of particles toward the center of the microchannel, although many of the particles remained relatively well dispersed across the microchannel width. As shown in Figure 5.6f, stimulation using both the in-plane and out-of-plane transducer resulted in significant particle trapping in the center of the microchannel resulting in a ~100 µm wide concentrated particle stream and surrounding disperse zone that extended ~90 µm from the wall. We note that the following observations of particle motion in the presence of acoustic stimulation differ from that observed in the absence of acoustic stimulation, which resulted in a uniformly dispersed continuously flowing suspension (see Figure 5.6g). Thus, the data in Figure 5.6d-g suggest that it is possible to move particles comparable to the size of suspended animal cells in a continuously flowing suspension in both the lateral (in-plane) and transverse (out-of-plane) direction as well as toward and away from the walls of a microfluidic channel by selective stimulation using in-plane and out-of-plane transducers, respectively. The data also show that although the PDMS-water acoustic impedance mismatch ($Z_{a,PDMS} = 1.0$ MRayl vs. $Z_{a,water} = 1.5$ MRayl) is less than silicon-water systems ($Z_{a,si} = 21.3$ MRayl vs. $Z_{a,water} = 1.5$ MRayl),\textsuperscript{56, 57} particle trapping was still observed. The development of transparent printable materials with high acoustic impedance or PDMS-based impedance matching techniques\textsuperscript{56} could potentially improve the
trapping effects. The ability to move continuously flowing suspended particles toward and away from the microchannel walls could potentially enable next-generation continuous flow-based whole cell biosensing platforms considering the walls of the microfluidic channels are often the locations of sensors. Furthermore, the ability to control the 3D orientation of acoustic transducers offers potentially novel opportunities in acoustic-based material design.\textsuperscript{[58, 59]}
**Figure 5.6** a) Schematic of the fabrication steps associated with integration of a 3D printed microchannel in between the in-plane and out-of-plane piezoelectric transducers with corresponding photographs. b) Schematic showing the microchannel orientation with respect to the integrated piezoelectric transducers and corresponding photograph after dissolution and washout of the printed eutectic Gallium-Indium. c) Schematic showing the principle of exciting both the in-plane and out-of-plane transducer during continuous flow of suspended particles through the microchannel. Flow visualization studies showing continuously flowing 6 µm particles in the presence of stimulation from the in-plane transducer (d), presence of stimulation from the out-of-plane transducer (e), presence of simultaneous stimulation from both transducers (f), and absence of acoustic stimulation (g) (white arrow indicates the direction of flow).

5.5. Conclusions

Here, we showed additive manufacturing processes composed of robotic pick-and-place functionality with multi-material 3D printing enable the fabrication of microfluidic-based 3D MEMS devices. We demonstrate that piezoelectric transducers could be embedded with 3D configurations consisting of orthogonal in-plane and out-of-plane configurations using a combination of vacuum- and adhesion-based embedding techniques, and showed that these transducers exhibit multiple resonant modes over the 0 – 20 MHz frequency range using electrical impedance spectroscopy. Additive manufacturing was also used to interweave microchannels between the 3D transducer configurations. We found suspended particles could be selectively concentrated in both the transverse (out-of-plane) and lateral (in-plane) directions as well as toward the walls or center of the microchannel depending on the transducer and transducer combination used for stimulation. Overall, this work suggests that additive manufacturing offers unique opportunities for the fabrication of 3D MEMS devices with 3D configurations of embedded piezoelectric components, interconnects, and microfluidic channels.
5.6. References


5.7. Supporting Information

These supporting information include: photographs of the multi-material pick-and-place 3D printing process for fabrication of ceramic-based electronics (Figure S5.1), a magnified view of the profilometry data described in Figure 5.2 of the main text (Figure S5.2), calculated transverse displacement profiles in the width direction of the PZT chip upon excitation at each resonant mode (Figure S5.3), data regarding the accuracy and precision of the robotic pick-and-place approach relative to hand positioning techniques (Figure S5.4), a video of the 3D printing process (Video S5.1), and a video of the droplet-based particle manipulation studies for the first resonant mode (1.9 MHz) using the representative 6 µm particles (Video S5.2).
Figure S5. 1) Multi-material pick-and-place 3D printing process for fabrication of ceramic-based electronics consisting of a print stage, extrusion nozzles, suction-based pick-and-place tool, 3-axis robot, vision system, and pressure regulators. 2) Magnified view of the print heads and pick-and-place tool.
**Figure S5.2** Magnified view of the profilometry data of 3D printed MEMS devices during the printing process showing: the 3D printed Ag conductive pathway (a), the robotically placed PZT chip on top of the Ag conductive pathway (b), the 3D printed thermosetting epoxy anchor (c), and the second 3D printed Ag conductive pathway on top of the PZT chip (d).
Figure S5.3 Calculated transverse displacement profiles in the width direction (dashed line) of the PZT chip upon excitation at each resonant mode (a – d) and corresponding displacement profiles (e – h).
Figure S5. 4  a) Representative micrographs comparing the accuracy and precision of PZT chip placement via the robotic pick-and-place approach vs. hand positioning. b) Schematic showing the percent error in chip placement relative to a target location (e.g., a chip that is centered on a printed trace of adhesive material). c) Experimental data showing the chip placement accuracy and precision of each approach corresponding to panel (b).
Chapter Six: Process- and Bio-inspired Hydrogels for 3D Bioprinting of Soft Free-standing Neural and Glial Tissues

6.1. Abstract

A bio-inspired hydrogel for 3D bioprinting of soft free-standing neural tissues is presented. The novel filler-free bioinks were designed by combining natural polymers for extracellular matrix biomimicry with synthetic polymers that endow desirable rheological properties for 3D bioprinting. Crosslinking of thiolated Pluronic F-127 with dopamine-conjugated (DC) gelatin and DC hyaluronic acid through a thiol – catechol reaction resulted in thermally gelling bioinks with Herschel-Bulkley fluid rheological behavior. Microextrusion 3D bioprinting was used to fabricate free-standing cell-laden tissue constructs. The bioinks exhibited flattened parabola velocity profiles with tunable low shear regions. Two pathways were investigated for curing the bioink: chelation and photocuring. The storage modulus of the cured bioinks ranged from 6.7 – 11.7 kPa. The iron (III) chelation chemistry produced crosslinked neural tissues of relatively lower storage modulus than the photocuring approach. In vitro cell viability studies using the 3D bioprinted neural tissues showed that the cured bioink was biocompatible based on a minimal cytotoxic response observed over seven days in culture relative to control studies done using alginate hydrogels. Rodent Schwann cell-, rodent neuronal cell-, and human glioma cell-laden tissue constructs were printed and cultured over seven days and exhibited comparable viability relative to alginate bioink controls. The ability to fabricate soft, free-standing 3D neural tissues with low modulus has far-reaching implications in the biofabrication of microphysiological neural
systems for drug discovery and disease modeling applications as well as neural tissues and innervated tissues and organs for regenerative medicine.

6.2. Introduction

Additive biomanufacturing (BioAM), or 3D bioprinting, is a rapidly developing field focusing on the fabrication of miniaturized tissues, organs, and organ systems for use in fundamental research and drug discovery applications as well as macro-scale tissues and organs for regenerative medicine.\[1-4\] Among the various types of 3D bioprinting techniques that are used, including stereolithography, microextrusion printing, inkjet printing, and laser-assisted bioprinting, microextrusion 3D printing has shown flexibility regarding multi-material integration.\[1\] While microextrusion 3D bioprinting has been used to fabricate connective and muscle tissues,\[5, 6\] it has been used relatively less for fabrication of neural tissue as well as innervated muscle and connective tissues. Bioinks for neuroscience, neural engineering, and nerve regeneration can potentially be used for applications in regenerative medicine, such as fillers for regeneration of neural tissue in the central or peripheral nervous system, or drug discovery, such as 3D mini-tissues for drug screening.\[7-9\] Importantly, an ideal bioink for neural tissues should not only mimic the chemistry and modulus of native neural tissue, but also exhibit the desirable rheological properties and curing mechanisms to support the fabrication of high quality free-standing tissue constructs.

Neural tissue extracellular matrix (ECM) is primarily comprised of structural proteins, such as collagen and other proteoglycans and glycoproteins.\[10\] The glycosaminoglycan hyaluronic acid is also an important component found in neural tissue ECM, and has been shown to play an important role in nerve regeneration.\[11, 12\] Unlike bone,
connective, and muscle tissue that exhibit moduli ranging from 0.1 – 15,000 MPa\cite{13, 14}, the modulus of neural tissue in the central nervous system ranges from 0.3 to 27 kPa.\cite{14} Peripheral nerve tissue exhibits a modulus near 0.5 MPa.\cite{15} Given many emerging applications require stem cells, it is also useful to consider that substrate stiffness has been reported to affect differentiation.\cite{14, 16, 17} For example, it was found that substrates from 0.5 to 1 kPa favored differentiation of adult neural stem cells into neurons, while the relatively stiffer substrates from 1 – 10 kPa favored glial cells.\cite{16, 17}

Microextrusion 3D printing processes enable the fabrication of a wide range of tissues, including neural tissues.\cite{1} Material deposition via extrusion imposes design constraints on the rheological properties of the printed material, commonly referred to as a ‘bioink’ or cell-laden hydrogel.\cite{18} A desirable bioink for microextrusion 3D bioprinting exhibits rheological properties that enable the formation of free-standing macroscopic constructs (e.g., yield stress or thixotropic behavior) and minimize shear stress. However, the natural polymers that comprise the native neural tissue ECM components do not produce hydrogels that exhibit such desirable rheological behavior without additional formulation and additives (e.g., fillers and rheological modifiers).\cite{19} Current state-of-the-art bioinks are typically curable aqueous solutions of biomacromolecules, such as gelatin methacrylate (gel-MA) or alginate. The two approaches for fabricating 3D bioprinted tissue constructs are: 1) printing of a bioink within a thermoplastic scaffold,\cite{20, 21} or 2) printing of bioinks that contain rheological modifiers, such as nanofibrillated cellulose or clay nanoparticles, which endow desirable properties for formation of free-standing structures.\cite{19, 22-24} 3D bioprinting of self-supporting (i.e., free-standing) hydrogel constructs has recently been identified as an
area of importance in materials design for 3D bioprinting.[24] In both cases, a crosslinking mechanism, such as photocuring, is often implemented as a post-processing step to cure the printed bioink.[18, 19] While the use of scaffolds and fillers could be advantageous for the fabrication of load-bearing tissues that exhibit high modulus (e.g., bone, connective, and muscle), they increase the cured construct’s modulus relative to filler-free systems, thereby impeding the fabrication of soft neural tissues.[19, 25, 26]

While 3D printing has been used to fabricate scaffold-based microphysiological neural systems and scaffolds for nerve regeneration,[20, 27] its use in fabrication of soft neural tissues has remained limited.[7, 27, 28] Neuron- and glia-laden hydrogels have been printed successfully with high viability, but the rheological properties have not been optimized for the fabrication of soft, free-standing structures.[8, 9] For example, Gu et al. used a microextrusion 3D bioprinting process with a polysaccharide-based bioink to fabricate neural stem cell-laden tissues, which could be differentiated in situ and formed synapses.[9] Lozano et al. used a gellan gum-based bioink to 3D print layered brain-like structures for studying brain injuries and neurodegenerative disease.[8] While the aforementioned studies demonstrate the scope of bioink design for 3D printed neural tissues, a critical review of 3D bioprinted free-standing neural tissues reveals a tradeoff between ink printability and mimicry of the native tissue’s mechanical properties. As a result, it remains a challenge to 3D print free-standing biomimetic neural tissues that mimic the native tissue with respect to both chemistry and mechanical properties.

Here, we show a chemically and mechanically biomimetic filler-free bioink for 3D bioprinting of soft neural tissues. As shown in Figure 6.1, the bioink design was based on
combining natural polymers found in neural tissue ECM for biomimicry of tissue chemistry with synthetic polymers that endow both biomimicry of neural tissue mechanical properties as well as desirable rheological properties for 3D printing. To mimic the ECM composition of neural tissue, gelatin and hyaluronic acid were selected as the natural polymers. Pluronic F-127 was selected as the synthetic polymer based on its ability to form low modulus, thermoreversible gels that exhibit Herschel-Bulkley-type rheological behavior. Dopamine, which has recently been used to improve hydrogel adhesion, flexibility, and reactive properties for 3D cell culture,[29-32] was conjugated onto hyaluronic acid and gelatin as a group for crosslinking. Dopamine-based materials, specifically polydopamine, have also been examined for modifying neural electrodes,[30] suggesting they are compatible with neural tissues and provide useful biomaterials for neural interface design. A polymer network was created by reacting thiolated Pluronic F-127 with dopamine-conjugated (DC) gelatin and DC hyaluronic acid through a thiol – catechol reaction, which resulted in thermally gelling bioinks that also exhibited Herschel-Bulkley type rheological behavior. Two curing pathways were investigated: chelation and photocuring. In vitro viability studies using the 3D bioprinted rodent Schwann cell-laden constructs showed the cured bioink was biocompatible based on a minimal cytotoxic response observed over seven days in culture relative to control studies done using alginate hydrogels. Additionally, rodent neuronal cells and human glioma cells were printed using the bioink and cultured for seven days. The ability to fabricate free-standing chemically and mechanically biomimetic 3D soft neural tissues has far-reaching implications in the manufacturing of microphysiological neural
systems for drug discovery and disease modeling as well as the manufacturing of neural tissues and innervated tissues and organs for regenerative medicine applications.

Figure 6. 1 a) Neural tissue bioink design for biomimicry and processability. Native extracellular matrix components of neural tissue were combined with a synthetic polymer to achieve biomimicry of neural tissue chemistry and mechanical properties as well as desirable rheological properties for microextrusion 3D bioprinting of soft, free-standing neural tissues. b) The slightly crosslinked hydrogel network creates a highly printable bioink capable of forming free-standing structures. c) The printed bioink could be chelated or photocured to produce a cured chemically and mechanically biomimetic 3D bioprinted neural tissue.

6.3. Materials and Methods

6.3.1. Materials

Microbially-produced hyaluronic acid (60 kDa) was purchased from LifeCore (Chaska, MN, USA). Pluronic F-127 (PF127), dichloromethane (DCM), alginic acid sodium salt from brown algae, calcium chloride, 4-nitrophenyl chloroformate, sodium sulfate, sodium
chloride, diethyl ether, phosphate buffered saline (PBS), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), dopamine hydrochloride, 2-(N-morpholino)ethanesulfonic acid (MES), gelatin (50-100 kDa, from porcine skin, 300 g bloom), N-hydroxysuccinimide (NHS), mouse nerve growth factor (NGF; 26 kDa; 2.5S), and poly-L-lysine (PLL) (70-150 kDa, 0.01%) were from MilliporeSigma. Hydrochloric acid (12M; HCl), Dulbecco’s PBS (DPBS), Dulbecco’s Modified Eagle’s Medium (DMEM), Dulbecco’s Modified Eagle’s Medium/F12 (DMEM/F12), heat-inactivated horse serum (HS), lipophilic dye (DiI), AlamarBlue, fetal bovine serum (FBS), and penicillin-streptomycin (PS) were from ThermoFisher. RPMI-1640 medium was obtained from ATCC.

6.3.2. Bioink Synthesis and Preparation

Thiolated PF127 (PF127-SH) was synthesized as previously described.[33] Briefly, 10 g of PF127 was dissolved in 70 mL of DCM containing 221 µL triethylamine. This solution was then added dropwise over 30 minutes to a 10 mL DCM solution that contained 960 mg 4-nitrophenyl chloroformate. Subsequently, the solution was stirred at room temperature for 48 hours. The activated PF127 was then washed with sodium chloride saturated DI water and the DCM phase was further dried with sodium sulfate for 30 minutes. The suspension was then filtered with a Büchner funnel to remove the sodium sulfate. Ice cold diethyl ether was then used to precipitate the product from DCM. The precipitate was then separated using a Büchner funnel, washed with additional ice cold diethyl ether, and dried under vacuum. Activated PF127 (5 g) was then dissolved in 50 mL of DCM with 420 mg cysteamine and the solution was then stirred at room temperature for 24 hours. The PF127-SH was then purified via the same precipitation-based separation approach used for the activated PF127.
The PF127-SH was then dissolved in 100 mL DI water and dialyzed against DI water with daily water changes for two days. Following dialysis, the product was lyophilized (BT85; Millrock Technology) for two days until bone dry. The lyophilized PF127-SH product was then stored at -20 °C.

Dopamine-conjugated hyaluronic acid (DC-HA) was synthesized as previously described.[33] Briefly, 1 g of hyaluronic acid was dissolved in 100 mL of 0.1 M PBS. The pH was then adjusted to 5.5 by the dropwise addition of 1 M HCl solution. 474 mg of EDC and 390 mg dopamine hydrochloride were added and stirred gently for four hours to conjugate dopamine to carboxylic groups along the HA backbone. The pH was monitored during the reaction and maintained at 5.5 using a 1 M HCl solution. Following the reaction, the solution was dialyzed against DI water at pH = 5.0 for two days followed by 24 hours against DI water at pH = 7. The DC-HA product was then lyophilized and stored at -20 °C.

Dopamine-conjugated gelatin (DC-Gel) was synthesized as previously described.[34] Briefly, 350 mg gelatin was dissolved in 60 mL of 50 mM MES buffer. The pH was then adjusted to pH 4.5 by dropwise addition of 1 M HCl. To this, 150 mg NHS, 191 mg EDC, and 103 mg dopamine hydrochloride were added. This solution was reacted in the dark with gentle stirring for 48 hours at 40 °C to conjugate dopamine to carboxylic groups along the gelatin backbone. Following the reaction, the solution was then dialyzed against DI water at 40 °C for 48 hours and lyophilized for 2 days. The product was stored at -20 °C.

DC-Gel was then methacrylated (DC-Gel-MA) as previously described.[27, 32] Briefly, 5 g of DC-Gel was dissolved in 50 mL PBS at 60 °C. The temperature was then reduced to 50 °C and 10 mL methacrylic anhydride was added dropwise to conjugate photocurable acrylate
groups to amine groups along the DC-Gel backbone. The solution was reacted at 50 °C for 1 hour with continuous mixing. To stop the reaction, 200 mL of 40 °C PBS was added to the solution. The product was then dialyzed at 40 °C for one week with daily water changes followed by lyophilization for two days. The dry product was stored at -20 °C.

To prepare the bioink PF127-SH and DC-HA were dissolved in PBS at 4 °C overnight to maintain the cold solution phase. The PF127-SH DC-HA solution was then heated to 65 °C using a waterbath to form a warm solution phase for dissolving the gelatin. The solution was then allowed to cool to 37 °C into the gel phase and stored at 37 °C until used. Samples with final concentrations of 6, 9, 12, or 15% PF127-SH, 1% DC-HA, and 2% DC-Gel or 5% DC-Gel-MA were prepared. The bioinks were aged a minimum of 12 hours to allow the thiolated end groups of the PF127-SH to react with the catechol groups present on dopamine within the DC-HA, DC-Gel, and DC-Gel-MA through a Michael-type reaction.[33]

6.3.3 Characterization of Bioink Thermal Gelation

The gelation temperature of all formulations was measured using differential scanning calorimetry (DSC) (Q2000; TA Instruments). A heat-cool-heat cycle was used to remove thermal history in the sample with a heating rate of 10 °C/min and a cooling rate of 20 °C/min. Samples were run from 5 – 40 °C. Gelation temperatures were determined by peak fitting to the endotherm using the vendor-provided software (TRIOS software, TA Instruments).

6.3.4 Bioink Curing via Chemical Chelation and Photopolymerization

Two mechanisms for curing were examined: 1) covalent crosslinking through photocuring, and 2) physical crosslinking through chelation. Photocuring was conducted on
the bioink formulations containing 5 wt% DC-Gel-MA and 0.1 wt% photoinitiator (DMPA) through 10-minute exposure to UV light (365 nm, 6 W; UVP, LLC). Physical crosslinking was conducted on the bioink formulations containing 2 wt% DC-Gel through a 2-minute exposure to 50 mM ferric nitrate solution followed by rinsing with PBS.

6.3.5. Rheological Characterization of Slightly Crosslinked Bioinks

Characterization of uncured bioink rheological behavior was done using a parallel plate rheometer (25 mm plate; AR 2000, TA Instruments). Frequency and stress sweeps were run at 37 °C. Frequency sweeps were conducted with 1% strain from 0.01 to 100 Hz. Stress sweeps were run from 0.1 to 50,000 Pa at 1 Hz.

The bioink rheological properties were determined using a Herschel-Bulkley fluid model given as:

\[ \tau = \eta \dot{\gamma} \]  

(1)

where \( \eta \) is the effective viscosity defined as:[35]

\[ \eta = \begin{cases} \eta_o, & \dot{\gamma} \leq \dot{\gamma}_c \\ \tau_o \dot{\gamma}^{-1} + k \dot{\gamma}^{n-1}, & \dot{\gamma} \geq \dot{\gamma}_c \end{cases} \]  

(2)

where \( \tau_o \) is yield stress, \( k \) is the flow consistency index, \( n \) is the flow behavior index, \( \eta_o \) is the constant viscosity for low strain rates, and \( \dot{\gamma}_c \) is a critical strain rate below which viscosity is treated as a constant given as:

\[ \dot{\gamma}_c = \frac{\tau_o}{\eta_o} \]  

(3)

where the \( k \) and \( n \) were obtained by fitting Equation (1) to the experimentally measured complex viscosity vs. shear rate data. The \( \tau_o \) was calculated from the stress sweep data using the tangent method.[36]
6.3.6. Finite Element Modelling

A commercially available finite element modeling software (COMSOL Multiphysics; Vers. 5.3a) was used to model the steady-state two-dimensional (2D) velocity profile of the bioink under the typical microextrusion 3D bioprinting conditions (nozzle diameter = 400 µm; nozzle length = 10 mm). The velocity profile was obtained as the solution to the equations of motion for an incompressible fluid assuming the fluid obeys a Herschel-Bulkley fluid relationship. An average inlet velocity of 1 mm/s, a typical printing speed, was applied at the inlet boundary and an atmospheric pressure boundary condition was applied at the outlet. The walls were modeled using a no-slip boundary condition. Model parameters were obtained from the experimental data as described in Section 2.5. The computational domain was discretized using a physics controlled mesh resulting in 27,088 elements. The fraction of the bioink experiencing low shear was taken as the fraction of the pipe diameter within $u \geq 0.95u_{max}$.

6.3.7. Mechanical Characterization of Cured Bioinks

Mechanical characterization of the cured bioink was done using a dynamic mechanical analyzer (DMA) (Q800, TA Instruments). The samples were prepared for compressive testing by casting disks with 10 mm diameter and 2 mm thickness in a machined mold. Exact sample dimensions were confirmed for each sample using digital calipers. The discs were then cured as described in Section 2.4. A controlled-force mode was used to probe mechanical properties. A force ramp rate of 0.02 N/min was used at 37 °C with a 5 minute equilibration period. The elastic modulus was determined from the 5 – 10%
strain region of the stress-strain plot. The standard deviations reported correspond to \( n = 5 \) samples.

6.3.8. Cell Culture and Expansion

Schwann cells from rat sciatic nerve (ATCC, S16Y) were cultured in DMEM containing 10% FBS and 1% PS according to previous protocols.\[^{27}\] Tissue culture flasks (75 cm\(^2\)) were coated with PLL by allowing a 15 µg/mL PLL solution (0.1 ml/cm\(^2\)) to adsorb for 2 h. Subsequently, the plates were rinsed thrice with DPBS and dried for 30 min. The cells were then cultured at 37 °C and 5% CO\(_2\). The cells were expanded by twice-weekly passaging at a 1:3 subcultivation ratio to generate a biomass that was sufficient for the 3D bioprinting studies. Neuronal cells from the rat adrenal gland (PC-12, ATCC) were cultured in RPMI-1640 containing 10% HS, 5% FBS, and 1% PS in a 37°C 5%-CO\(_2\) incubator following the vendor-provided protocols.

For visualization of the non-fluorescent lines (PC-12 and S16Y), a lipophilic dye (DiI) was used for labeling following the vendor-provided protocol. In short, the cells were counted and subsequently suspended in culture media containing the DiI solution for 20 minutes at 37°C. The cells were then washed with fresh medium and recounted. We note that instead of the standard growth medium, the PC-12 cells were resuspended in a ‘differentiating medium’ that contained high glucose DMEM with 1% HS, 1% PS and 100 ng/mL NGF at \( 2.4 \times 10^7 \) cells/mL. The S16Y cells were resuspended at \( 2.0 \times 10^6 \) cells/mL in DMEM/F12 with 10% FBS.

Human glioma cells (D54-MG, glioblastoma; Dr. D. Bigner, Duke University) made into a stably expressing enhanced green fluorescent protein (eGFP) cell line\[^{37}\] were maintained in
DMEM/F12 supplemented with 7% FBS in a 37°C 10%-CO₂ incubator. As the D54-MG cell line was already fluorescent via eGFP expression, these cells were counted with a hemocytometer and resuspended at $2.4 \times 10^7$ cells/mL in DMEM/F12 with 7% FBS.

6.3.9. 3D Bioprinting

All bioink preparation and bioprinting steps were done in a biosafety cabinet (SterilGARD II; Baker). To prepare the bioinks for bioprinting studies, all lyophilized polymers were sterilized via treatment with UV and 200 proof ethanol spray. To prepare the DC-Gel containing bioink, 2.4 g PF127-SH and 0.2 g DC-HA were dissolved in 9 g DPBS at 4 °C. The mixture was then heated to 65 °C in a waterbath to dissolve 0.4 g DC-Gel-MA before cooling to 37 °C. The bioink was subsequently aged for 12 hours. To prepare the DC-Gel-MA containing bioink, 2.4 g PF127-SH and 0.2 g DC-HA were dissolved in 8.4 g DPBS at 4 °C. The mixture was then heated to 65 °C in a waterbath to dissolve 1 g DC-Gel-MA before cooling to 37 °C. The bioink was subsequently aged for 12 hours. This resulted in bioinks that were more concentrated than the final concentration used for 3D bioprinting to accommodate the subsequent addition of a cell suspension to the bioink. 8 mL of the resulting concentrated bioink was then briefly cooled to ~15 °C into the cold liquid phase and mixed with 8 mL of a cell suspension that contained $8 \times 10^6$ Schwann cells/mL in fresh DMEM for a final bioink concentration of $4 \times 10^6$ Schwann cells/mL. The cell-laden bioink was then warmed back to the gel phase at 37 °C.

The addition of cell suspension diluted the two bioinks to the desired concentrations for bioprinting: 1) a formulation that contains no photocurable groups consisting of a final bioink composition of 12% PF127-SH, 1% DC-HA, 2% DC-Gel, and $3.2 \times 10^6$ Schwann...
cells/mL (referred to as the ‘chelating neural bioink’), and 2) a photocurable formulation consisting of a final bioink composition of 12% PF127-SH, 1% DC-HA, 5% DC-Gel-MA, and 3.2 × 10^6 Schwann cells/mL (referred to as the ‘photocured neural bioink’).

The cell-laden bioink (37 °C) was then loaded into 5 cc syringes. Microextrusion 3D bioprinting was done using a custom system comprised of a desktop computer, three-axis robot (MPS; Aerotech), and a digital dispenser (Ultimus V; Nordson EFD). Tissue constructs were printed into uncoated six well plates using a 27 gauge tapered tip at a speed of 1 mm/s with an extrusion pressure ranging from 2 – 10 psi. Subsequently, the constructs were cured as described in Section 2.4 and used for cell viability studies (we note that the ferric nitrate solution was first syringe filtered prior to use and sterile DPBS was used instead of PBS).

For imaging studies, the same protocol described above was implemented to prepare bioinks that contain Schwann cells, glioma cells, or PC-12 cells at 2 × 10^6, 4 × 10^6, or 4 × 10^6 cells/mL, respectively. The tissue constructs were printed using a commercially available bioprinter (Inkredible+; CELLINK) using the same printing parameters (i.e., extrusion pressure, print speed, and nozzle diameter) to better demonstrate the utility of the bioink

6.3.10. Cell Viability Studies

The biocompatibility of the bioink was examined using an alamarBlue (AB) assay, a viability assay based on the reducing power of living cells. Single-layer tissue constructs (25 mm² squares; thickness = 200 µm) were printed using: 1) the chelating neural bioink, 2) the photocured neural bioink, and 3) a control composed of 1.5 wt% alginate (3.2 × 10^6 cells/mL). Following printing, the alginate construct was cured by exposure to a syringe-filtered 100 mM CaCl₂ solution followed by rinsing with DPBS. Following curing, the
bioprinted tissue constructs were then cultured at 37 °C and 5% CO₂ in phenol-free DMEM (2 mL/well) for one week. Viability measurements were made on days 1, 3, and 7 following printing (n = 4 samples per day, per bioink) using an adapted AB assay as follows. AB was first added to each well at 10% by volume in a biosafety cabinet. Subsequently, the plate was then returned to the incubator for 18 h (37 °C and 5% CO₂). 100 µL aliquots were then removed from each well and transferred to a 96 well plate. The absorbance intensity of each well was then scanned from 300 to 700 nm with 10 nm steps against a phenol-free DMEM blank using a well-plate reader (Synergy H1; BioTek). The percent AB reduction, which relates to cell viability, was calculated as follows\[^{[38]}\]:

\[
\%AB_{\text{reduced}} = \frac{(\varepsilon_{\text{OX}}\lambda_1) - (\varepsilon_{\text{OX}}\lambda_2)}{(\varepsilon_{\text{RED}}\lambda_1) - (\varepsilon_{\text{RED}}\lambda_2)} \times 100
\]

(4)

where \(\varepsilon_\lambda_1\) and \(\varepsilon_\lambda_2\) are molar extinction coefficients of AB at 570 and 600 nm, and \(\varepsilon_{\text{OX}}\) and \(\varepsilon_{\text{RED}}\) indicates the oxidized or reduced form. \(A_\lambda_1\) and \(A_\lambda_2\) indicate the measured absorbance at 570 and 600 nm, and \(A'\) indicates the absorbance in a negative (cell-free) control.\[^{[38]}\] A Student’s t-test was used to quantify the difference in sample means.

6.3.11. Imaging of Bioprinted Tissue Constructs

Fluorescence microscopy of the 3D bioprinted tissue constructs containing Schwann, glioma, and neuronal cells was conducted at days 1, 3 and 7 using a laser scanning microscope (A1R; Nikon). All measurements were made using a Plan Apo 10×/NA 0.45 air objective and a Plan Fluor 40×/NA 1.30 oil objective.
6.4. Results

6.4.1. Phase Transition Behavior of the Biomimetic Neural Bioinks

The PF127-SH/DC-HA/DC-Gel and PF127-SH/DC-HA/DC-Gel-MA bioinks formed brown gels due to the DC-Gel content. We found that both the chelating and photocuring neural bioinks formed a solution below 20 – 30 °C and above 50 – 60 °C. The bioinks formed a gel phase from approximately 25 – 55°C. DC-Gel or DC-Gel-MA was added to the PF127-SH/DC-HA solutions at ~65 °C in the warm liquid phase as gelatin exhibited the slow dissolution kinetics in the cold liquid phase. The DC-Gel or DC-Gel-MA components exhibited high adhesion to glassware. The fully prepared bioinks could be briefly cooled to 4 °C in the cold liquid phase, but underwent phase separation following more extended storage periods (i.e. overnight).

Having shown that the bioinks form thermoreversible slightly crosslinked hydrogels, we next examined the effect of bioink composition on flow characteristics and hydrogel stability. The chelating bioinks contained 1 wt% DC-HA, 2 wt% DC-Gel, and either 6, 9, 12, or 15 wt% PF127-SH. The photocurable bioinks contained 1 wt% DC-HA, 5 wt% DC-Gel-MA, and either 6, 9, or 12 wt% PF127-SH. All of these formulations resulted in hydrogels that flowed out of an extruder nozzle under a typically applied extrusion pressure (1 – 20 psi). Die swelling effects were observed at high flow rates. We found that the slightly crosslinked bioinks dissolved when submerged in water at 37 °C after 24 – 48 hours, suggesting the need for the additional curing step to maintain a crosslinked construct to support long term tissue culture.
6.4.2. Effect of Bioink Composition on Gelation Temperature

The gelation temperature of a bioink is an important parameter to inform the processability and practicality of the bioink for use in microextrusion 3D bioprinting. For example, sol-gel transitions are often used to facilitate the removal of sacrificial printed materials from 3D bioprinted constructs, commonly referred to as fugitive inks. Gelation of PF127 solutions arises due to micelle formation due to the hydrophilic-hydrophobic-hydrophilic triblock structure of the polymer.\textsuperscript{[39, 40]} The concentration at which the polymers in solution will form micelles, resulting in gelation, is referred to as the critical micelle concentration (CMC) and occurs at approximately 20% for PF127.\textsuperscript{[39, 40]} As shown in Figure 6.2, the chelating and photocuring bioink formulations exhibited depressed CMCs compared to pure PF127 solutions, forming gels at PF127-SH content as low as 6%. This depression due to slight crosslinking has been previously reported.\textsuperscript{[33]} The data show that the gelation temperature was proportional to PF127-SH content, and decreased from 21 to 16 °C over the PF127-SH concentration range examined. We found the gelatin content did not significantly affect the gelation temperature over the range examined (from 2 to 5%).
Figure 6.2 Gelation temperatures of the bio-inspired PF127-based neural bioinks obtained from DSC show an inverse relationship with PF127-SH content.

6.4.3. Rheological Characterization of Fluid Properties

The rheological properties of the uncured bioinks were investigated at 37 °C. The results of oscillatory stress sweeps and frequency sweeps are shown in Figure 6.3. The presence of both a yield stress and a shear thinning viscosity indicate a Herschel-Bulkley fluid. The associated rheological parameters ($\tau_0$, $k$, and $n$) were extracted from the rheology data and are presented in Table S6.1 of Supporting Information. As shown in Figure 6.3a, similar yield stresses were observed between 6 and 9% and between 12 and 15% PF127-SH for the chelating bioinks. The data also show the expected result that yield stress increased with increasing PF127-SH content. The stress sweep data for the photocurable bioinks is shown in Figure 6.3b. The photocurable bioink containing 6% PF127-SH exhibited a relatively lower yield than those containing 9 and 12% PF127-SH. Figures 6.3c and d show that all samples were shear thinning. The data also show that the bioink viscosity increased with PF127-SH content. The lowest yield stress was 103 Pa and occurred from the 6%
PF127-SH chelating formulation. The 15% PF127-SH chelating formulation exhibited the highest yield stress at 2,534 Pa.

Figure 6.3 Rheological data of for the chelating (a and c) and photocuring (b and d) neural bioink formulations that contain 2 wt% DC-Gel and 5 wt% DC-Gel-MA, respectively, 1 wt% DC-HA, and varying concentrations of PF127-SH ranging from 6 – 15 wt%. Stress sweeps (a and b) show the yield stress as indicated by the sharp drop in relative modulus. Frequency sweeps (c and d) show the shear thinning fluid behavior as indicated by the decrease in complex viscosity with shear rate.
Having examined the effect of formulation on the bioink’s rheological properties and verified that the bioinks exhibit desirable Herschel-Bulkley-type rheological behavior for microextrusion 3D bioprinting, we next calculated the velocity profiles associated with the different formulations under typical microextrusion printing conditions. Figure 6.4a shows the two-dimensional velocity profiles at the middle of a 10 mm long and 400 µm diameter pipe (i.e., nozzle) for the 9% PF127-SH photocuring and chelating formulations. As shown in Figure 6.4a, the bioinks exhibit a flattened parabola profile, commonly associated with Herschel-Bulkley fluids. The flattened velocity profile near the center of the pipe indicates solid-like behavior and very low shear. As shown in Figure 6.4b, the bioink in a large fraction of the extruder (greater than 50% in all cases) experiences low shear.
Figure 6.4 a) Velocity profiles calculated using finite element analysis associated with continuous bioink microextrusion through a 400 µm nozzle diameter. b) Fraction of the nozzle in which the bioink experiences relatively low shear stress.

6.4.5. Mechanical Characterization of Cured Bioinks

As shown in Figure 6.5a, two pathways for curing were investigated: chelation and photocuring. Unlike the slightly crosslinked bioinks, which dissolved in water after 24–48 hours, we found that the chelated and photocured bioinks did not flow under stress or dissolve when submerged in water over several days, indicating the curing approaches
produced a fully crosslinked gel. Curing of the chelating bioinks via exposure to ferric nitrate led to a change in the hydrogel’s color from brown to dark green.

As shown in Figure 6.5b, there was a direct correlation between PF127-SH content and elastic modulus of the cured bioinks. The elastic modulus of the bioinks ranged from 6.7 – 11.7 kPa. The chelating bioinks were softer than photocured bioinks. We also found that the 6 and 9% PF127-SH chelating formulations did not form robust gels upon chelation. The photocured 6% PF127-SH and chelated 12% PF127-SH samples exhibited similar moduli of 6.7 and 6.9 kPa. The photocured 9% PF127-SH formulation exhibited a modulus of 9.5 kPa. The photocured 12% PF127-SH and chelated 15% PF127-SH samples yielded similar moduli of 11.7 and 11.0 kPa. A statistically significant difference in elastic modulus (p<0.05) was observed among all groups, except between the photocured 6% PF127-SH and chelated 12% PF127-SH groups, and between the photocured 12% PF127-SH and chelated 15% PF127-SH groups.
Figure 6.5 a) Schematic showing two curing pathways: photocuring of formulations containing methacrylated gelatin in the presence of a photoinitiator through UV light exposure, and chelation of dopamine groups with iron (III). b) Compressive elastic modulus results of inks cured through UV exposure or chelation show an increasing modulus with increasing PF127-SH content. The chelating bioinks were relatively softer than photocured bioinks.

6.4.6. 3D Bioprinting and Cytotoxicity Analysis of Glial Tissues for Engineering of Peripheral Nerve Tissue

The fabrication of peripheral nerve tissue models and scaffolds for peripheral nerve regeneration and artificial neural tissue is critical to the regeneration of peripheral nerve
injuries, drug discovery, neural disease modeling, and personalized neuroscience. As shown in Figures 6.6a–c, free-standing Schwann cell-laden tissue constructs could be printed in various geometries. The cell-laden bioinks were highly viscoelastic due to the high polymer content. We found that the uncured, slightly crosslinked bioinks were robust enough to form free-standing bridges across gaps of up to 1 mm, which is an important aspect for creating 3D heterogeneous cell-laden tissues, such as vascularized and innervated tissue, as the void space facilitates the formation of peripheral nerve pathways, fluid flow, or blood vessels.

Having demonstrated the ability to 3D bioprint free-standing peripheral nerve tissue constructs, we next examined the cell viability in cured single-layer square printed constructs over a 7-day period relative to an alginate control. Alginate was selected as a control bioink given its standard use in the tissue engineering and bioprinting communities; however, unlike the neural bioink, it requires the addition of fillers to produce desirable rheological properties for 3D bioprinting applications.\[41-44] Nonetheless, it provides a useful control for a 3D bioprinted cell culture environment to assess the viability of cells within the bioprinted tissue constructs. We found that incubation of the tissue construct with the AB reagent yielded a peak in the absorbance spectrum of the medium at 570 nm after an 18 h incubation period, indicating reduction by living cells. The viability data shown in Figure 6.6e, as indicated by percent reduction of AB, show the photocured constructs exhibited higher viability on day 1 than the chelated constructs (p < 0.05), but were otherwise comparable to the viability observed in the control constructs made from alginate. No significant difference in viability among the constructs made using the three different bioinks was observed on day 3. The data in Figure 6e show that after one week in culture
the chelated constructs exhibited higher viability than the alginate constructs (p < 0.001) that were otherwise comparable to the viability observed in the photocured constructs. All bioinks showed an increase in %AB reduction with time over the 7-day culture period.

Figure 6. Printed bioinks (cross-hatched structure (panels a and c) and the Virginia Tech logo (panels b and d); height = 2 and 4 mm, respectively) shown from prismatic (a and b) and top-down views (c and d). e) Percent alamarBlue (% AB) reduction in the bioprinted

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Schwann cell-laden tissue constructs cured through chelation or photocuring. Alginate was used as a control. On all days, n = 4 samples per bioink were sacrificially tested.

6.4.7. 3D Bioprinting and Imaging of Neural and Glial Tissues for Engineering of Brain and Spinal Cord Tissue

After showing that the bioink could be printed with high fidelity and exhibited low cytotoxicity to Schwann cells, its suitability for 3D culture of additional cell types found in the nervous system was investigated. Given Schwann cells are found in the peripheral nervous system, we next focused on bioprinting of tissues containing cells found in the central nervous system (e.g., neurons and astrocytes). Human glioma cells, rat neuronal cells, and rat Schwann cells were individually mixed with 9% PF127-SH chelating bioink to provide final cell concentrations of $4 \times 10^6$ cells/mL for the glioma and neuronal cell-laden bioinks and $2 \times 10^6$ cells/mL for the Schwann cell-laden bioinks. The cell-laden bioinks were subsequently printed into single layer constructs cured with ferric nitrate, and cultured for 7 days. As shown in Figure 6.7, fluorescence micrographs of the bioprinted tissue constructs on day 7 showed that live cells were observed in the bioinks and that the cells exhibited reasonable morphology associated with cells in 3D hydrogel matrices. Micrographs associated with the tissue constructs taken at days 1, 3, and 7 are available in Figure S6.1 of Supplementary Information Figure S6.1, which show that the population of viable cells in the bioink remained high over the culture period. These data also suggest that the cells were well distributed throughout the hydrogel and that the bioprinting and curing processes did not adversely affect the cell viability and morphology.
Figure 6.7 Fluorescence micrographs of 3D bioprinted neural and glial tissue constructs using the gelatin-hyaluronic acid-Pluronic F-127 (Gel-HA-PF127) bioink containing rodent Schwann cells (a), human glioma cells (b), and rodent neuronal cells (c) after seven days in culture.

6.5. Discussion

Pluronic F-127 was selected as the synthetic component for the neural bioink due to its known Herschel-Bulkley fluid properties and biocompatibility.[33,45-47] The micelle-based PF127 hydrogel is often used for 3D printing removable scaffolds due its thermoreversibility and excellent printability,[48-50] however, it lacks groups for cell binding and rapidly dissolves when submerged in water, which impedes its use as a bioink. Gelatin and HA were selected given their presence in the native ECM of neural tissue.[10,11] Gelatin provides a structural polymer, while HA is an important ECM component for cell proliferation and migration.[10,11] The experimentally observed brownish color of the dopamine-containing gelatin is consistent with previously reported studies.[34] Similar to the behavior of PF127-water systems, the slightly crosslinked bioinks exhibited a cold solution phase, a warm gel phase,
and a hot solution phase, where the gel phase exists between approximately 20 and 50 °C. We observed that the bioink rheological properties changed throughout the aging interval. This behavior is consistent with a bioink network consisting of thiolated PF127 micelles that are conjugated to the backbones of dopamine conjugated structural biomolecules (see Figure 6.8). The observed thermal phase transition behavior and change in rheological properties during the aging process suggest the bioink formed a thermoreversible slightly crosslinked hydrogel prior to curing.

**Figure 6.8** Schematic of slight crosslinking through covalent bonding of dopamine (DA) conjugated gelatin (DC-Gel) and hyaluronic acid (DC-HA) with thiolated Pluronic F-127 (PF127-SH) through a thiol (SH) – catechol reaction resulting in a micelle based hydrogel with slightly crosslinked biomacromolecules (note: DA contains a catechol group).

As shown in Figure 6.3, the yield stresses in the bioinks containing 6 to 15% PF127-SH at 37 °C ranged from 103 to 2.534 Pa. In comparison, PF127-water gels containing 19% PF127 at 35 °C exhibit a yield stress of 242 Pa, indicating that the slight crosslinking with
biomacromolecules both increased the yield stress and lowered the CMC of the network.\[46\] We found \( k \) and \( n \) ranged from 12 – 325 Pa-s\(^n\) and 0.29 – 0.46, respectively, which agree reasonably with previously reported values obtained from 19% PF127-water gels. The data in Figure 6.4 suggest that neural bioinks that exhibit Herschel-Bulkley rheological behavior are optimal for bioprinting applications as they establish low-shear zones in the nozzle. Strategies for reducing the shear stress on bioinks during printing are important in bioprinting applications, as high shear is a known cause of cell death in tissue printing.\[51, 52\]

Curing was used to prevent flow or dissolution of printed structures. Two curing mechanisms often used in biocompatible hydrogels were examined: photocuring and chelation. To chelate the gels, the well known affinity of catechols for iron (III) was exploited by exposing the inks to dilute ferric nitrate solution. The color change from brown to dark green observed in the chelating bioinks upon curing is indicative of bis-coordination between two catechol groups and the Fe\(^{3+}\) ion. We found that 2 and 5% gelatin were the minimal polymer content needed to establish a fully crosslinked hydrogel using the chelating and photocuring bioinks, respectively. Gelation did not occur when the gelatin concentration was less than these thresholds, indicating that the chelation curing approach required relatively less gelatin to achieve a fully crosslinked hydrogel, likely due to the fact that the chelation pathway could utilize both the dopamine conjugated gelatin and hyaluronic acid components. Bioink formulations based on these thresholds were chosen to minimize the modulus of the resultant hydrogel such that it matched the low modulus of neural tissue. The elastic modulus of the bioinks, which ranged from 6.7 – 11.7 kPa, compared reasonably with the reported modulus of neural tissue.\[14\]
Understanding the mechanisms that drive the mechanical property change of cell-laden hydrogels and 3D bioprinted tissues, remains an active area of research. The mechanical properties of hydrogels used for 3D cell culture have been reported to change with time potentially due to a combination of factors, including swelling, cell growth, production of extracellular matrix, and degradation of polymer components within the bioink. For example, it was reported that chondrocyte-seeded agarose hydrogels exhibited an increase in Young’s modulus over two months in culture. That study also showed dynamic loading led to a near 2-fold increase in material properties relative to free-swelling controls. While some cell-laden hydrogels exhibit increases in mechanical properties over time in culture, the mechanical properties of cell-laden hydrogels have also been shown to degrade with time. A detailed discussion of mechanobiology of cell-laden hydrogels is beyond the scope of this paper, but we note that when interpreting the dynamic mechanical property changes of cell-laden tissue constructs one should also consider a number of other parameters, including cell type and seeding density, metabolic state, and crosslinking mechanism. Here, while examining dynamic changes in tissue modulus was beyond the scope of this study - which established the ability to print high quality free-standing tissue constructs of comparable chemistry and modulus to native neural tissue – our microscopy studies shown in Figure 6.7 also showed no substantial change in construct geometry over the course of seven days in culture.

Alginate was selected for a control for cytotoxicity as it is a commonly used hydrogel for 3D cell culture. However, it does not form 3D free-standing structures without the need to actively cure the object during printing or by the addition of rheological modifiers.
We found through DMA testing that the storage modulus of pure alginate hydrogel (1.5 wt%) was 55 kPa, and the addition of a filler, such as nanofibrillated cellulose (1.5 wt%) to make the alginate capable of forming 3D printable free-standing structures raised the modulus to 159 kPa, which was greater than one order of magnitude larger than the neural bioinks examined here (6.7 – 11.7 kPa). Overall, the data in Figures 6.6 and 6.7 suggest that the novel biomimetic neural bioinks enable the ability for fabrication of soft, free-standing tissue constructs with biocompatibility, mechanical properties, and geometry that are otherwise challenging to achieve. The design and synthesis of materials, such as this, that replicate neural ECM properties and support 3D cell culture is important for the further advancement of neural tissue engineering and neuroscience.

The bioink has been utilized with multiple cell types found in the central and peripheral nervous system to demonstrate its potential for use in multiple applications, including fundamental neuroscience research as well as clinical applications in nerve regeneration and drug screening for treatment of neurological diseases and disorders. Schwann cells were selected based on their ability to promote regeneration of peripheral nerve through scaffolds (i.e., nerve guidance conduits),\textsuperscript{[61, 62]} which are commonly seeded onto the conduit lumenal walls or incorporated into hydrogel or foam-based conduit luminal fillers. In addition, the fabrication of personalized and anatomical pathways for peripheral nerve regeneration is an important emerging clinical application area of 3D bioprinting processes.\textsuperscript{[27, 63]} Thus, the Schwann cell results suggest the bioink could potentially be used as a biomimetic cell-laden luminal filler in future 3D printed hydrogel-based anatomical nerve repair technology.
We note that in addition to applications in regenerative medicine, the application of 3D bioprinting to drug discovery and pharmaceutical engineering remains an important area of research.\cite{7,64,65} Brain tumor cells were used to demonstrate the potential for the bioink’s use in clinical disease modeling and drug screening applications toward future personalized pharmacology paradigms for treatment of neurological diseases and disorders enabled by 3D bioprinting. Glioma cells were specifically selected based on the need to establish new therapies for treatment of gliomas, one of the most common and aggressive types of brain tumors.\cite{66} For example, glioblastoma patients only have a median survival time of 10-18 months, with a survival rate as low as 3.4%.\cite{66} Additionally, while pharmacological targets have been discovered, clinical response to target inhibition has shown little improvements in patient outcomes leading to a strong need for improved treatment.\cite{67}

Neuronal cells were implemented on the basis that both Schwann and glioma cells are glial cells. Thus, their use supports the claim that the bioink can also support the growth of tissues that contain neuronal cells. We note that the incorporation of neuronal cells into bioprinted tissue scaffolds and constructs remains an active area of research.\cite{8,68} Recent work has shown the ability to organize compartmentalized neural systems containing a combination of neuronal and non-neuronal cells found both inside and outside of the nervous system\cite{28} as well as the penetration of neuronal cells through hydrogel matrices, such as methylcellulose- and gellan gum-based gels.\cite{8,9}
6.6. Conclusions

While the field of additive biomanufacturing (i.e., 3D bioprinting) has developed greatly over the past two decades regarding relatively high modulus structural tissues, the area of neural tissue biofabrication has lagged due to a lack of chemically and mechanically biomimetic neural bioinks. Novel biomimetic bioinks for 3D bioprinting of free-standing soft neural tissues were created based on the combination of natural polymers for ECM biomimicry (gelatin and HA) with a synthetic polymer (PF127) that endows desirable rheological properties for 3D bioprinting. Slight crosslinking based on thiol–catechol chemistry resulted in thermally gelling inks with Herschel-Bulkley type rheological behavior and enabled the fabrication of soft, free-standing neural tissues. The bioinks could be cured through chelating and photocuring pathways, resulting in hydrogels with similar moduli to native neural tissue ECM. High viability was observed among Schwann, glioma, and neuronal cells over seven days in culture. These bioinks enable 3D bioprinting of soft, free-standing neural tissues for wide-ranging applications in fundamental neuroscience research, disease modeling, drug discovery, and regenerative medicine.

6.7. References


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6.8. Supporting Information

These supporting information contain a table of the best-fit Herschel-Bulkley model parameters obtained from the data shown in Figure 3 of the main text and micrographs of schwann, glioma, and PC12 cells on days 1, 3, and 7.

**Table S6. 1** Best-fit Herschel-Bulkley parameters.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>$\tau_o$ (Pa)</th>
<th>$k$ (Pa.s$^n$)</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>6% PF127-SH, 2% DC-Gel, 1% DC-HA</td>
<td>103</td>
<td>12</td>
<td>0.36</td>
</tr>
<tr>
<td>9% PF127-SH, 2% DC-Gel, 1% DC-HA</td>
<td>131</td>
<td>93</td>
<td>0.46</td>
</tr>
<tr>
<td>12% PF127-SH, 2% DC-Gel, 1% DC-HA</td>
<td>2520</td>
<td>224</td>
<td>0.42</td>
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<tr>
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<td>274</td>
<td>0.42</td>
</tr>
<tr>
<td>6% PF127-SH, 5% DC-Gel-MA, 1% DC-HA</td>
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<td>24</td>
<td>0.3</td>
</tr>
<tr>
<td>19% PF127-SH, 5% DC-Gel-MA, 1% DC-HA</td>
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</tr>
<tr>
<td>12% PF127-SH, 5% DC-Gel-MA, 1% DC-HA</td>
<td>2001</td>
<td>325</td>
<td>0.29</td>
</tr>
</tbody>
</table>
Figure S6. 1 Fluorescence micrographs (10x) of Schwann cell- (a, d, g), glioma cell- (b, e, h), and neuronal (c, f, i) cell-laden bioprinted tissue constructs on days 1 (a-c), 3 (d-f), and 7 (g-i).
Chapter Seven: 3D Printed Multiplied Competitive Migration Assays with Spatially Programmable Release Sources

7.1. Abstract

Here, we report a 3D-printed multiplexed, competitive migration assay for characterizing chemotactic response in the presence of multiple spatially-distributed chemoattractants. The utility of the assay was demonstrated by examining the chemotactic response of human glioblastoma cells to spatially-opposing chemotactic gradients of epidermal growth factor (EGF) and bradykinin (BK). Competitive migration assays involving spatially-opposing gradients of EGF and BK that were optimized in the absence of the second chemoattractant showed that 46% more glioblastoma cells preferentially migrated toward EGF sources. The migration velocities of human glioblastoma cells toward EGF and BK sources were reduced by 7.6 ± 2.2 % and 11.6 ± 6.3 % relative to those found in the absence of the spatially-opposing chemoattractant. This work provides new insight to the chemotactic response associated with glioblastoma-vasculature interactions and a versatile, user-friendly platform for characterizing the chemotactic response of cells in the presence of multiple spatially-distributed chemoattractants.
7.2. Introduction

Chemotaxis is the net movement of cells in response to an extracellular chemical gradient. This driving force for cell transport is essential to many critical biological processes.\textsuperscript{[1, 2]} In multicellular organisms, chemotaxis plays a central role in various physiological processes, such as embryogenesis, angiogenesis, host immune responses, and brain development. Additionally, chemotaxis is highly important in directing wound healing. Fibroblasts play a central role in wound healing through the production of collagen and other extracellular matrix proteins. This process requires the rapid movement of these cells to the site of injury—making fibroblasts highly motile and responsive to various chemoattractants. For example, fibroblast growth factor (FGF) and the consequential activation of FGF receptors drive multiple cellular processes of fibroblasts, including chemotactic migration.\textsuperscript{[3, 4]}

In addition to driving regenerative processes, chemotaxis is a contributing factor to the progression of many diseases. The unregulated chemotaxis of immune cells contributes to inflammatory conditions, such as asthma and arthritis.\textsuperscript{[2, 5]} Furthermore, chemotaxis has been implicated in cancer, specifically the migration of malignant cells within the primary organ site, or hematogenously in the case of metastasis.\textsuperscript{[6]} For example, glioblastomas are drawn from the primary tumor to secondary structures in the brain via chemotaxis, making them highly invasive Grade IV brain tumors.\textsuperscript{[7]} Glioblastomas are the most common malignant primary brain tumors in humans. In the United States, approximately 13,000 people are annually diagnosed with glioblastoma, and unfortunately, approximately 85% of
these patients lose their lives within two years. This limited survival time can largely be associated with the extensive migration of these tumor cells away from the primary tumor site.

Among the secondary structures involved in glioblastoma chemotaxis, the influence of chemical signals from vascular structures on the chemotactic migration of glioblastoma cells toward blood vessels is not yet fully understood. Several molecules that originate from blood vessels or other vascular structures are known to induce glioblastoma migration. One such molecule is bradykinin (BK), a peptide important in blood pressure regulation and inflammatory reactions, which stimulates the movement of glioblastoma cells to the vascular network—a well-documented avenue of glioblastoma migration. Another chemoattractant provided by the vascular network and believed to play an important role in glioblastoma migration toward vasculature is epidermal growth factor (EGF). EGF plays a significant role in glioblastoma biology, largely due to disease-associated alterations in the EGF receptor (EGFR) via mutation or amplification that lead to constitutive activation of EGFR. EGF is present in blood serum, is able to cross the Blood-Brain-Barrier, and has been associated with enhanced glioblastoma proliferation, invasion, and chemotactic migration responses.

While various migration assays exist, they can be broadly classified as chamber- or microfluidic-based migration assays. Chamber-based migration assays, such as the commonly utilized Boyden chamber, involve the imaging of migrating cells from a cell-containing chamber through a porous material into a chemoattractant containing chamber. Similar designs include the Zigmond and Dunn chambers, which yield
improved cell counting and imaging resolution capabilities, but exhibit time-dependent gradients with relatively short transients (i.e., time over which the gradient is present).\cite{19-21} Additionally, while under-agarose assays have been used to study the effect of opposing chemotactic gradients on neutrophil migration,\cite{22} under-agarose and micropipette chemotactic assays also exhibit time-dependent gradients with relatively short transients.\cite{17, 23} Ultimately, while chamber-based methods are user-friendly and pose limited technical challenges to use, they constrain the type and duration of gradients that can be studied. Alternatively, while chamber-based assays typically create time-dependent gradients, microfluidic-based assays offer the ability to examine time-independent gradients (i.e., non-uniform steady-state concentration profiles), which are sustained by convective flow of chemoattractant-containing aqueous solutions.\cite{17, 19, 23, 24} For example, a three-dimensional (3D) multilayered hydrogel-based microfluidic device was designed to characterize chemotaxis in a biomimetic 3D environment.\cite{25} In addition to creating time-independent gradients, microfluidic-based migration assays offer the ability to examine the effect of multiple spatially-opposing gradients of different chemoattractants on cell migration response. For example, a microfluidic-based migration assay for studying the effect of multiple chemoattractant sources was recently established for studying migration of mesenchymal stem cells towards cytokines present in arthritis.\cite{26} Additionally, microfluidic-based migration assays have also been established based on actively controlled concentration gradients.\cite{27, 28} For example, a microfluidic system was recently developed that enabled the study of chemotactic response to single-pulse or periodic chemical gradient signals.\cite{27} While not yet leveraged for the study of cell migration, it should also be noted
that 3D printing has also been used to create drug release technology for active gradient control in bioprinted tissue constructs based on stimuli-responsive materials,[29] suggesting that active concentration control is not limited to microfluidic systems and 2D migration processes. However, while these devices can generate steady, non-uniform concentration profiles (i.e., gradients) over long time periods, technical barriers posed by the use of traditional microfabrication processes, microscale fluid handling, and actuator integration impede widespread implementation outside of engineering groups.[30] Thus, a migration assay that combines the advantages of chamber- and microfluidic-based designs (i.e., user-friendliness, robustness, and gradient design flexibility) could provide a novel high-throughput approach for studying the effect of competing chemotactic signals on cell migration, such as spatially-opposing gradients of multiple chemoattractants (i.e., multiple chemoattractants with different spatially-distributed sources).

Additive manufacturing, commonly referred to as 3D printing, has recently emerged as a promising bio-fabrication approach for creating artificial axons,[31] neural and glial tissues,[32] brain tumor models,[32] conduits for nerve regeneration,[33],[34],[35] cuffs for neural modulation and neural interface,[36] and neural systems-on-a-chip.[37] Simultaneously, 3D printing now offers a complementary manufacturing process to soft lithography for fabricating microfluidic devices. For example, 3D printing has been used to fabricate 3D microfluidics,[38] microfluidic-based microelectromechanical systems (MEMS),[39] organ-conforming microfluidics,[40] and tissue chip systems.[41] Microextrusion 3D printing recently enabled a breakthrough in fabrication of a chamber-based neural system on a chip that contained 3D printed microchannels for studying the role of Schwann cells on uptake of
pseudorabies virus in the nervous system. In addition to neural engineering and microfluidics, 3D printing has been used to construct spatially-distributed controlled-release systems that contain chemoattractants as well as other biologics (e.g., enzymes) and molecular species (e.g., drugs). The use of microextrusion 3D printing to construct nerve guidance conduits for regeneration of bifurcating, mixed nerve injuries that contained path-specific chemoattractants of nerve growth factor (NGF) and glial-derived neurotrophic factor (GDNF) (i.e., spatially-distributed end sources) provides a recent example.

Therefore, 3D printing could facilitate the fabrication of user-friendly migration assays for studying competing chemoattractants with spatially-distributed sources.

Here, we leverage 3D printing to construct a user-friendly, multiplexed migration assay for studying the effect of spatially-opposing chemotactic gradients on glioblastoma migration to provide new insights into the chemical signaling underlying glioblastoma migration toward vasculature. The platform was first validated using single-source migration assays with a well-documented fibroblast-FGF (cell-chemoattractant) system. Three dimensional (3D) finite element analysis (FEA) simulations of chemoattractant mass transport were performed to characterize the spatiotemporal profile throughout the two-day assay under varied source loading conditions and chemoattractant transport properties. Multiplexed migration assays with on-chip controls were leveraged to better understand the roles of EGF and BK in migration of glioblastoma toward vasculature. We found that spatially-opposing gradients of BK have a significant effect on the migration velocity and trajectory of human glioblastoma cells toward EGF-containing sources, which differ from those observed in the presence of uniform BK distributions. This work provides new insight
to the chemical signaling that attracts glioblastoma to vasculature and the migration response of glial cells in the presence of spatially-distributed chemoattractants. These insights are useful for a variety of healthcare applications, including cancer treatment and regenerative medicine.

7.3. Experimental

7.3.1. Materials

Silicone (SI 595 CL) was purchased from Ellsworth Adhesives. Alginic acid sodium salt from brown algae, calcium carbonate, hexadimethrine bromide, bradykinin acetate salt (BK; MW = 1.1 kDa), fluorescein isothiocyanate (FITC)-labeled dextran (MW = 10 kDa), and glucono-δ-lactone (GDL) were purchased from Sigma-Aldrich. Dulbecco’s modified Eagle’s medium/F12 (DMEM/F12), bovine serum albumin (BSA), penicillin-streptomycin (10,000 U/mL), recombinant human basic fibroblast growth factor (FGF; AA 1-15; MW = 17.2 kDa), recombinant human epidermal growth factor (EGF; MW = 6.2 kDa), and antibiotic-antimycotic (Anti-Anti, 100X) were purchased from Thermo-Fisher. Fetal bovine serum (FBS) was from Aleken Biologicals.

7.3.2. 3D Printing

A custom microextrusion 3D printer comprised of a desktop computer, a gantry robot (F5200N.1, Fisnar), and a digital dispenser (Nordson EFD, Ultimus V) was used for fabrication of the migration assays. Silicone was loaded into a 5 cc syringe with a 27 ga tapered nozzle. A print speed of 2 mm/s was implemented with a dispensing pressure of 18 psi. Three migration chambers were printed per cell culture imaging dish (35 mm; μ-dish glass bottom; Ibidi) using a single g-code program via using vendor provided software.
(RoboEdit, Fisnar). A 2D DXF file stacked in the z direction with a 300 µm step for a final height of 2.1 mm was imported for the path data. Following 3D printing, the devices were cured at room temperature prior to use.

7.3.3. Finite Element Simulations

A commercially-available FEA software (COMSOL Multiphysics, version 5.3a) was used to model the mass transport of the chemoattractants in the device during cell migration. A transient, 3D simulation using the transport of diluted species physics module was executed over a 3D domain comprised of hydrogel and water sub-domains, which solved the following set of equations:

\[
J_i = -D_i \nabla c_i \quad (1)
\]

\[
\frac{\partial c_i}{\partial t} + \nabla \cdot J_i = 0 \quad (2)
\]

where \(c_i\) is the concentration of \(i^{th}\) chemoattractant, \(t\) is time, \(J_i\) is diffusive flux, and \(D_i\) is the diffusion coefficient, subject to a set of boundary and initial conditions specified by the assay design (e.g., the chemoattractant loading concentration). As shown in Equation (1), the model was solved assuming Fickian diffusion of the chemoattractant in both sub-domains and neglecting hydrogel swelling effects. Diffusion coefficients of peptides with similar MW to BK were implemented in the model. The following diffusion coefficients were used in the hydrogel domain: \(D_{\text{FGF/Gel}} = 3.5 \times 10^{-7} \text{ cm}^2/\text{s}\) \[44\] \(D_{\text{EGF/Gel}} = 5.2 \times 10^{-7} \text{ cm}^2/\text{s}\) \[45\] and \(D_{\text{BK/Gel}} = 2.2 \times 10^{-8} \text{ cm}^2/\text{s}\) \[46\]. In the water domain the diffusion coefficients were: \(D_{\text{FGF/Water}} = 1 \times 10^{-6} \text{ cm}^2/\text{s}\) \[47\] \(D_{\text{EGF/Water}} = 1.6 \times 10^{-6} \text{ cm}^2/\text{s}\) \[45\] and \(D_{\text{BK/Water}} = 3.5 \times 10^{-6} \text{ cm}^2/\text{s}\) \[48\]. The initial concentration of the hydrogel domain were defined by the experimental values (5, 10, 25, 50, and 100 ng/mL FGF; 25, 50, and 100 ng/mL EGF; and 50, 100, 200, and 400 µM BK). The
initial concentration in the water domain was zero. The concentration was calculated in terms of molarity using the following vendor-provided molecular weights. A flux continuity condition was implemented between the hydrogel and water domain, and a no flux condition was applied on all other boundaries. The model was discretized using a fine physics-controlled mesh resulting in 2081 elements and solved with a 0.1 hr time step for 48 hr. The output of the simulation was the 3D spatiotemporal concentration profile of the chemoattractant in the device (i.e., each computational domain).

7.3.4. Experimental Measurement of Passive Drug Release from a Hydrogel Source

The method for measuring temporal passive drug release from the hydrogel system was adapted from our previous report.[34] Alginate solutions were prepared at 1.5 w/v% in a 100 µM FITC-dextran solution. A droplet (5 µL) was then deposited at the edge of a well in a 96-well plate and allowed to cure. 100 µL of DI water was then added to the well. The fluorescence signal of the well was subsequently recorded every 15 minutes using a fluorescence well-plate reader (Biotek Synergy H1, 488 nm excitation 525 nm emission). The measurement was repeated in triplicate. Concentration was interpreted as the normalized fluorescence intensity defined as the change in fluorescence divided by the total change in fluorescence.

7.3.5. Cell Culture

Human glioblastoma cells (D54-MG, WHO Grade IV; Dr. D. Bigner, Duke University, Durham, NC), which had previously been made into a stably expressing eGFP cell line,[49] were maintained in DMEM/F12 supplemented with 7% FBS in a 37°C 10%-CO2 incubator. Mouse embryonic fibroblasts (NIH/3T3, ATCC) were obtained and a stable eGFP expressing
cell line was generated as follows. Lentivirus particles (CSCGW2-eGFP-IG, MGH Vector Core) were applied at a multiplicity of infection (MOI) of 20.0 with 8 μg/μL hexadimethrine bromide to 3T3 cells for 24 hr. Approximately 72-96 hrs after viral transduction, the cells were sorted into eGFP positive and negative populations using fluorescence-activated cell sorting (FACS, Sony SH800). The resulting 3T3-eGFP cells were maintained in DMEM/F12 supplemented with 100 U/mL penicillin-100 μg/mL streptomycin and 10% FBS in a 37°C 5%-CO₂ incubator.

7.3.6. Hydrogel Preparation

Sterile alginate hydrogel solutions were prepared immediately prior to use. GDL (0.2% w/v) was added to 5 mL of 1.5% w/v alginate solution and vortexed for 30 seconds. Calcium carbonate (0.3% w/v) was then added and vortexed for 1 minute to homogenize the mixture. This resulted in a hydrogel solution that cured in approximately 2 hrs.

7.3.7. Migration Assay

Following 3D printing, the devices were sterilized with 70% ethanol spray, triple-rinsed with sterile DPBS, and allowed to dry in a biosafety cabinet with 30 minutes of UV exposure. 3T3-eGFP cells were plated by depositing 10 μL of cell suspension that contained 500 cells/μL at the center of the cell seeding chamber. D54-eGFP cells were plated by depositing 10 μL of cell suspension that contained 1000 cells/μL (single-chemoattractant experiments) or 500 cells/μL (competitive chemoattractant experiments) at the center of the cell-seeding chamber. To maintain a constant contact area between the droplet and the petri dish, the 10 μL droplet of cell suspension was supplemented with 1 μL of culturing media approximately every hour until the start of the time-lapse migration experiment. Two
hours after cells were plated in the migration chamber, the hydrogel solution was prepared and the chemoattractant was added at the specified concentration (FGF for 3T3 cells; EGF and BK for D54eGFP cells). The resulting solutions were then pipetted into the migration chamber and allowed to crosslink for approximately 2 hrs. At 4 hrs following cell plating in the cell-seeding chamber, the initial plating media was removed by the addition of a second droplet of cell culture medium (~30 μL) to the initial droplet, followed by aspiration of all the medium. Subsequently, a low-nutrient migration buffer (DMEM/F12 + 0.025-0.1% BSA+1X Anti-Anti) was added to fill the entire device (70 μL total volume). The migration chamber dish was then placed in a closed-system imaging chamber (Micro-Incubator Platform, Warner, DH-40iL) within an environmental incubator (Weather Station) on an inverted Olympus IX81 microscope outfitted with a Hamamatsu ORCA-ER digital camera, which afforded localized control of atmospheric CO₂ concentrations (5%-CO₂ 3T3, 10%-CO₂ D54; Okolab DCTC02BX) and thermal regulation (Weather Station). Multipoint time-lapse images were acquired every 20 min for 48 hr with differential interference contrast (DIC) and fluorescent imaging using Slidebook 6 software and an encoded Prior Stage. In the non-competitive migration assays, the image data associated with the cell tracking analysis were acquired at the entrance of the cell-seeding chamber for each time point. In the competitive migration assays, the image data associated with the cell tracking analyses were acquired at three locations for each time-point (the center of the cell-seeding chamber and both entrances of the cell-seeding chamber). These areas were selected to mitigate any potential bias in the migration analyses that could be caused by an imbalance in the imaging locations. In all cases, the imaging field was fixed.
7.3.7. Data Analysis

Time-lapse data were analyzed using the NIS-Elements 2D Tracking Module (v5.0) in combination with the Spot Detection and Binary applications, which provided a migration velocity for each cell. The relative migration velocity was then obtained as the ratio of the migration velocity in the presence of the chemoattractant to that observed in negative control studies in which the chemoattractant was absent that were conducted in the same petri dish (i.e., with a multiplexed configuration). Only cells that were present throughout the entire time-lapse period (48 hr) were analyzed and no additional cells were excluded. In fibroblast and glioma tracking studies, the number of tracked cells ranged from 14 – 73 to 54 – 124, respectively.

7.3.8. Statistical Analysis

Experiments were performed in triplicate. Statistical analysis was performed using OriginPro (version 8.0). Error bars and +/- symbol indicate standard deviation. A Student’s t-test was used to determine significance among the relative migration velocity datasets. A two-proportion z-test was used to determine significance in the chemoattractant trajectory data acquired from the competitive experiments. Statistical calculations were executed with 95% confidence intervals and two tailed distributions. Error bars indicated standard deviation.
7.4. Results

7.4.1. Fabrication and Characterization of the 3D Printed Migration Assays for Spatially-distributed Sources of Multiple Chemoattractants

As shown in Figure 7.1a, microextrusion 3D printing enables the fabrication of a migration assay platform for analysis of competing chemotactic signals that consist of chambers for cell seeding and chemoattractant loading separated by microchannels in which cell migration and chemoattractant transport occurs. Following addition of cell culture medium to the device, the chemoattractant establishes a gradient in the microchannel that the cells encounter via passive release from a crosslinked hydrogel, which served as the chemoattractant source (see Figure 7.1b). This fabrication approach and design facilitated rapid prototyping (i.e., study of alternative signaling networks) and multiplexing within a single petri dish. The schematic associated with a design for spatially-opposing chemoattractants is shown in Figure 7.1c.

As shown in Figure 7.1d, the cell-seeding chamber was 6 mm in diameter, which enabled the manual deposition of a 10 µL droplet of cell suspension in the center of the chamber that remained free of contact with the walls. Micrographs showing cell seeding and initial movement are provided in Figure S7.1 of Supporting Information. The cell-seeding chamber was connected to the chemoattractant-loading chamber by a 1 mm wide and 5 mm long microchannel. The chamber’s dimensions were chosen to synergize with the length scales associated with the anatomy of critical tissues in small animals and humans (e.g., peripheral nerves and tumors). Thus, the millimeter-scale dimensions were selected to demonstrate that the assay could be applied to broad fundamental and translational
research, such as that involving 3D bioprinted tissues. The diameter of the chemoattractant-loading chamber was 2 mm, which facilitated manual deposition of the chemoattractant-containing hydrogel solution and prevented the hydrogel solution from flowing into the microchannel prior to crosslinking. The chamber and microchannel walls were 2 mm high to provide a sufficient volume of cell culture medium support cell migration throughout the two-day assay. Based on the resolution of microextrusion 3D printing processes, this 3D printing approach could also enable the fabrication of microscale chamber-based migration assays, which could potentially create larger driving forces (i.e., larger gradients) for eliciting chemotactic response. Micrographs of the cell-seeding chamber, microchannel, and chemoattractant-loading chamber are shown in Figures 7.1f-h. A micrograph showing a side-profile of the device wall has been provided in Figure S7.2 of Supporting Information to highlight the height uniformity of the device. The micrographs show that the microextrusion 3D printing process produced watertight walls that enabled medium isolation within the structure (see Figure S7.3 of Supporting Information). The artifact in the chemoattractant-loading chamber wall thickness observed in Figure 7.1h was consistent across all devices and resulted from lifting of the extrusion nozzle at the end of the printing process. However, this printing artifact did not affect the watertight seal. As shown in Figure 7.1i, the approach enabled multiplexing in single petri dishes for integration of on-chip negative controls as well as improving the assay throughput via simultaneous testing of multiple chemoattractant loading concentrations (we remind the reader that 3D printing itself is a rapid prototyping process[50]). As shown in Figure 7.1i, we found that up to three devices
could be integrated into a 35 mm dish using the single chamber-microchannel design examined here.

Figure 7. 3D printed migration assays for high-throughput study of spatially-distributed chemotactic signals (e.g., spatially-opposing sources). a) Schematic of fabrication via 3D printing into 35 mm petri dishes. b) Schematic of cell seeding and chemoattractant loading steps via manual pipetting. c) Schematic of a migration assay design with multiple spatially-distributed sources for studying the effect of spatially-opposing chemotactic signals on
glioblastoma migration. d) Dimensions of the device highlighting the cell-seeding chamber (radius = 3 mm), microchannel (5 mm long), and chemoattractant-loading chamber (1 mm radius). e) Photograph of a device printed in a 35 mm petri dish. Micrographs of the cell-seeding chamber (f), microchannel (g), and chemoattractant-loading chamber (h). i) Photograph demonstrating the potential for multiplexing, showing the integration of three devices in a 35 mm dish (all single-source designs).

7.4.2. Characterization of Chemoattractant Spatiotemporal Concentration Profiles during Migration via Finite Element Analysis

Having designed a user-friendly, multiplexed migration assay that accommodates repeatable manual cell seeding and chemoattractant loading, we next carried out finite element simulations of the chemoattractant release and transport processes to obtain the spatiotemporal concentration profiles associated with a given chemoattractant loading condition (i.e., initial concentration of chemoattractant in the hydrogel). It was of interest to calculate both the concentration and gradient of the concentration profile at the location of migrating cell front, as these values are fundamental parameters associated with chemotactic-driven migration flux.[51] Figure 7.2a shows 3D and top-down schematics of a single-source migration assay. Figure 7.2b schematically represents the temporal evolution of the chemoattractant concentration profile in the microchannel. As shown in Figures 7.2c and d, passive release of chemoattractant from the hydrogel source established a gradient across the microchannel.

Surface plots of the chemoattractant concentration in the device over time are shown in Figure 7.2c (chemoattractant loading of 50 ng/mL). Under these conditions, the FGF concentration in the source decreased from 43 to 28 ng/mL from 4 to 48 hrs. As shown in Figure 7.2d, as the concentration in the chemoattractant source decreased, the concentration
at the migrating cell front increased. However, it is important to note that this did not occur immediately upon addition of cell culture medium to the device, but required temporal evolution of the gradient. We found that the temporal evolution of the concentration profile in the microchannel was dependent on the chemoattractant loading concentration. As shown in Figure S7.4 of Supporting Information, the chemoattractant penetration time, defined here as the time at which the concentration of the chemoattractant at the entrance of the cell-seeding chamber exceeded 1 pM, ranged from 7 to 4 hrs for FGF loading concentrations that ranged from 5 to 100 ng/mL, respectively. This suggests that modeling of the chemoattractant spatiotemporal profile, such as the magnitude of the gradient at the migrating cell front and chemoattractant penetration time, are needed to correctly compare results from single-source migration studies with different chemoattractants as well as interpret migration responses involving multiple chemoattractants (e.g., spatially-opposing chemoattractants).

We next examined the dependence of the gradient characteristics (e.g., penetration time, concentration at the migrating cell front, and concentration gradient at the migrating cell front) on the chemoattractant transport properties. As shown in Figure 7.2, we found that the temporal evolution of the concentration profile was also dependent on the transport properties of the chemoattractant. This is an important consideration in this study, given EGF and BK differ in size, as EGF is a globular protein and BK is a peptide (6.2 vs. 1.1 kDa, respectively). Thus, they exhibit different diffusivities in hydrogel and cell culture medium (see Materials and Methods Section). We found that the penetration times for EGF and BK ranged from 2.8 to 2.5 hrs over a loading range of 25 to 100 ng EGF/mL and 0.5 to 0.2 hrs.
over a loading range of 50 to 400 µM BK, respectively. The model was validated experimentally using fluorescently-labeled dextran with similar diffusivity to BK.\textsuperscript{[52]} The experimentally measured penetration time compared within 10% with the penetration time associated with BK (0.22 vs. 0.2 hrs, respectively). The 10% variation is attributed to potential: 1) differences between the experimental diffusivity and the reported value; and 2) two-dimensional effects that may be present in the well plate-based measurement format used for experimental validation. The penetration time for FGF, EGF, and BK at all tested loading conditions is shown in Figure S4 of Supporting Information. We found that the hydrogel density had a relatively insignificant effect on chemoattractant release based on a 1.8% change in penetration time observed between 2 and 4 wt% alginate (see Figure S5 of Supporting Information)
Figure 7.2 Sensitivity analysis of chemoattractant gradient characteristics using FEA simulations, including the magnitude of the concentration gradient at the migrating cell front and the chemoattractant penetration time. a) 3D and top-down schematics showing the simulation domain, including the cell-seeding chamber, microchannel, and chemoattractant-loading chamber (8 mm dashed line indicates line plot location corresponding to data in panel d). b) Schematic showing the temporal evolution of the spatial concentration profile. c) Surface plots of FEA simulations using a basis of 50 ng/mL fibroblast growth factor (FGF) loaded in a 1.5% alginate hydrogel at $t = 4, 8, 24,$ and $48$ hr ($c_{\text{max}} = 1.3 \mu$M (4 hr), $1.1 \mu$M (8 hr), 740 nM (24 hr), and 540 nM (48 hr)). d) Line plots corresponding to panels a and c showing the concentration profiles in the device at 4, 8, 24, and 48 hr. The center of the cell-seeding chamber and the entrance to the chemoattractant-loading chamber correspond to the positions of $x = 0$ and 8 mm, respectively.
7.4.3. Effect of EGF and BK Loading Concentration on Glioblastoma Migration Response in the Absence of Competing Chemoattractants

Prior to utilizing the model for studying glioblastoma migration toward spatially-distributed EGF and BK sources, we first validated the migration assay using a well-established fibroblast-FGF (cell-chemoattractant) system, an important aspect of wound healing processes as shown in Figure 7.3a. Migration studies repeated at various FGF source loading concentrations ranging from 0 to 100 ng/mL showed three important features (see Figure 7.3b): 1) the onset of chemotactic response; 2) a maximum in chemotactic response; and 3) a saturation effect. As shown in Figure 7.3b, the minimum FGF loading concentration for which a chemotactic response occurred was 15 ng/mL. The FGF loading concentration that resulted in a maximum fibroblast migration velocity was 50 ng/mL (see Video S7.1 of Supporting Information), at which the velocity was increased by a factor of 1.51 +/- 0.40 relative to the negative control in which the chemoattractant was absent. A saturation effect was observed above 50 ng/mL, at which the migration velocity was increased relative to the negative control by 1.33 +/- 0.37. FEA simulations enabled calculation of the temporal FGF concentration profile at the location of the migrating cell front, here taken as the entrance of the cell-seeding chamber, for the range of FGF loading concentrations examined (see Figure 7.3c). These results compared well with previously reported values\textsuperscript{[53]} as discussed further in the Discussion Section, suggesting that this platform could provide meaningful data regarding chemotactic migration behavior of other cell-chemoattractant systems, such as the chemical signals that drive glioblastoma migration toward vasculature.
Figure 7.3 Fibroblast migratory response to a FGF gradient. a) Schematic of fibroblast chemotaxis due to FGF gradients during wound healing. b) Migration velocity relative to the negative control for FGF loading concentrations ($c_{load}$) of 5, 10, 15, 25, 50, and 100 ng/mL showing an optimal concentration at 50 ng/mL. c) FEA results showing the temporal evolution of the FGF concentration in the cell-seeding chamber (location indicated by the red dot) for FGF loading concentrations of 10, 25, 50, and 100 ng/mL (n = 3, Student’s t-test, P-value notation: * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001).

Having validated the migration assay using a well-established fibroblast-FGF cell-chemoattractant system, we next examined the glioblastoma-EGF and -BK systems using a single-source assay configuration, as such data served as the control studies for the subsequent experiments involving migration in the presence of EGF and BK gradients generated by two spatially-opposing sources (see Figure 7.4a). As shown in Figures 7.4b-e, similar features in the migration response were observed over the range of tested chemoattractant loading concentrations for both the glioblastoma-EGF and -BK systems. EGF loading concentrations of 25, 50, 75, and 100 ng/mL were investigated. The concentration at the onset of chemotactic response and the concentration that produced the maximum migration velocity were 50 and 75 ng/mL, respectively. As shown in Figure 7.4b, the migration velocity at an EGF loading concentration of 75 ng/mL was increased by a factor of $1.44 +/- 0.26$ relative to the velocity obtained in the control studies in which the
chemoattractant was absent (see Video S7.2 of Supporting Information). The migration velocity was relatively decreased above this optimal concentration (i.e., at 100 ng/mL). The corresponding EGF temporal concentration profile at the entrance to the cell-seeding chamber as obtained via FEA simulations is shown in Figure 7.4c. Similarly, BK loading concentrations of 50, 100, 200, and 400 µM were investigated (see Figure 7.4d). The concentration at the onset of chemotactic response and the concentration that produced the maximum migration velocity were 50 and 100 µM, respectively. As shown in Figure 7.4d, the migration velocity at a BK loading concentration of 100 µM was increased by a factor of 1.30 +/- 0.43 relative to the negative control (see Video S3 of Supporting Information). The migration velocity was relatively decreased above this optimal concentration (i.e., at 200 and 400 µM). The FEA data shown in Figure 4e associated with the temporal concentration profile of BK at the entrance to the cell-seeding chamber was validated experimentally using a 10 kDa FITC-dextran at 100 µM. As shown in Figure S7.6 of Supporting Information, the FEA data agreed reasonably with the experimental results regarding the release time delay and duration of the transient period. As shown in Figure S7.7 of Supporting Information, image processing also facilitated the analysis of the trajectory (i.e., direction) associated with the migrating glioblastoma cells. For example, 26% of imaged cells migrated toward the EGF source, defined as the octant at 0° (i.e., consisting of a +/- 22.5° region found in a rose plot's first and fourth quadrants). We note that analysis of migration trajectory becomes a critical aspect of understanding the migration response of glioblastoma cells toward spatially-opposing EGF and BK gradients, and is discussed further in the following section.
Figure 7.4 Glioblastoma migratory response toward single sources of chemoattractants in the absence of competing gradients. a) Schematic describing the spatially-opposing chemotactic signals that glioblastoma cells encounter in vivo through exposure to spatially-varying vascular microenvironments. b) Migration velocity relative to a negative control in which EGF was absent for loading concentrations of 25, 50, 75, and 100 ng/mL showing an optimal EGF loading concentration of 75 ng/mL. c) FEA results showing the temporal evolution of the EGF concentration in the cell plating area for loading concentrations of 25, 50, 75, and 100 ng/mL. d) Migration velocity relative to a negative control in which BK was absent for loading concentrations of 50, 100, 200, and 400 µM showing an optimal BK loading concentration of 100 µM. e) FEA results showing the temporal evolution of the BK concentration in the cell plating area for loading concentrations of 50, 100, 200, and 400 µM (n = 3, Student’s t-test, P-value notation: * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001).

7.4.4. Effect of Spatially-Opposing BK Gradients on Chemotactic Migration Response toward EGF Sources

The chemotactic response of glioblastoma cells toward EGF and BK sources in the absence of the other chemoattractant identified the loading concentrations that produced the highest migration velocities (75 ng/mL EGF and 100 µM BK, respectively; see Figure 7.4).
We next examined the effect of spatially-opposing EGF and BK gradients on the migration response of glioblastoma cells using a multi-end-source assay configuration (see Figure 7.5a). The loading concentrations that produced the maximum relative migration velocity in the single-source experiments, also referred to as the ‘optimal’ loading levels, were selected as the basis concentration for the competitive migration assay with spatially-distributed sources (i.e., \( c_{\text{loading}} = 75 \text{ ng/mL and } 100 \mu\text{M for the EGF and BK sources, respectively} \)).

Given the migration rate towards a chemotactractant source depends on both the concentration of the chemoattractant and gradient of the chemoattractant’s concentration profile,\(^{[51]}\) it is of interest to examine their temporal response at the location of the migrating cells (the entrance to the cell seeding chamber). The migration trajectory is shown in Figure 7.5b in terms of a rose plot. As shown in Figure 7.5c, while the concentration at the entrance of the cell seeding area increased throughout the entire experiment, the concentration gradient reached a maximum during the 4 – 8 hr interval, suggesting that the cells would experience a relatively strong driving force associated with cell migration during this period. Cells found in the region of \( 0 \pm 22.5^\circ \) were classified as migrating toward the EGF source, while those in the region of \( 180 \pm 22.5^\circ \) were classified as migrating toward the BK source (see Figure 5b). As shown in Figure 7.5d, 46\% more cells migrated toward the EGF source than the BK source based on the ratio of cells migrating toward each source (20.7 ± 3.2\% vs. 14.2 ± 2.9\%, respectively; \( n = 3 \) repeated experiments). As shown in Figure 7.5e, the relative migration velocity toward the EGF source was also higher than toward the spatially-opposing BK source, which was consistent with the single-source studies shown in Figure 7.4. However, the relative migration velocity toward both sources was decreased compared
to the measured value in the absence of the other chemoattractant (see Figure 7.5f). We note that while the presence of a spatially-opposing BK gradient decreased the migration velocity toward the EGF source relative to that observed in the absence of BK, the observed effect differed from that caused by uniformly-distributed BK (i.e., BK that was integrated with the cell culture medium). For example, we found that studies involving an EGF gradient established by a source loading concentration of 75 ng/mL that contained 700 nM BK in the cell culture medium decreased the migration velocity toward the EGF source to a greater extent than the spatially-opposing BK gradient (see Figure S7.8 of Supporting Information). We note that the bulk BK concentration of 700 nM was selected because it was the associated concentration at the migrating cell front at $t = 48$ hr caused by the optimal BK gradient loading conditions. The cells migrated toward the EGF source at a relative velocity of $1.10 \pm 0.29$ in the presence of bulk BK, which was lower than the migration velocity toward the EGF source both in the absence of BK and in the presence of a spatially-opposing gradient of BK (see Figure S8).
Figure 7. 5 Competitive migration assay results associated with glioblastoma migratory response to spatially-opposing gradients of EGF and BK. a) Photograph and schematic of a 3D printed migration assay with spatially-opposing chemoattractant sources. b) Rose plot showing the direction and magnitude of cell migration (the EGF and BK sources located at 0 and 180°, respectively). Highlighted regions indicate octants around each source location. c)
FEA results showing the temporal evolution of the spatial concentration profile at \( t = 4, 8, 24, \) and 48 hr. d) Analysis of the rose plot shown in panel b in terms of the percentage of cells heading toward the EGF and BK octants. e) Relative migration velocity of cells heading toward the EGF and BK sources compared to that measured in the absence of EGF or BK, which served as the negative control. f) Relative migration velocity towards the EGF and BK sources in an experiment with competing chemoattractant sources compared to those measured with a single chemoattractant source (\( n = 3, \) z-test (d), Student’s t-test (e and f), P-value notation: * = \( p \leq 0.05, \) ** = \( p \leq 0.01, \) *** = \( p \leq 0.001)\).

7.5. Discussion

While various assay formats exist for studying chemotaxis of adherent mammalian cells, such as glial cells, they can be classified in terms of biomimicry, type of gradient (i.e., time-dependent or –independent), and technical complexity. Chamber-based assays, such as Boyden, Zigmond, and Dunn chambers are user-friendly and compatible with manual pipetting techniques. However, they typically only allow for single-source experiments with short gradient timescales. Alternatively, microfluidic-based assays can establish time-independent gradients and be used for competitive migration assays, but they require relatively increased manual fabrication and assembly steps, technical complexity associated with their use, and cost because of the use of relatively larger fluid volumes (e.g., of chemoattractant-containing solutions). Thus, while it is the opinion of the authors that chamber- and microfluidic-based migration assays offer unique advantages, a comparison of results obtained from each platform should consider the spatiotemporal concentration profile and the substrate on which migration is occurring.

The 3D printed migration assay examined here incorporates advantages from each design to facilitate the study of multiple chemoattractants with spatially-distributed sources with gradients that can persist longer than 24 hours. This device also allows for in situ
spatiotemporal cell tracking to provide trajectory information beyond that of traditional chamber-based devices, which typically rely on cell-counting to quantify the chemotactic response. Additionally, the device was designed for compatibility with manual plating techniques to facilitate user-friendliness and high-throughput studies, a challenge that was recently identified in the migration assay literature.[54] The use of a biofabrication approach here, 3D printing, as opposed to traditional microfabrication processes has further implications in on-chip multiplexing, which can offer improved throughput and measurement confidence, rapid prototyping, and interface with bioprinted tissues, an additional challenge that has been identified in the 3D-bioprinting literature.[55] As shown in Figure S7.9 of Supporting Information, we demonstrate that it is possible to easily alter the spatial distribution of the chemoattractant release sources and increase the number of potential competitive chemoattractants. We also show that the approach can potentially be used to study chemotaxis in bioprinted tissue constructs using one-pot fabrication approach. The features of migration response over the range of loading concentrations examined, specifically, the onset, maximum, and saturation of chemotactic response, are consistent with those observed in previous studies using other migration assays.[56, 57] The results shown in Figures 7.3 and 7.4 associated with the FEA simulations of chemoattractant release and transport provide insight into the effect of assay parameters, such as the loading concentration and chemoattractant transport properties, on resultant gradient characteristics (e.g., the concentration or the magnitude of the concentration gradient at the migrating cell front as well as the time for the chemoattractant to reach the migrating cell front, referred to here as the chemoattractant penetration time).
The results obtained using the fibroblast-FGF cell-chemoattractant system agreed reasonably with the results of previous in vivo studies. For example, the highest migration velocity of fibroblasts occurred using an FGF loading concentration of 50 ng/mL, which corresponded to a concentration of 120 pM FGF at the migrating cell front at $t = 48$ hr based on our FEA simulations. This value was in the FGF concentration range found in fluid around actively healing wounds (80 – 130 pM FGF).[53]

The glioblastoma cells exhibited the highest migration velocity to BK loading concentrations that were higher than physiological levels. For example, we found that the BK loading concentration that caused the largest migration velocity was 100 µM, which corresponded to a concentration of 700 nM BK at the migrating cell front at $t = 48$ hr. However, the physiological levels of BK in plasma range from 2 to 4 pM.[58] The EGF loading concentration that caused the highest migration velocity was 75 ng/mL, which corresponded to a concentration at the migrating cell front of 500 pM at $t = 48$ hr. This value is slightly higher than the physiological plasma levels of EGF, which range from 94 to 122 pM.[15] However, as shown in Figure 7.4b, we also observed chemotactic response of glioblastoma cells to EGF loading concentrations of 50 ng/mL, which corresponded to concentrations of EGF at the migrating cell front that were within the physiological plasma EGF levels (see Figure 7.4c).

Given the experimentally measured migration velocity could be affected by cell proliferation, it was of interest to consider the doubling times of the cells used in comparison to the duration over which the cell migration was monitored. For example, previous studies have imposed cell selection criteria for purposes of measuring cell migration velocity in
cases when a substantial fraction of cells were either proliferating or blebbing during the cell tracking interval (e.g., ~20% of total cells). While the doubling time of the cells used in this study were comparable to the duration of the cell tracking interval (for example, the doubling time of 3T3 cells has been reported near 24 hours, and the doubling time for the D54 glioma cell line has been reported as 46 hr in serum-free media, proliferation effects were not observed in this study, which is attributed to the use of a low-nutrient migration buffer. Thus, no cell selection criteria were imposed in this study.

These experimental results and information regarding physiological levels of EGF suggest the chemotactic response that occurs in vivo under physiological conditions is below the saturation limit of the glioblastoma-EGF and -BK chemotactic response. The χ parameter is a fundamental parameter relating chemotactic flux to the characteristics of the chemoattractant’s concentration gradient. Using the normalized migration velocity data from the migration assays and the concentration gradient obtained from the FEA simulations shown in Figures 4b and c, respectively, an effective χ parameter of $\chi_e = 5.2 \, \mu m^4/nmol$ was obtained for the glioblastoma-EGF system (supporting analysis provided in Figure S7.10 of Supporting Information). While $\chi_e$ was estimated here assuming that the concentration and gradient were constant throughout the migration interval for the sake of demonstrating the value of integrating the results of finite element simulations and experimentation (i.e., measured migration velocities), this approach could potentially enable calculation of a time-dependent χ parameter. However, such a calculation would require use of an instantaneous migration velocity as opposed to a time-averaged migration velocity that was used for calculation of $\chi_e$ and is beyond the scope of this study. Overall, these experimental and
computational results obtained from the single-source chemotactic assays provide useful data for understanding the chemotactic responses of glial cells\footnote{51} and creating devices for chemotactically-driven tumor cell isolation\footnote{62}.

The results from the competitive migration assays with spatially-opposing EGF and BK gradients provide new insight into the chemical signaling that drives glioblastoma toward vasculature. While EGF appeared to elicit a ‘stronger’ chemotactic response based on migration velocity, 14\% of cells still migrated toward the BK source, which differed from the value observed in control studies (10\%; \(p < 0.01\)). This result demonstrates the challenges associated with preferentially guiding cells \textit{in vivo} using a chemotactic signal – that is, random migration and chemotaxis toward other chemoattractant sources. Thus, these results are potentially useful for understanding a variety of higher-order physiological and pathophysiological processes as well as tissue engineering applications associated with chemotaxis. For example, tissue scaffolds often contain gradients of multiple chemoattractants to facilitate the regeneration of complex tissues.\textsuperscript{34, 63, 64} Understanding and designing spatiotemporal concentration profiles of chemoattractants, such as growth factors, has been identified as a major challenge for tissue engineering and regenerative medicine.\textsuperscript{55} For example, it has been suggested that mass transport processes in bioprinted tissue constructs must be studied to create optimally-engineered tissue systems that mimic native tissue, facilitate cellular ingrowth, and control complex heterogeneous tissues.\textsuperscript{55}
7.6. Conclusions

We report on new insights to the chemotactic migration of glioblastoma cells toward vasculature. Specifically, a user-friendly, multiplexed migration assay enabled a study of the effect of spatially-opposing gradients of the peptide BK on the migration response of glioblastoma cells toward EGF sources. Competitive migration assays involving spatially-opposing gradients of EGF and BK at their respective optimal levels determined from single-source and -chemoattractant assays showed that glioblastoma cells exhibited a relatively increased migration velocity and extent of migration toward EGF sources. However, a substantial fraction of cells migrated toward the BK source, which was the relatively ‘weaker’ chemoattractant. The migration velocities toward spatially-distributed sources in the presence of spatially-opposing EGF and BK gradients were reduced relative to those found in the absence of the competing chemoattractant. This work provides new insight to the chemical signaling that attracts glioblastoma to vasculature and valuable information for improving the efficacy of 3D-printed scaffolds that contain multiple spatially-distributed chemoattractants.

7.7. References


7.8. Supporting Information

This supporting information contains: Micrographs of seeded glioblastoma cells exposed to an EGF source with initial concentration of 75 ng/mL at 0, 6, 12, and 24 hours (Figure S7.1), side-profile micrograph of the device walls (Figure S7.2), demonstration of device’s water-tight nature (Figure S7.3), penetration times calculated using FEA for all concentrations of FGF, EGF, and BK used experimentally (Figure S7.4), temporal evolution of FGF corresponding to different alginate concentrations (Figure S7.5), FITC-dextran release data for FEA validation (Figure S7.6), demonstration of device functionality for tracking migration velocity and trajectory (Figure S7.7), chemotactic response of glioblastoma cells to an EGF gradient in the presence of uniform and gradient BK distributions (Figure S7.8), a demonstration of rapid prototyping capability and proof-of-concept for integration with bioprinted tissue constructs in a one-pot fabrication approach (Figure S7.9), determination
of an effective $\chi$ parameter from experimental and simulation data (Figure S7.10), and videos of cell migration in the presence of the optimal source concentration (Videos S7.1-3).

**Figure S7. 1** Micrographs of seeded glioblastoma cells exposed to a 75 ng/mL EGF source at 0, 6, 12, and 24 hours.
Figure S7. 2 Side-profile micrograph of the 3D-printed wall of the migration device (taken from the microchannel region of the device).
Figure S7. 3 Demonstration of water-tight nature of the multiplexed 3D-printed migration assays. Blue dye was added for contrast.
Figure S7. 4 Penetration time defined as the time at which the concentration of the chemoattractant at the entrance of the cell seeding chamber exceeds 1 pM determined by FEA for FGF (a), EGF (b), and BK (c).
Figure S7. 5 Effect of hydrogel concentration on chemoattractant gradient characteristics in terms of the concentration at the entrance to the cell seeding chamber. Location indicated by schematic. FEA simulation results using reported diffusion coefficients of FGF in 2 and 4 wt% alginate hydrogels.[1]
Figure S7.6 Release profile of FITC-dextran loaded at 100 μM in 1.5 wt% alginate hydrogels measured by fluorescence intensity.
Figure S7. 7 Demonstration of device functionality for examining migration trajectory. Schematic showing microscope viewing area used for time-lapse imaging of cell movement, including coordinates used in cell traces and rose plots. Movement traces with the initial cell position set to the origin for 10 glioblastoma cells at $t = 48$ hrs in response to no source (b) and an EGF source that initially contained 50 ng/mL located in the $y$-direction (c). Colors correspond to individual cells. Rose plot indicating migration trajectory and distance of glioblastoma cells with no source loading (d) and a 50 ng/mL EGF source located in the $0^\circ$ direction (e).
Figure S7. 8 Chemotactic response of BK (i.e., migratory response toward BK gradients) compared with that observed in the presence of uniformly-distributed BK. a) Spatiotemporal chemoattractant concentration profiles corresponding to an ‘optimal’ EGF gradient established by an EGF source initially loaded with 75 ng/mL EGF in cell culture medium that contains 0.7 µM BK. b) Relative migration velocity of human glioblastoma cells toward an ‘optimal’ EGF source in: 1) a single-source assay configuration (i.e., containing no competing chemoattractant), 2) the presence of a competing BK gradient, and 3) in the presence of uniformly-distributed BK. P-value notation: * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001.
Figure S7. Demonstration of rapid prototyping capability regarding the spatial distribution of competitive chemoattractants shown using a four-channel model (a) and ability to integrate a 3D bioprinted tissue construct (1% alginate crosslinked using a saturated calcium chloride solution) using a one-pot fabrication approach (b; red dye added to enhance contrast of the bioprinted tissue construct).
Determination of an Effective Chemoattractant Parameter $\chi_e$ from Experimentally Measured Chemotactic Migration Data and FEA Simulations of Chemoattractant Transport

The $\chi$ parameter relates the chemotactic migration (i.e., cellular) flux to the concentration profile of the chemoattractant (e.g., the gradient). Assuming the chemotactic migration response provides the dominant driving force for cell migration during the assay, the chemotactic cellular flux can be expressed as:

$$J = \chi(c)n \cdot \nabla c$$  \hspace{1cm} (1)

where $J$ is cellular flux, $n$ is the cell concentration, and $c$ is the chemoattractant concentration. While we assume here that $\chi(c) = \chi_o$ for the sake of simplicity in the mathematical argument, $\chi$ can also be concentration-dependent, including logarithmic and kinetic expressions. Assuming the cell concentration remained constant throughout the assay, Equation (1) can be expressed as follows:

$$J = \bar{v}n$$  \hspace{1cm} (2)

$$\bar{v} = \chi_e \cdot \nabla c_{|x=0}$$  \hspace{1cm} (3)

where $\bar{v}$ is relative migration velocity and $x = 0$ indicates the imaging location on the device (i.e., the entrance to the cell seeding chamber). This relationship enabled the calculation of $\chi_e$ from the experimental and simulation data, which provided $\bar{v}$ and $\nabla c$, respectively.
Figure S7. 10 Determination of an effective $\chi$ parameter for the glioblastoma-EGF system using experimental and simulation data shown in Figure 4 of the main text ($\chi_e = 5.2 \mu m^4/nmol$).

References


8.1 Abstract

3D bioprinting is an emerging biofabrication process for the production of adherent cell-based products, including engineered tissues and foods. While process innovations are rapidly occurring in the area of process monitoring, which can improve fundamental understanding of process-structure-property relations as well as product quality by closed-loop control techniques, in-line sensing of the bioink composition remains a challenge. Here, we show that hollow multifunctional fibers enable in-line impedimetric sensing of bioink composition and exhibit selectivity for real-time classification of cell type, viability, and state of differentiation during bioprinting. Continuous monitoring of the fiber impedance magnitude and phase angle response from $10^2$ to $10^6$ Hz during microextrusion 3D bioprinting enabled compositional and quality analysis of alginate bioinks that contained fibroblasts, neurons, or mouse embryonic stem cells (mESCs). Fiber impedimetric responses associated with the bioinks that contained differentiated mESCs were consistent with differentiation marker expression characterized by immunohistochemistry. 3D bioprinting through hollow multifunctional fiber impedimetric sensors enabled classification of stem cells as stable or randomly differentiated populations. This work reports an advance in monitoring of 3D bioprinting processes in terms of in-line sensor-based bioink compositional analysis using fiber technology and provides a non-invasive sensing platform for achieving future quality-controlled bioprinted tissues and injectable stem-cell therapies.
8.2 Introduction

The increasing demands for products capable of repairing injuries and treating diseases have driven the growth of the regenerative medicine industry.\cite{1-6} While various biofabrication processes have been examined for tissue engineering, demands for product prototyping and personalization have driven the creation of computer-aided biofabrication processes, such as 3D bioprinting processes. 3D bioprinting of cell-laden tissue constructs involves a number of pre-processing operations, including cell differentiation and expansion processes\cite{7,8} cell separation processes\cite{8,9} and bioink preparation steps. Thus, while there are various process parameters that are important toward the fabrication of repeatable bioprinted tissues, such as bioink rheological properties, there is a need for real-time in-line bioink compositional analysis, including concentration, cell type, and cell viability, for scalable and quality-controlled of 3D bioprinted constructs.

3D bioprinting processes have various process parameters, each of which exhibit a unique measurement strategy and challenge. One particularly challenging aspect from a process monitoring approach is the use of animal cells, often human cells, as a processed material, which have been referred to as “living materials” from a processing perspective. While sensors are emerging for compositional analysis in cell expansion bioreactors\cite{10} there remains a need for sensing platforms capable of continuous in-line monitoring of bioink composition and cell quality measures, such as viability and state of differentiation, during bioprinting. For example, in addition to potential variability in cell expansion, differentiation outcome, and bioink preparation steps, processing defects such as nozzle clogging can also lead to inconsistency among 3D bioprinted tissue constructs.
Impedimetric-based sensing platforms have emerged as an attractive non-destructive testing approach for monitoring cell health, proliferation, and activity in both suspensions and adherent cultures.[11] For example, Nordberg et al demonstrated an impedance-based cell assay that monitored the impedance magnitude at 40 kHz in human adipose stem cell (hASC) cultures from young, middle aged, and elderly donors during proliferation and osteogenic differentiation.[12] In that study, sensor response enabled prediction of osteogenic potential in hASC populations, a result which has implications in quality-controlled biomanufacturing of stem cell-based therapies.[12] In another example, Sharma et al used impedance spectroscopy to investigate the viability of NS0 murine myeloma cells, an important cell line for production of therapeutic proteins, and found that impedance response enabled a more rapid detection of cell death than a Trypan blue as well as potential for continuous viability monitoring.[13] Narayanan et al used dielectric impedance spectroscopy for monitoring the quality of 3D bioprinted constructs.[14] This work showed that monitoring of permittivity change, Cole-Cole slope factor, and critical polarization frequency correlated with cell viability and proliferation and were significantly affected by printing parameters such as processing time and temperature.[14] Thus, an impedimetric sensing approach that could be integrated with the bioink deposition tool (e.g. nozzle) of the 3D bioprinting process may be particularly useful for in-line real-time bioink compositional and quality analysis throughout the material deposition process.

Hollow multifunctional fibers have emerged as attractive platforms for chronic neural monitoring applications given their integrated sensing and microfluidic-based drug delivery capabilities.[15, 16] For example, Canales et al hollow polymer fibers which allowed
simultaneous drug delivery, optical stimulation, and neural measurements for long-term experiments in transgenic Thy1-ChR2-YFP mice.\cite{17} In this study, CNQX, an AMPA receptor antagonist, was injected through the fibers and electrophysiological measurements were made during optogenetic stimulation before and after the injection demonstrating successful injection, stimulation, and measurement.\cite{17} In another study, Park et al injected viral vectors containing carrying opsin genes into mouse brains, then optically stimulated the brain and monitored neural electrophysiological response.\cite{18} This demonstrated ability to simultaneously inject a fluid and obtain electrical measurements makes microfluidic multifunctional fibers an attractive platform for impedimetric sensing of bioink composition and quality during continuous extrusion.

Here, we present a fiber-based impedimetric sensor for monitoring of bioink composition and quality during continuous microextrusion. Extrusion through electrode-functionalized hollow fibers enabled continuous monitoring of cell-laden solution or bioink impedance magnitude $|Z|$ and phase angle $(\phi)$ of the extrudate. Studies using fibroblasts, neuronal cells, and stem cells showed that impedimetric sensing enabled detection of cell type, state of differentiation, and viability. This report provides an advance in sensor-based monitoring of 3D bioprinting processes based on bioink compositional and quality analysis using hollow multifunctional fiber technology. Overall, hollow multifunctional fibers provide an attractive platform for controlled delivery and fabrication of cell therapies.

8.3 Experimental

8.3.1 Materials: Alginic acid sodium salt from brown algae (alginate) and calcium chloride ($\text{CaCl}_2$) were purchased from Sigma-Aldrich. Nanofibrillated cellulose (NFC) was purchased
from the University of Maine Product Development Center. RPMI-1640 was from American Type Culture Collection (ATCC). Horse serum was from Gibco. Fetal bovine serum (FBS) was from Aleken Biologicals. Penicillin-streptomycin, Dulbecco’s Modified Eagle Medium (DMEM)/F12 medium, DPBS, Trypsin EDTA, Hoechst 33342, calcein AM, and ethidium homodimer-1 (EthD-1) were from ThermoFisher. High-glucose DMEM was from GE Healthcare Life Sciences. ES-cell qualified FBS was from ZenBio (Research Triangle, NC). Leukemia inhibitory factor (LIF; 1000 U/ml) was from PeproTech (Rocky, NJ). Nonessential amino acids (NEAA) was from Quality Biological Inc. (Gaithersburg, MD). 2-mercaptoethanol (2-ME) was from Sigma-Aldrich (St. Louis, MO). L-glutamine was from Quality Biological Inc. Penicillin-streptomycin (1X) was from (VWR) Radnor, PA. Polycarbonate (PC) film was from Laminated Plastics. Medical epoxy (EA M-121HP) was from Henkel Loctite. Silver paint was from SPI Supplies. Vinyl acetate tubing (0.5 mm inner diameter) and polyvinylidene fluoride was from McMaster-Carr. 5-minute epoxy was from Devcon.

8.3.2 Hydrogel Preparation: Alginate solutions were prepared by dissolving alginate in DPBS at room temperature under constant stirring for 24 hours. An alginate concentration of 1 wt% was prepared for fibroblast and neuronal cell experiments, and a concentration of 0.25 wt% was prepared for embryonic stem cell (mESC) experiments. Following preparation, 2 wt% NFC was added to the 1 wt% alginate solution used for fibroblast and neuronal cell 3D printing and homogenized under sonication for 90 seconds (FB705 with microtip accessory, Fisher Scientific). All solutions were autoclaved at 121 °C for 30 minutes prior to use.
8.3.3 Cell Culture and Differentiation: Neuronal cells from the rat adrenal gland (PC-12, ATCC) were cultured in RPMI-1640 supplemented with 10% v/v horse serum, 5% v/v FBS, and 100 U mL−1 penicillin-100 μg mL−1 streptomycin at 37 °C and 5% CO2. The PC-12 cells grew as a suspension. The cell medium was changed twice weekly. PC-12 cells for low-viability bioinks were prepared via nutrient deprivation for one week, which was initiated when the culture reached confluence.

Mouse embryonic fibroblasts (NIH/3T3, ATCC) were cultured in DMEM/F12 supplemented with 100 U mL−1 penicillin-100 μg mL−1 streptomycin and 10% v/v FBS at 37°C and 5% CO2. The 3T3 cells grew as adherent cultures. Passaging was performed using treatment with Trypsin-EDTA solution at 90% confluency.

The mouse embryonic stem cells (mESCs) (C57BL/6; Cat. SCRC-1002; ATCC) was cultured in 0.1% gelatin-coated plates at 37°C, 21%O2 and 5%CO2 with high-glucose DMEM supplemented with 15% ES-cell qualified FBS, 1000 U/ml leukemia inhibitory factor, 0.1 mM nonessential amino acids, 0.1 mM 2-mercaptoethanol, 2 mM L-glutamine, and 1X penicillin streptomycin with daily medium change. To induce spontaneous differentiation, mESCs were washed twice with phosphate-buffered saline (PBS) and then cultured in the same medium, except in the absence of LIF. Medium was changed every 2 days.

8.3.4 Fabrication of Hollow Multifunctional Fibers: Similar to previous functional fibers,[17] PC films were first wrapped round a Teflon rod (diameter = 6.35 – 12.7 mm) and placed in a heated oven (T = 200 °C). After the consolidation of the materials by heating, the Teflon rod was removed resulting a uniform PC tube. Single grooves (diameter = 3.175 – 4.37 mm) were then machined on opposite sides of the tube surface. A thin layer of PVDF (thickness =
A 0.12 mm thick layer of PC (thickness = 3 mm) was then rolled on the machined PC tube. The assembly was then placed in the vacuum oven to facilitate material forming and consolidation. A copper wire (16 gauge) was passed through each of the two channels that remained from the machining process to complete the fabrication of the preform. Hollow multifunctional fibers were subsequently drawn from the preform using a thermal drawing process, which was performed under controlled temperature and stress using a custom furnace (temperatures in the pre-heating section, heating section, and cooling section were 150, 285, and 110 °C, respectively). Fiber drawing was done at constant velocity (85 cm/min).

Upon initial production of the fiber, the copper electrodes were exposed on both ends of the fiber. To prevent contact between the electrodes and the extrudate at both the entrance and exit to the fiber, the exposed electrodes at the entrance were blocked with medical epoxy. This ensured the two electrodes would only contact the material at one location, at the exit of the fiber. To provide an opening for connecting leads to the copper wires embedded in the fiber, a scalpel was used to remove material along a section of the outer walls of the fiber exposing the embedded copper wire. Silver paint was used to connect the exposed copper wire in the fiber to copper leads. To build the fluidic connection, the fiber was inserted into the ethylene vinyl acetate tubing (0.5 mm inner diameter) and the whole device was affixed with 5-min epoxy. The tubing was slid onto 18-gauge syringe tips to form a tight seal (see Supplementary Figure S1 for a schematic illustrating the assembly process).
8.3.5 3D Bioprinting of Cell-laden Bioinks: Following cell resuspension in fresh pre-warmed growth medium, cell-laden bioinks were then created by combining cell suspensions and 2 wt% alginate solutions at a 1:1 ratio by volume. 2 mL of 1 wt% cell-laden alginate bioinks with 2 wt% NFC were loaded into sterile 5 cc syringe barrels with 500 µm diameter tips that contained the hollow fibers. Tips and fibers were sterilized with 70% ethanol prior to use. The PC-12 and 3T3 cells were collected as a single-cell suspension in phosphate-buffered saline (PBS, Fisher) following dissociation with Trypsin-EDTA (3T3) or Accutase (PC-12, Sigma). The cell concentration and percent of viable cells was determined using Trypan Blue (VWR) following manufacturer’s instructions. Based on the Trypan blue counts, bioinks containing 5 x 10^5 and 1 x 10^6 PC-12 cells/mL, 1 x 10^6 3T3 fibroblasts/mL, and 5 x 10^5 dead PC-12 cells/mL were prepared. All tissue constructs were printed into 6-well plates using a commercial 3D bioprinter (Inkredible+, CELLINK) using extrusion pressures ranging from 2 to 10 psi at a print speed of 1 mm/s print speed. Following printing, 500 µm of sterile 100 mM CaCl₂ was applied to each sample and allowed to cure for 120 s before triple rinsing with 2 mL sterile DPBS. All bioprinting was done in a biosafety cabinet. All studies were done using single-layer square tissue constructs (10 x 10 mm^2). Each experimental group contained (n = 6) replicates.

Syringe Extrusion of Cell-laden Bioinks: 2 mL of 0.25 wt% cell-laden alginate bioinks containing 10^6 cells/mL were prepared and loaded into 5 mL syringes. Suspensions containing stable stem cells (+ LIF) and spontaneously differentiated cells (– LIF) were prepared at the same concentration. Sterile tips that contained the hollow fibers (diameter = 500 µm) were then attached to the syringe. Cell-laden bioinks were hand printed based on
a previously reported technique\cite{19} into petri dishes and subsequently cured with 500 µL of sterile 50 mM CaCl$_2$ by exposure for 120 s followed by triple rinsing with 2 mL sterile DI water.

8.3.6 Sensing Principle and Data Acquisition: The configuration of the hollow multifunctional fiber sensor is based on impedance analysis of the circuit composed of the two fiber copper electrodes and the extruded material, which is in the middle of the copper electrodes. The electrical impedance response of the system was done using a potentiostat (Interface 1010E, Gamry Instruments) using a two-electrode format in which one copper electrode served as the working electrode, the other copper electrode served as the counter electrode using an AC voltage of 10 mV and zero DC offset over a range of 0.100 – 100 kHz in a logarithmic sweep with 10 points per decade.

8.3.7 Immunocytochemistry: The mESC and differentiated mESC cells were fixed with 4% paraformaldehyde (PFA) at room temperature (RT) for 30 minutes and then cytopun onto glass tissue slides at 1100 revolutions per minute (RPM) for 4 minutes. The resulting cells were then washed twice with PBS, followed by blocking in 1.5% BSA and 0.2% Tween20 buffer for 1 hour at room temperature. The cells were then incubated overnight with 1:250 anti-stage specific embryonic antigen-1 (SSEA-1, Santa Cruz Biotech, Dallas, TX), washed thrice, and stained with a secondary antibody, goat anti-mouse 594, at a concentration of 1:1000 in blocking buffer. Hoechst 33342 was counterstained to label cell nuclei in all groups. An additional set of coverslips were stained with only secondary antibody as a negative control. Fluorescent micrographs were acquired using an Olympus IX73 microscope equipped with a DP70 CCD camera (Olympus, Center Valley, PA). The images
were then analyzed using ImageJ software (NIH). At least 1,100 Hoechst cells were analyzed in each group to calculate percent of SSEA-1-positive cells over total number of cells.

8.3.8 Live/Dead Assay of PC-12 and 3T3 Cells: At days 1 and 7 following printing, cell viability was assessed with a LIVE/DEAD Assay (ThermoFisher) following manufacturer’s instructions. In brief, the bio-printed parts were incubated in a 2μM calcein AM and 4 μM EthD-1 solution for 30 minutes at 37°C with 5% CO₂. The bio-printed parts were then moved into fresh cell medium for imaging using a laser-scanning microscope (A1R; Nikon) equipped with a Plan Apo 10×/N.A 0.45 air objective. Two independent fields of view were imaged per sample for each condition. Quantification of the percentage of cells that were viable was performed with ImageJ Fiji software. In short, the total volume of cells (green signal from calcein AM plus and red signal from EthD-1) was calculated by creating a binary representation of the fluorescent signals. Subsequently, the percentage of viable cells was obtained by dividing the calcein AM (green signal) volume from the calculated total volume.

8.3.9 Live/Dead Assay of mESCs and Spontaneously Differentiated Cells: Cured alginate hydrogels were dissolved with the addition of 0.1M EDTA. Bioprinted mESC and differentiated mESC cells were centrifuged at 1100 RPM for 4 minutes and then suspended in 1 ml culture medium to remove alginate. Cells were then plated in a 0.1% gelatin-coated 48-well plate (1 x 10⁵ cells per well) and allowed 2 hours to attach to the bottom of the culture dish. Afterward, cells were washed with PBS and incubated with 2 μM calcein AM, 1.25 μM EthD-1, and 1 μM Hoechst 33342 for 20 minutes. Only live cells with intracellular esterase activity could digest non-fluorescent calcein AM into fluorescent calcein. Dead or dying cells containing damaged membranes allowed the entrance of EthD-1 to stain the
nuclei. Cells were imaged with Olympus IX73 microscope described above and analyzed using ImageJ software. At least 800 Hoechst cells were analyzed in each group to calculate percentage of dead cells over total number of cells.

The calcein AM/EthD-1 staining was also performed for cells 72 hours following 3D bioprinting, except the cells were cultured and imaged in the hydrogel. The percentage of dead cells was calculated by dividing total red (representing dead cells) fluorescent intensity over total blue (representing total number of cells) fluorescent intensity for each image.

8.3.10 Statistics: Statistical analysis was completed in Origin Pro 2016. Student’s t-test were used for live/dead and SSEA-1 assays. Chi-squared tests were used for Trypan blue counts. *, **, and *** indicate a p-value (p) less than 0.05, 0.01, and 0.001, respectively. Error bars and ± indicate standard deviation. Number of replicates (n) is specified for each experiment and was greater than three in all cases.

8.4 Results and Discussion

8.4.1 Principle of In-line Bioink Compositional Analysis for 3D Bioprinting Processes using Hollow Multifunctional Sensors

Impedimetric sensing is a useful characterization technique for enabling in-line monitoring of cell-laden bioink composition and quality, given it can be acquired non-invasively via boundary electrodes and can be performed with high-sampling rates. As shown in Figure 1a, the flow of alternating current through cell-laden materials, such as tissues, is dependent on the frequency of the applied electrical potential.\textsuperscript{[20]} At low frequency,
the current is dominated by charge transfer around cells through the extracellular matrix (ECM) or extracellular fluid (ECF), while current also arises from charge transfer through cells at high frequency.\textsuperscript{[20]} To date, impedimetric characterization of cell-laden materials has enabled the characterization of cell shape, size, density, and integrity.\textsuperscript{[11, 20]} As shown in Figure 1b, hollow multifunctional fibers enable impedimetric characterization of extruded materials, such as a cell-laden bioink, using the two copper fiber electrodes. Thus, multifunctional fibers potentially enable real-time monitoring of the bioink impedance magnitude ($|Z|$) and phase angle ($\phi$) frequency response during extrusion. As shown in Figure 1c, hollow multifunctional fibers facilitate in-line sensing of 3D bioprinting processes, particularly real-time bioink compositional analysis. Photographs of the nozzle-sensor integration process are provided in Supplementary Figure S2. Multifunctional fibers potentially provide an attractive sensing platform for understanding the effect of bioink composition and process parameters, such as extrusion flow rate, on the properties and quality measures of 3D bioprinted tissue constructs.

**Figure 8.**  
(a) Schematic representation of impedimetric monitoring of cell-laden materials using a two-electrode format showing the paths of current when driven by low- ($f \rightarrow 0$) and high-frequency ($f \rightarrow \infty$) applied potentials. (b) Schematic showing the impedimetric sensing principle for bioink compositional analysis implemented in hollow multifunctional fibers. (c)
Schematic describing the concept of sensor-based in-line bioink compositional analysis of 3D bioprinting processes using multifunctional fibers.

8.4.2 Fabrication of Hollow Multifunctional Fibers for Extrusion of Cell-laden Bioinks

Given the majority of microextrusion 3D bioprinting is done using extrusion nozzles of diameter ranging from 100 – 500 μm,[21-23] we first examined the ability to tune the fiber channel diameter by variation of the preform design or thermal drawing process parameters. As shown in Figures 2a, the thermal drawing process produced hollow fibers of conserved cross-sectional geometry and composition. The copper wires were confined within their initial grooves and remained adjacent to the hollow channel. We found that the size of the Teflon rod determined the inner diameter of the hollow channel in the as-drawn fiber. For example, the diameters of the Teflon rod used in this study were 6.35, 9.525, and 12.7 mm, which produced the corresponding hollow channel diameters in as-drawn fibers of 181, 272, and 362 µm, respectively. The corresponding reduction in area (r) defined as \((A_0 - A_f)/A_0\) was 0.999, where \(A_0\) and \(A_f\) are the initial and final fiber cross-sectional areas, respectively. The strain in drawing (ε) defined as \(\ln [1/(1-r)]\) was 7.11%. The ability to fabricate hollow multifunctional fibers with a range of fiber dimensions is shown in Figures 2b-d. As shown in Figure 2d, we found that the use of the largest diameter PC tube (inner diameter = 12.7 mm) with the smallest machined grooves resulted in fibers that exhibited the relatively largest hollow channel (500 µm diameter). In addition to modifying the fiber channel diameter through the dimensions of the Teflon rod used in the preform fabrication process, we also found that the channel diameter could be further tuned by adjusting the draw ratio defined as the ratio of initial to final diameter. We found that fibers with a range
of commonly used inner diameters of 100, 200, 300, 400, and 500 µm could be fabricated by adjusting of the draw ratio used for the three aforementioned preform geometries, and micrographs of all fabricated fiber diameters can be found in Supplementary Figure S3. Thus, the thermal drawing process enables the fabrication of hollow multifunctional fibers with a range of channel dimensions that are commonly employed for 3D bioprinting processes. As shown in Figure 2e, the volumetric flow rate \( Q \) increased nonlinearly with respect to increasing fiber channel diameter at a constant pressure, an expected result considering the Hagen-Poiseuille equation for shear-thinning power-law fluid (e.g., alginate solutions):

\[
Q = \frac{\pi R^2}{n+3} \left( \frac{\Delta P R}{2LK} \right)^{1/n}
\]

where \( n \) is the flow behavior index, \( K \) is the flow consistency index, \( R \) is the nozzle radius, \( \Delta P \) is the pressure drop, and \( L \) is the length of the nozzle. The frictional head loss \( h_f \), an important parameter relating to shear stress-induced cell death and injury in 3D bioprinting processes, was calculated using Bernoulli’s equation assuming negligible contributions of potential energy effects as:

\[
h_f = \frac{P_{\text{appl}} - P_{\text{atm}}}{\rho g} - \frac{v_{\text{out}}^2}{2g}
\]

where \( P_{\text{appl}} \) is the applied pressure (here, 5 psi), \( P_{\text{atm}} \) is atmospheric pressure, \( v_{\text{out}} \) is the average velocity at the outlet, \( g \) is the acceleration due to gravity, and \( \rho \) is the density of the fluid. We note that Equ. (2) ignores the effect of head loss due to contraction effects, which is a valid assumption in laminar flow regimes. The head loss per unit length \( (S = h_f/L) \) can be related to the Darcy friction factor \( (f_0) \) through the Darcy-Weisbach equation as:
\[ f_D = \frac{\pi^2 g b^5 \xi}{8Q^2} \]  

(3)

The mean wall shear stress \( (\tau) \) that cells are exposed to during the 3D bioprinting process can subsequently be estimated in terms of the Darcy-Weisbach friction factor as: \[\text{[28]}\]

\[ \tau = \frac{1}{6} f_D \rho v_{out}^2 \]  

(4)

As shown in Figure 2e, the head loss per length ranged from 98.9 ± 3.4 to 53.7 ± 2.0 over the diameter range 100 to 500 μm. While this study provides insight into the roughness of the fiber channel walls and illustrates the ability to understand 3D bioprinting quality in terms of quantitative estimates of the shear stress experienced by cells, it should be noted that the shear stress generated in practice can be reduced by decreasing the average outlet velocity of the printed bioink (i.e., the speed of the 3D bioprinting process) as shown in Equ. 4. As shown in Figure 2f, hollow multifunctional fibers enabled continuous extrusion of alginate bioinks with minimal die swell at the average outlet velocities (i.e., extruder feed rates) for the 3D bioprinting studies discussed in the following sections.
8.4.3 Detection of Cell Viability Differences among Extruded Cell-laden Bioinks via Fiber-based Impedimetric Monitoring

Understanding the effects of material (i.e., bioink) properties and process parameters, such as bioink rheological properties and average velocity of extruded bioink, respectively, on resultant tissue quality remains an active area of research within the biofabrication field. For example, material innovations, such as those based on novel shear-thinning bioinks, are continuing to emerge that reduce the shear stress-induced cell damage and death during 3D bioprinting. Given, the viability of deposited cells is among the most important characteristics to quantify and monitor from a process and quality control perspective, we
next examined if the hollow multifunctional fibers could detect the viability of cells within extruded cell-laden bioinks. As shown in Figures 3a and b, alginate-NFC bioinks containing PC-12 cells with either high or low viability at the same concentration of 5 x 10^5 cells/mL exhibited different impedance responses over the frequency range of 0.1 to 100 kHz and different phase angle responses over the range of 0.1 to 2 kHz. Live/dead stained images taken following bioprinting can be seen in Figure 3c. As shown in Figure 3d, the bioinks containing populations of cells with either high or low viability exhibited 89.8 ± 2.8% and 0% viability. As shown in Figure 3a, bioinks that contained a high population of viable cells exhibited a higher impedance than those that contained a high population of dead cells across the 0.1 to 100 kHz frequency range. The maximum relative difference in impedance between the two bioinks of 118% was observed at 12 kHz. This result agrees with previous impedimetric results, and is attributed to the integrity of the cell membranes.[29, 30] As shown in Figure 3b, bioinks that contained a high population of viable cells exhibited a higher phase angle at relatively low frequencies ranging from 0.1 to 2 kHz. Differences in phase angle between the two bioinks was not distinguishable above 2 kHz. The maximum difference in phase angle between the two bioinks of 13.6 degrees was observed at 0.16 kHz. Thus, fixed-frequency impedimetric monitoring of extruded cell-laden bioinks at 160 Hz or 12 kHz could potentially enable real-time monitoring of cell viability in cell-laden 3D-bioprinted materials (e.g., bioinks) at high sampling rates. Bioprinted constructs fabricated using the viable bioink were cultured for seven days. We found that the cell viability in the bioprinted tissue constructs was 76.5 ± 8.1% after the curing process (day 1) and did not significantly differ
from the cell viability on day 7 (81 ± 2.8%) (see Supplementary Figure S4 for associated live/dead stains).

**Figure 8.** Electrical impedance spectra of hollow multifunctional fibers during 3D bioprinting of cell-laden alginate-NFC bioinks containing $5 \times 10^5$ cells/mL shown in terms of the impedance magnitude (a) and phase angle (b) responses (error bars correspond to the standard deviation of measurements obtained from bioprinting of $n = 3$ constructs). c) Fluorescence micrographs associated with a live/dead stain of bioinks containing populations of PC-12 cells with either high or low viability. d) Viability study of bioinks containing populations of PC-12 cells with either high or low viability ($n = 3$ biological replicates, *** indicates $p < 0.001$).
8.4.4 Detection of Cell Type Differences among Extruded Cell-laden Bioinks via Fiber-based Impedimetric Monitoring

Having shown that 3D bioprinting using hollow multifunctional fibers enables detection of cell viability differences among cell-laden bioinks, we next examined whether they could also characterize differences in cell type. While many bioprinted constructs are composed of a single cell type, applications of 3D bioprinting to fabrication of organ-on-a-chip system requires bioprinting of multiple cell types on a single substrate.[31-33] In addition to fabrication of organ-chip platforms, applications of 3D bioprinting for fundamental study of cell migration[34] and cell-laden scaffolds for repair of complex injuries requires[33] bioprinting of multiple cell types in a single construct. As shown in Figures 4a and b, alginate-NFC bioinks containing either PC-12 cells or fibroblasts at the same concentration of 1 x 10^6 cells/mL exhibited different impedance responses over the frequency range of 0.1 to 100 kHz. Live/dead stained images taken following bioprinting can be seen in Figure 4c. As shown in Figure 3d, the PC-12 cell- and 3T3 cell-laden bioinks exhibited cell viabilities of 70.5 ± 5.8 and 92.5 ± 4.2%, respectively. As shown in Figure 4a, bioinks that contained PC-12 cells exhibited a higher impedance than those that 3T3 cells across the 0.1 to 100 kHz frequency range. The maximum relative difference in impedance between the two bioinks of 153% occurred at 0.1 kHz, which was different than the location of the maximum impedance difference associated with sensing of bioink cell viability (12 kHz) (see Figure 3a). As shown in Figure 4b, bioinks that contained PC-12 cells exhibited a higher phase angle than those that contained 3T3 cells across the 0.1 to 100 kHz frequency range. The maximum difference in phase angle between the two bioinks of 10.8 – 11.7 degrees was observed.
across the 1 – 5 kHz frequency range. Thus, fixed-frequency impedimetric monitoring of extruded cell-laden bioinks at 0.1 kHz or a fixed frequency within the 1 - 5 kHz could range potentially enable real-time monitoring of cell type in cell-laden 3D-bioprinted materials (e.g., bioinks) at high sampling rates. Bioprinted constructs fabricated using the viable bioink were cultured for seven days. The 3D bioprinted PC-12 cell- and 3T3 cell-laden bioprinted tissue constructs exhibited day-7 cell viabilities of 74.6 ± 7.4 and 87.0 ± 2.1%, respectively (see Supplementary Figure S5 for associated live/dead stains).

**Figure 8.** Electrical impedance spectra of hollow multifunctional fibers during 3D bioprinting of PC-12 cell- and 3T3 cell-laden alginate-NFC bioinks containing 1 x 10^6 cells/mL shown in terms of the impedance magnitude (a) and phase angle (b) responses.
(error bars correspond to the standard deviation of measurements obtained from bioprinting of \( n = 3 \) constructs). c) Fluorescence micrographs associated with a live/dead stain of bioinks containing viable PC-12 or 3T3 cells. d) Viability study of PC-12 or 3T3 cell-laden bioinks (\( n = 3 \) biological replicates, ** indicates \( p < 0.01 \)).

8.4.5 Signal Response to Stem Cells and Differentiated Cells

In addition to the ability to detect changes in cell viability and types, the ability to classify the state of 3D bioprinted cells remains an important process monitoring challenge, particularly for biofabrication of quality 3D bioprinted stem cell therapies. Given stem cell pluripotency and stability are required for effective function of associated 3D bioprinted stem cell therapies, it remains a present biomanufacturing challenge to preserve pluripotency and maintain stem cell stability during processing based on the tendency of stem cells to spontaneously differentiate into terminal cell types.\(^{[35]}\) Having shown that 3D bioprinting using hollow multifunctional fibers enables detection of cell viability and cell type differences among cell-laden bioinks, we next examined if they could also characterize differences in the extent of differentiation among stem cells comprising a stem cell-laden bioink. Embryonic stem cells can be stabilized in a self-renewing state through the application of leukemia inhibitory factor (LIF).\(^{[36, 37]}\) As shown in Figures 5a and b, alginate bioinks containing mESCs at \( 1 \times 10^6 \) cells/mL cultured in the presence or absence of LIF exhibited distinguishable impedance responses across the 0.1 – 100 kHz frequency range. As shown in Figures 5a and b, bioinks that contained a high population of stem cells (94.1% stem cells; + LIF) exhibited a lower impedance and phase angle than those in which a significant fraction of stem cells (56.8% differentiated cells; – LIF) had undergone differentiation across the 0.1 to 100 kHz frequency range. As shown in Figure 5a, the
difference in impedance between the two bioinks of ranged from 69% at 100 kHz to 136% at 1 kHz, but the differentiated group (- LIF) exhibited higher impedance across the 0.1 to 100 kHz frequency range. The highest signal to noise level was achieved above 30 kHz. As shown Figure 5b, the largest difference in phase angle between the bioinks occurred in the 0.1 – 5 kHz frequency range, with the maximum difference of 17.1 degrees occurring at 0.1 kHz.

As shown in Figure 5c and d, mouse embryonic stem cells (mESCs) exhibited significant differences in expression of stage specific embryonic antigen-1 (SSEA-1), an established stemness marker, after culture in the presence or absence of LIF for 14 days (+ LIF and – LIF, respectively). As summarized in Figure 5e, stem cells cultured in the presence and absence of LIF (i.e., + LIF and – LIF, respectively) tested 94.1 ± 2.1 and 43.2 ± 7.6% positive for SSEA-1, respectively. As shown in Videos S1 and S2 of Supporting Information, mESCs cultured in the absence of LIF entered mesodermal differentiation towards cardiac differentiation. The presence of these contractile cells (see Videos S1 and S2) provides further evidence that the cells cultured in the absence of LIF are no longer stem cells.

To investigate the impact of extrusion through the hollow multifunctional fiber on stem cell viability, the viability of stem cells in 3D bioprinted constructs was compared with that in molded constructs, which served as the control group. Micrographs associated with the live/dead assay in the 3D bioprinted and molded stem cell-laden tissue constructs are shown in Figures 5f-i. As shown in Figure 5j, the extruded + LIF and – LIF stem cell-laden 3D bioprinted constructs exhibited similar viability (86.8 ± 3.7% and 88.6 ± 2.7%, respectively) and extrusion of stem cell-laden bioinks through the hollow multifunctional fiber had a
minimal effect on cell viability relative to the molding process (86.8 ± 3.7% vs. 89.7 ± 2.9 for the + LIF group, respectively, and 88.6 ± 2.7% vs. 92.6 ± 1.2% for the − LIF groups, respectively). As shown in Supplementary Figure S6, the stem cell-laden tissue constructs exhibited no significant differences in cell viability by day 3. These results suggest that hollow multifunctional fibers provide an attractive form factor for in-line monitoring of stemness and extent of differentiation in stem cell-laden bioinks during extrusion and assembly using 3D bioprinting processes.
**Figure 8.** Impedance magnitude (a) and phase angle (b) of stem cells (+ LIF) and spontaneously differentiated cells (– LIF), averages of n = 3 samples shown. Merged fluorescent micrographs (200x) associated with expression of SSEA-1, a mESC specific marker, in mESC cultures stabilized in a stem cell state (+ LIF) (c) and allowed to spontaneously differentiate (– LIF) (d), which served the basis of the stem cell-laden bioinks. e) Percentage of stem cells present in a stem cell state based on SSEA-1 expression data
following 14 days of culture in +LIF and – LIF media (i.e., panels c and d) (** indicates $p < 0.001$, $n = 3$). Fluorescent micrographs (100x) associated with live/dead assays of stem cells in a stem cell state (+ LIF) (f and g) and in a spontaneously differentiated state (– LIF) (h and i) in both bioprinted and molded constructs using calcein AM, EthD-1, and Hoechst 33342 (nuclei). j) Summary of stem cell viability after following processing (* indicates $p < 0.05$, $n = 3$).

8.5 Conclusions

Here, we demonstrate the capability of in-line monitoring of cell-laden bioink composition and quality during 3D bioprinting processes via impedimetric sensing. 3D bioprinting using hollow electrode functionalized fibers with controlled inner diameter enabled impedance spectroscopy of cell-laden bioinks during extrusion across the $10^2$ to $10^6$ Hz frequency range. Monitoring of fiber electrical impedance was shown to enable sensing of bioink compositional characteristics, such as cell viability, type, and extent of stem cell differentiation. This work shows that microextrusion 3D bioprinting using hollow multifunctional fiber sensors provides a potential path to improving process monitoring capability of 3D bioprinting processes, particularly regarding in-line monitoring of deposited cell viability, type, stemness, and extent of differentiation. This work also suggests that fiber-based sensors may provide useful platforms for controlled delivery of cell therapies, such as injectable stem cell therapies.

8.6 References


8.7 Supplementary Information

These supporting materials contain: a schematic illustrating the wire bonding process that enables interface between the electroded multifunctional fiber and the potentiostat (Figure S1), photographs of a sensor-integrated 3D bioprinting microextrusion nozzle (Figures S2), micrographs of hollow multifunctional fibers fabricated with various internal channel diameters (Figure S3), micrographs associated with live/dead assay and corresponding cell viability of PC-12 cell cultures (5 x 10^5 cells/mL) cultures on days 1 and 7 (Figure S3), micrographs associated with live/dead assay and corresponding cell viability of PC-12 (10^6 cells/mL) and 3T3 (10^6 cells/mL) cultures on days 1 and 7 (Figure S4), micrographs associated with live/dead assay and corresponding cell viability of mESC and spontaneously differentiated cultures (10^6 cells/mL) on day 3 (Figure S6), and videos of contractile cells observed after 14 days of mESC culture without LIF (-LIF) indicating differentiation (Videos S1 and S2).
Figure S8. 1 Schematic illustrating the wire bonding process that enables interface between the electroded multifunctional fiber and the potentiostat. Step 1: Electrodes at the distal end of the fiber near the 3D bioprinting nozzle were blocked with medical epoxy. Cladding material was subsequently removed from the outer walls of the fibers to expose the embedded copper wire. Step 2: Copper leads were subsequently bonded to the exposed copper wires using a silver epoxy. Step 3: The fiber was inserted into tubing. Step 4: 5-minute epoxy was then used to seal and reinforce the tubing-fiber connection and the bonded copper leads. The bioprinting nozzle was subsequently inserted in the tubing forming a tight seal that prevented the backflow of bioink.
**Figure S8.2** a) Photograph of nozzle-fiber sensor assembly. b) Photograph illustrating 3D bioprinting through the hollow multifunctional fiber impedimetric sensor.
Figure S8. Micrographs of fiber cross sections demonstrating the ability to fabricate a range of inner channel diameters commonly used for 3D bioprinting: 100 (a), 200 (b), 300 (c), 400 (d), and 500 µm (e).
Figure S8. 4 Fluorescence images of $5 \times 10^5$ cell/mL cultures of PC-12 cells in a cured alginate-NFC hydrogel on day 1 (a) and 7 (b) with a live/dead stain. c) Live/dead assay results show no significant difference between days 1 and 7.
Figure S8.5 Fluorescence images of 10^6 cells/mL cultures of PC-12 (a and b) and 3T3 (d and e) cells in a cured alginate-NFC hydrogel on day 1 (a and d) and 7 (b and e). c) Live/dead assay results, * indicates p < 0.05.
Figure S8. 6 Merged fluorescence images of stable mESCs cultured with LIF (a and d) and differentiated cells cultured without LIF (b and e) cultured in an alginate hydrogel on day 3. Both the negative control (a and b) and experimental (d and e) groups are shown. c) Live/dead results showed no significant difference between groups.
Chapter Nine: Real-time Characterization of Hydrogel Viscoelastic Properties and Sol-Gel Transitions using Cantilever Sensors

9.1 Abstract

Here, we report for the first time that resonance in dynamic-mode cantilever sensors persists in hydrogels and enables the real-time characterization of hydrogel viscoelastic properties and the continuous monitoring of sol-gel phase transitions (i.e., gelation and dissolution processes). Real-time tracking of piezoelectric-excited millimeter cantilever (PEMC) sensor resonant frequency \( f_{\text{air}} = 55.4 \pm 8.8 \text{ kHz; } n = 5 \text{ sensors} \) and quality factor \( Q; Q_{\text{air}} = 23.8 \pm 1.5 \) enabled continuous monitoring of high-frequency hydrogel shear storage and loss moduli \( G'_{\gamma} \text{ and } G''_{\gamma}, \) respectively) calculated by sensor data and fluid-structure interaction models. Changes in the sensor phase angle, quality factor, and high-frequency shear moduli obtained at the resonant frequency \( G'_{\gamma} \text{ and } G''_{\gamma} \) correlated with low-frequency moduli obtained at 1 Hz using dynamic mechanical analysis (DMA). Characterization studies were performed using physically and chemically crosslinked hydrogel systems, including gelatin hydrogels \((6 - 10 \text{ wt\%})\) and alginate hydrogels \((0.25 - 0.75 \text{ wt\%})\). The sensor exhibited a dynamic range from the rheological properties of inviscid solutions to hydrogels with high-frequency moduli of 80 kPa and low-frequency moduli of 26 kPa. The sensor exhibited a limit of detection of 1.9 kPa for changes in hydrogel storage modulus \( E' \) based on the sensor’s quality factor response. We also show that sensor data enables quantitative
characterization of gelation process dynamics using a modified Hill model. This work suggests that cantilever sensors provide a promising platform for sensor-based characterization of hydrogels, such as quantification of viscoelastic properties and real-time monitoring of gelation processes.

9.2 Introduction

Hydrogels are crosslinked polymer networks that contain high water content. The past two decades have seen a sharp rise in fundamental research involving hydrogels and the development of novel hydrogels for various applications in energy storage and biotechnology.[1-4] The need for controlled drug release systems was among the earliest driving forces for hydrogel research in the pharmaceutical sciences.[5] In addition to the ability to incorporate drugs, the ability to incorporate animal cells has led to the widespread use of hydrogels in 3D cell culture and tissue engineering applications.[6, 7] For example, a number of studies have shown that the gene expression profiles of cells differ in monolayer tissue culture environments (i.e., 2D substrates) vs. 3D matrices, which is often attributed to differences in cell-cell and cell-matrix interactions.[6] Hydrogels have emerged as attractive materials for regenerative medicine applications, including 3D-bioprinted tissues and injectable scaffolds for wound healing and tissue regeneration.[8-11] In combination with their use as energy storage devices (e.g., gel electrolytes for lithium ion batteries),[2-3] passive drug release systems,[5, 12] tissue scaffolds,[13, 14], and sensors sensors,[15, 16] emerging applications to soft actuators, active drug release systems, and complex engineered tissues have driven research on stimuli-responsive hydrogels – for example, in soft robotics and 4D bioprinting fields.[17, 18] In such applications, the characterization of hydrogel structure,
physical properties, and rheological properties (e.g., crystal structure, dielectric properties, and viscoelastic properties) serve as important indicators of the material’s processability, performance, quality, and response to stimuli. Therefore, identifying new paradigms for the characterization of hydrogels and other gel-based materials is central to accelerating the pace of gel-based materials research and improving the processability and quality of gel-based therapeutics, devices, and other products.

While emerging synthetic techniques, such as automated synthesizers and synthetic biology, are now being developed to produce materials with unprecedented throughput, characterization loops represent major bottlenecks in accelerated molecular and material discovery workflows.\textsuperscript{[19-21]} The challenges associated with a lack of complementary, high-throughput characterization techniques are also compounded by the breadth of structure and property information that could be useful in assessing material performance and quality across different applications.\textsuperscript{[12, 22, 23]} In particular, the characterization of hydrogel viscoelastic properties, as well as of other soft materials, presents significant rate-limiting steps because of the requirement for manual sample preparation steps and the time-intensive nature of the tests. The gold-standard instruments for characterization of hydrogel viscoelastic properties are rheometers and dynamic mechanical analyzers.\textsuperscript{[24]} While such instruments are robust and provide reliable information regarding the viscoelastic properties of a sample over a range of strain rates and temperatures, the samples must be manually prepared and measured to high tolerances, and experiments can take upwards of 0.5 - 1 day per sample, depending on the complexity and type of scan being performed. Additionally, such instruments are difficult to integrate with processes, which impedes
applications that require viscoelastic property sensing or monitoring. As a result, it remains a pressing challenge to eliminate characterization bottlenecks from accelerated material discovery paradigms. In contrast to traditional characterization methods (e.g., DMA), sensor-based characterization techniques offer measurement advantages associated with sensors, which include process integration through miniaturization and the ability for real-time process monitoring and control. Sensors can also offer improved sensitivity, limit of detection, throughput, and measurement repeatability relative to traditional methods through the use of sensitive miniaturized transducers and the elimination of manual sample preparation steps. Therefore, sensor-based techniques for characterization of hydrogel viscoelastic properties could provide useful tools for eliminating characterization bottlenecks that currently limit the pace of hydrogel materials research and development.

While various sensors have been created to measure the physical and rheological properties of liquids,[25-28] milli- and micro-electromechanical systems have enabled the characterization of viscoelastic properties based on fluid-structure interaction effects.[29] Thickness shear mode (TSM) resonators, such as quartz crystal microbalances (QCMs), were among the first sensors leveraged for viscoelastic property characterization. While TSM resonators enable the characterization of the viscoelastic properties of semi-infinite and thin layers of viscoelastic liquids, viscoelastic properties are obtained using equivalent circuit models, which imposes the requirement of sensor calibration. In addition to shear-mode resonators, dynamic-mode cantilevers have been extensively examined for sensor-based rheological and compositional analysis of liquids, such as viscosity monitoring, chemical sensing, and biosensing.[30-35] Analysis of cantilever sensor response is done using fluid-
structure interaction models, which are the same physics that drive the sensor response, in contrast to equivalent circuit models, which are useful for modeling measurements based on impedance responses but are not directly representative of the physical phenomenon. The earliest applications of dynamic-mode cantilevers for characterizing the physical properties of liquids were focused on density monitoring using the well-known inviscid result. In parallel with liquid density monitoring applications, rheological measurements (i.e., viscosity monitoring) were also performed using cantilever sensors by incorporating the frequency- and mode number-dependent hydrodynamic function. An explicit theoretical relationship between cantilever resonant frequency, quality factor, and the viscosity of the surrounding media has been previously reported. Chon et al. validated the theoretical results of the cantilever hydrodynamic function using an atomic force microscope (AFM) cantilever submerged in a range of viscous fluids. Boskovic et al. demonstrated that an AFM cantilever with a known undamped natural frequency (i.e., resonant frequency) and mass per unit length allowed for the simultaneous calculation of the density and viscosity of both gasses and liquids. Mather et al. extended the use of cantilever sensors for characterization and monitoring of the viscoelastic properties of liquids through the use of mesoscale piezoelectric cantilevers. In that study, the complex shear modulus was replaced with the viscosity in the previous models developed by Sader, Maali et al., and Belmiloud et al., which yielded a system of equations for the determination of the shear storage and loss moduli of a material from the cantilever resonant frequency and quality factor responses (after accounting for the internal damping of the larger cantilever). While Mather et al. were able to characterize the viscoelastic properties of a polyacrylamide
solution, fluid damping effects impeded applications to characterization of more viscous or viscoelastic materials. Johnson and Mutharasan more recently showed that millimeter-scale cantilevers exhibited a sufficiently high cantilever Reynolds numbers to resonate in highly viscous liquids with dynamic viscosities as high as $10^3$ cP. These studies suggest that millimeter-scale cantilevers could enable sensor-based characterization of solutions that undergo gelation, materials that exhibit sol-gel phase transitions, and the viscoelastic properties of gel-based materials.

In this paper, we show that resonance in millimeter cantilevers persists in hydrogels and enables the characterization of hydrogel viscoelastic properties and the continuous monitoring of sol-gel phase transitions (i.e., gelation processes). Sensor signal changes and associated high-frequency viscoelastic properties of alginate and gelatin hydrogels over concentration ranges of 0.25 – 10 wt% are compared with low-frequency viscoelastic properties obtained from DMA studies (1 Hz). We obtain the sensor’s dynamic range and limit of detection based on the low-frequency viscoelastic moduli at which the sensor no longer exhibited resonance and the noise level associated with the sensor quality factor response, respectively. Ultimately, this work shows that cantilever sensors provide a complementary platform to traditional DMA for real-time monitoring and viscoelastic characterization of hydrogels and gelation processes and could serve to mitigate existing bottlenecks related to low-throughput characterization loops in accelerated material discovery workflows.
9.3 Materials and Methods

9.3.1 Materials

Alginic acid sodium salt, gelatin (300 g bloom from porcine skin), poly(ethylene glycol) diacrylate (PEGDA) (750 Da), 2,2-Dimethoxy-2-phenylacetophenone (DMPA), calcium chloride, and ethylenediaminetetraacetic acid (EDTA) were purchased from Millipore Sigma. Lead zirconate titanate (PZT-5A; 72.4 × 72.4 × 0.127 mm³) with nickel electrodes was purchased from Piezosystems (Woburn, MA). Borosilicate glass was purchased from VWR. Glass cylinders and ethanol (200 proof) were from Fisher Scientific. Polyurethane (Fast-Drying) was from Minwax. Epoxy (EA 1C-LV) and cyanoacrylate (409 Super Bonder) were from Loctite.

9.3.2 Fabrication of Piezoelectric-excited Millimeter Cantilever Sensors

Composite PEMC sensors (3 × 1 × 0.127 mm³) with a flush design consisting of a notch length = 2 mm were fabricated from lead zirconate titanate (PZT) as described in previous studies (see Figure 1a). Briefly, borosilicate and PZT sheets were diced into chips (2 × 1 × 0.16 mm³ and 5 × 1 × 0.127 mm³, respectively; American Dicing; Liverpool, NY). A borosilicate chip was first bonded symmetrically to one end of the cantilever using cyanoacrylate such that the front of both chips were aligned. Subsequently, 30-gauge copper (Cu) wires were soldered to the top and bottom faces of the nickel electrodes on the distal end of the PZT layer opposite to the glass layer. The cantilever was then potted in a glass cylinder (6 mm diameter) with a non-conductive epoxy resulting in a cantilever geometry (3 × 1 × 0.127 mm³). The distance between the anchor and the glass layer, referred to as the notch length, was 0.5 mm. The sensors were then coated with polyurethane via spin coating.
(1000 rpm for 2 min), which was then allowed to cure at room temperature. The sensors were then coated with parylene-c (10 μm thick) following vendor protocols (PDS 2010 Labcoter® 2; Specialty Coating Systems; Indianapolis, IN). Following parylene-c coating, the sensors were annealed for 1 hour at 75 °C.

9.3.3 Measurement Principle and Data Acquisition

The sensor resonant frequency ($f_n$), quality factor ($Q_n$), and phase angle at resonance ($\phi_n$), where $n$ indicates the mode number, were continuously monitored with a vector-network analyzer with impedance option (E5061b-005; Keysight). The sensor’s dynamical mechanical response, here, the frequency response, was obtained via electromechanical coupling effects using electrical impedance analysis, which provides electrical impedance magnitude ($|Z|$) and phase angle ($\phi$) spectra of the piezoelectric layer ($|Z|$ and $\phi$ vs. frequency ($f$), respectively). Electrical impedance analysis was performed using a stimulus amplitude of 100 mV AC and zero DC bias across a frequency range ($f_n - 10$ kHz $\leq f_n \leq f_n + 10$ kHz), which enabled resolution of the off-resonance impedance response and, thus, measurement of the frequency-width-at-half-maximum (FWHM). Sensor signals ($f_n$, $\phi_n$, FWHM, and $Q_n = f_n$/FWHM) were acquired using a custom MATLAB program based on continuous monitoring of electrical impedance spectra.

9.3.4 Hydrogel Preparation

Alginate solutions (0.25, 0.5, 0.75, 1, 1.5, and 2.0 wt%) were prepared by dissolving alginic acid sodium salt in deionized water (DIW) at room temperature with continuous stirring. The solutions (5 mL) were chemically crosslinked by depositing a 500 μL droplet of saturated calcium chloride on the surface of the polymer solution approximately 5 mm from
the submerged sensor. Gelatin solutions (6, 8, 10, and 12 wt%) were prepared by dissolving gelatin in DIW at 40 °C with continuous stirring. Following dissolution, the solution was maintained at 40 °C until use. PEGDA hydrogels were prepared by dissolving 1, 2, 3, or 4 g PEGDA in 18.9, 17.9, 16.9, or 15.9 g of DIW at room temperature, followed by the addition of 0.1 g of 20 wt% DMPA in ethanol for final solutions containing 5, 10, 15, and 20 wt% PEGDA with 0.1 wt% DMPA. PEGDA hydrogels were cured with exposure to 365 nm UV light for 10 minutes (1200 μW/cm² at 3 inches; UVGL-58).

9.3.5 Viscoelastic Property Characterization using Dynamic-mode Cantilever Sensors

The shear storage and loss moduli of the surrounding material, here hydrogels, at the sensor’s resonant frequency ($G'$ and $G''$, respectively) were calculated based on previously established fluid-structure interaction models for resonant cantilevers.[29, 37, 39-41, 44] The inertial and dissipative components of the drag force on a vibrating cantilever ($g_1$ and $g_2$, respectively) can be written in terms of the frequency-dependent viscoelastic moduli ($G'$ and $G''$) as:[29]

$$g_1 = \frac{\pi}{2} \frac{b_2 G''}{\omega} + \frac{\pi}{4\sqrt{2}} \sqrt{\rho b} \left[ (b_1 - a_2) \sqrt{G''^2 + G'''^2 + G'} + (a_2 + b_1) \sqrt{G''^2 + G'''^2 - G'} \right]$$

$$g_2 = \frac{\pi}{4} a_1 \rho b^2 + \frac{\pi}{2} \frac{b_2 G'}{\omega^2} + \frac{\pi}{4\sqrt{2}} \frac{\sqrt{\rho b}}{\omega} \left[ (a_2 + b_1) \sqrt{G''^2 + G'''^2 + G'} + (a_2 - b_1) \sqrt{G''^2 + G'''^2 - G'} \right]$$

where $a_1$, $a_2$, $b_1$, and $b_2$ are Maali’s parameters ($a_1 = 1.0553$, $a_2 = 3.7997$, $b_1 = 3.8018$, $b_2 = 2.7364$)[40], $\omega$ is the angular frequency (here, taken as $\omega = 2\pi f_n$), $\rho$ is the density of the surrounding material (e.g., fluid), and $b$ is the cantilever width. The components of the drag force can also be calculated from sensor data (i.e., $f_n$ and $Q$) as:[29]
\[ g_1 = \frac{\pi}{4} \rho b^2 \omega \left( \frac{(m_c + m_A)\omega}{Q} - c_i \right) \]  

where \( g_1 \) is the cantilever length, \( \mu = \rho c b t \) is the cantilever mass per unit length, \( \rho_c \) and \( t \) are the respective cantilever density and thickness, \( Q_0 \) and \( \omega_o \) are the respective quality factor and resonant frequency in the absence of fluid damping (i.e., resonating in vacuum with only internal damping effects present), \( m_c = \rho_c b t L \) is the cantilever mass, \( m_A = \rho \pi b^2 \Gamma'/4 \) is the added mass, \( \Gamma' \) is the real part of the hydrodynamic function, and \( c_i = m_c \omega_o/Q_o \) is the internal damping coefficient. In calculation of \( c_i \), \( \omega_o \) and \( Q_0 \) were approximated as \( \omega_o \sim 2\pi f_{n,\text{air}} \) and \( Q_0 \sim Q_{n,\text{air}} \), which were reasonable assumptions as discussed in the following sections. The hydrodynamic function was approximated using the relation \( \Gamma' = a_1 + a_2 \delta/b \), where \( \delta = [(2\eta/(\rho \omega)]^{1/2} \) is the thickness of the thin viscous layer surrounding the cantilever in which the velocity has dropped by a factor of \( 1/e \), and \( \eta \) is the viscosity of the fluid. The solution to the system of equations formed by Equations (1) – (4) provides the viscoelastic properties of the surrounding material based on cantilever sensor data.

9.3.6 Real-time Monitoring of Gelation Processes using Cantilever Sensors

9.3.6.1 Gelatin Hydrogels: Prior to all experiments, the sensor impedance spectra were characterized in air to obtain the resonant frequency and quality factor in air. Experiments began by adding 5 mL of gelatin solution to a 35 mm dish at 40 ºC. The gelatin solution was maintained at 40 ºC during solution phase studies to prevent gelation. The solution was then cooled to room temperature, which took approximately 5 minutes. Subsequently, the
cantilever was submerged in the solution to a depth that brought the top surface of the polymer solution 30 µm above the cantilever’s anchor. The sensor data acquisition program was subsequently initiated, which enabled continuous monitoring of the sensor signals as the gelatin solution spontaneously underwent a thermoreversible gelation process.

9.3.6.2 Alginate Hydrogels: Prior to all experiments, the sensor impedance spectra were characterized in air to provide $f_{n,\text{air}}$ and $Q_{\text{air}}$. Experiments began by adding 5 mL of room temperature alginate solution to a 35 mm petri dish. Subsequently, the cantilever was submerged in the solution to a depth that brought the top surface of the polymer solution 30 µm above the cantilever’s anchor. The sensor data acquisition program was subsequently initiated, which enabled continuous monitoring of sensor signatures. Following stabilization of the sensor signals, the alginate solutions were chemically crosslinked by manually applying a 500 µL droplet of saturated calcium chloride to the surface of the solution approximately 5 mm from the anchor of the cantilever. Addition of a 500 µL droplet of DIW served as an in situ negative control. Following the stabilization of sensor signals after chemical gelation, the hydrogels were dissolved by applying 3 mL of a 1M aqueous solution of EDTA across the surface of the hydrogel.

9.3.7 Characterization of Hydrogel Low-frequency Viscoelastic Moduli via Traditional Dynamic Mechanical Analysis Studies

Characterization of hydrogel low-frequency viscoelastic properties was done using a dynamic mechanical analyzer (Q800; TA Instruments). Cylindrical test specimens of alginate and gelatin hydrogels (diameter = 12.7 mm and thickness = 5 mm) were punched from 5 mm thick hydrogel sheets prepared using the same crosslinking techniques as previously
described for the sensor studies. All measurements were acquired by application of a 15 μm periodic displacement at a constant frequency (1 Hz) and 5 mN preload force in the compression mode. Temperature-dependent data for the thermally-responsive gel was acquired under the same conditions using a temperature sweep from 26 to 36 °C at a rate of 0.5 °C/min.

9.3.8 Characterization of Sol-Gel Rheological Properties and Gelation Processes via Traditional Rheology

A rheometer (Discovery DH-2, TA Instruments) was implemented with recessed concentric cylinder geometry. Gelatin solution (8 wt%) was loaded into the test geometry with a 1 mm gap. Testing conditions of 1% strain and 1 Hz were imposed. The sample was held at 40 °C then quenched to 25 °C at a rate of 5 °C/min, which mimicked the temperature treatment used in the sensor studies. Data collection began when the sample temperature reached 25 °C and continued for 90 minutes. The time of the gelation process as measured through sensor and rheometer data was normalized by the respective total gelation times. The data was truncated in both sensor and rheometer data to the point where $G'$ reached 95% of the maximum ($G'_{95}$) and subsequently normalized by $G'_{95}$ for comparison.

9.4. Results and Discussion

9.4.1 Characterization of Cantilever Frequency Response in Concentrated Solutions of Gel-forming Polymers

As shown in Figure 9.1a, PEMC sensors are actuated and sensed using the same piezoelectric layer, which is referred to as a self-exciting and self-sensing design. This
design enables the sensor’s mechanical frequency response to be characterized by the electrical impedance response of the insulated piezoelectric layer (photographs shown in Figures 9.1b-c). Thus, electrical impedance analysis enables real-time monitoring of the cantilever resonant frequency ($f_n$) and quality factor ($Q_n$) in various liquids. Having previously shown that resonance in PEMC sensors persists in highly viscous liquids of viscosity up to $10^3$ cP,[42] here, we examined if resonance in PEMC sensors persists in solutions of gel-forming polymers and resultant hydrogels. As shown in Figure 9.1d, the resonance of PEMC sensors is minimally damped by air ($f_{n,\text{vac}} = 44.6$ kHz and $Q_{n,\text{vac}} = 24.8$ and $f_{n,\text{air}} = 44.4$ kHz and $Q_{n,\text{air}} = 24.7$). Considering the dimensions and resonant frequency of millimeter-scale cantilever sensors in a highly viscous liquid (width ($b$) = 1 mm; $f_n \sim 25$ kHz), as well as previously reported rheological properties of gel-forming aqueous polymer solutions ($\eta \approx 200$ cP),[46] the cantilever Reynolds number ($Re_c = \rho \omega b^2 / 4 \eta$) is $\sim 150$, where $\rho$ is the fluid density, $\omega = 2\pi f_n$ is the angular frequency of the cantilever, and $\eta$ is the dynamic viscosity of the solution. The result of $Re_c > 1$ suggests millimeter-scale cantilever sensors should resonate in concentrated solutions of gel-forming polymers. As shown in Figure 9.1d, resonance in PEMC sensors indeed persisted in concentrated solutions of gel-forming polymers (data shown for a 10% gelatin solution).
Figure 9. 1 a) Schematic of piezoelectric-excited millimeter cantilever (PEMC) sensor self-sensing and self-exciting design for sensor-based characterization of hydrogel rheological properties and real-time monitoring of sol-gel phase transitions. Photographs of a PEMC sensor from top-down (b) and side-view (c) perspectives. d) Sensor frequency response acquired via electrical impedance analysis shown in terms of the phase angle response (inset shows photograph of the PEMC sensor submerged in a concentrated solution of gel-forming polymer; spectra in air and vacuum correspond to 1 and 0.3 atm (vacuum), respectively).
Effect of Hydrogel Composition and Low-frequency Viscoelastic Moduli on Cantilever Frequency Response

Having established that PEMC sensors resonate in concentrated solutions of gel-forming polymers, we next examined if resonance persisted in gels following the sol-gel phase transition (see Figure 9.2a), as opposed to being absent as a result of increased damping effects of a surrounding gel phase. Studies were conducted using gelatin, alginate, and PEGDA hydrogels based on their extensive use across a range of applications, including tissue engineering, food engineering, and bioprinting applications. As shown in Figures 9.2b-d, the second mode of cantilever sensors exhibited a resonant frequency of $55.4 \pm 8.8$ kHz and quality factor of $23.8 \pm 1.5$ in air ($n = 5$ sensors). These values agreed reasonably with Euler-Bernoulli beam theory and previous finite element studies.\textsuperscript{[47]} The second mode was selected based on its previous use for characterization of high-viscosity liquids.\textsuperscript{[42]} As shown in Figure 9.2b, submersion of the sensor in a 6 wt% gelatin solution caused decreases in the cantilever resonant frequency and quality factor to $33.8 \pm 0.3$ kHz and $17.4 \pm 0.1$, respectively. As shown in Figures 9.2c and d, similar changes in resonant frequency and quality factor were observed upon submersion in 0.25 wt% alginate and 10 wt% PEGDA solutions. The shoulder peak near 36 kHz is attributed to a torsional mode that is adjacent to the transverse mode used for tracking and rheological characterization.\textsuperscript{[48]} Corresponding impedance magnitude data over the same frequency range is presented in Figure S9.1 of Supplementary Information. Importantly, as shown in Figures 2b-d, resonance in PEMC sensors persisted in 6 wt% gelatin, 0.25 wt% alginate, and 10 wt% PEGDA hydrogels following crosslinking (i.e., network formation).
Figure 9. 2a) Schematic depicting the sensor-based sol-gel rheological characterization study and associated measurement principle (i.e., real-time monitoring of gelation processes)
via sensor signal tracking). Observed cantilever impedance phase angle over a 25 – 50 kHz sweep in air, solution (sol), and gel phases of 6 wt% gelatin (b), 0.25 wt% alginate (c), and 10 wt% PEGDA (d).

Given that applications may use hydrogels across a range of concentrations, we next examined the concentration at which cantilever resonance no longer persisted in the gel phase (i.e., the concentration at which the cantilever quality factor could not be monitored with suitable resolution for characterization and sensing applications). As shown in Figure 9.3, we found that resonance persisted in hydrogels over a wide concentration range from dilute polymer solutions up to 15 wt%. For example, as shown in Figures 9.3a-c we found that resonance was observable in gelatin, alginate, and PEGDA hydrogels up to 10, 1.5 and 15 wt%, respectively. These concentrations corresponded to low-frequency storage moduli ($E'$) of 11.9, 36.2, and 46.2 kPa, respectively, as characterized by traditional DMA studies. Similarly, these concentrations corresponded to complex moduli ($|E^*|$) of 11.9, 37.3, and 47.0 kPa, respectively. This result suggests that the dynamic range and limit of detection regarding sensing of viscoelastic property changes is material dependent. Inspection of Figure 9.3 also shows that while gelation caused a decrease in the sensor quality factor for all hydrogels and concentrations thereof examined, network formation in the polymer solution caused a consistent decrease in the quality factor but had varying effects on the resonant frequency.
Figure 9.3 Limits of resonance persistence in increasingly concentrated hydrogels. a) 6, 8, 10, and 12 wt% gelatin. b) 0.5, 1, 1.5, and 2 wt% alginate. c) 5, 10, 15, and 20 wt% PEGDA.
Having established that cantilever resonant frequency and quality factor could be measured across a range of concentrations in various hydrogel systems, we next characterized the low-frequency viscoelastic moduli ($E'$ and $E''$) for each hydrogel examined to establish the sensor’s dynamic range regarding low-frequency viscoelastic moduli sensing. As shown in Table 9.1, a positive correlation was observed among the cantilever quality factor ($Q_n$) and the low-frequency viscoelastic moduli ($E'$ and $E''$). Overall, the data in Figures 9.2 and 9.3 and Table 9.1 show that resonance in PEMC sensors persists in gelatin, alginate, and PEGDA hydrogels across a range of concentrations that have been used extensively in various applications.\cite{49, 50} This suggests that PEMC sensors could be used to characterize the viscoelastic properties of hydrogels based on calibration approaches that involve low-frequency DMA measurements as well as potentially enable real-time sensing of hydrogel viscoelastic property changes by continuous tracking of cantilever resonant frequency, phase angle, and quality factor.

**Table 9.1** Comparison among total changes in sensor signals resulting from sol-gel phase transition of gel-forming polymer solutions with the viscoelastic properties of the resultant hydrogel acquired using low-frequency DMA studies.

<table>
<thead>
<tr>
<th>Sample</th>
<th>PEMC (≈35 kHz)</th>
<th>DMA (1 Hz)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\Delta f$ [%]</td>
<td>$\Delta \phi$ [%]</td>
<td>$\Delta Q$ [%]</td>
</tr>
<tr>
<td>6 wt% gelatin</td>
<td>1.2 ± 0.8</td>
<td>-0.1 ± 0.1</td>
<td>-5.1 ± 0.1</td>
</tr>
<tr>
<td>8 wt% gelatin</td>
<td>1.6 ± 0.3</td>
<td>-0.1 ± 0.03</td>
<td>-10.8 ± 2.4</td>
</tr>
<tr>
<td>10 wt% gelatin</td>
<td>1.5 ± 0.1</td>
<td>-0.2 ± 0.1</td>
<td>-18.4 ± 5.0</td>
</tr>
<tr>
<td>0.25 wt% alginate</td>
<td>-0.06 ± 0.01</td>
<td>-0.3 ± 0.1</td>
<td>-9.1 ± 1.4</td>
</tr>
<tr>
<td>0.5 wt% alginate</td>
<td>-0.5 ± 0.3</td>
<td>-0.6 ± 0.1</td>
<td>-22.6 ± 3.7</td>
</tr>
</tbody>
</table>
9.4.3 Real-time Monitoring of Gelation Processes using Millimeter Cantilever Sensors

Having demonstrated that resonance in PEMC sensors persists in hydrogels formed through differing chemistry and that changes in sensor quality factor correlated with low-frequency viscoelastic moduli, it was next of interest to determine if PEMC sensors enable real-time monitoring of sol-gel phase transitions via continuous tracking of sensor signals. Gelatin solutions undergo a thermoreversible sol-gel phase transition at room temperature without the addition of a curing stimulus, resulting in a gel.[51] As shown in Figures 9.4a-c, gelation of gelatin solutions caused a continuous change in the resonant frequency, phase angle, and quality factor at 6, 8, and 10 wt% over 1,800 s (30 minutes), which is relatively consistent with previously reported gelation times[52] (data shown for 8 wt%). At all concentrations examined (6, 8, and 10 wt%), the resonant frequency underwent an immediate increase before stabilizing after approximately \( t = 1,100 \) s. As shown in Table 9.1 and Figure 9.4a for the case of 8 wt%, gelation of 6, 8, and 10 wt% gelatin solutions caused resonant frequency increases of 1.2 ± 0.8, 1.6 ± 0.3, and 1.5 ± 0.1% (\( n = 3 \) studies). In contrast to the resonant frequency increase, which was an immediate effect, both the phase angle and quality factor remained relatively stable for the first 400 s, before ultimately decreasing exponentially until stabilizing at \( t = 1,270 \) and 960 s, respectively, defining the stabilization time as that at which 95% of the final change in sensor signal had been reached. As shown in Figures 9.4d-f and Table 1, the sensor signals changes upon gelation correlated with the
concentration and low-frequency viscoelastic moduli ($E'$ and $E''$), of the surrounding hydrogel. As shown in Figure 9.4d, the resonant frequency increased by approximately 1 to 1.5% upon gelation at all concentrations. The phase angle decreased by 0.1 ± 0.03 and 0.2 ± 0.1% at 8 and 10 wt% gelatin, respectively (a significant change in phase angle upon gelation of 6 wt% gelatin solutions was not observed) (see Figure 9.4e). As shown in Figure 9.4f, the quality factor decreased by 5.1 ± 0.1, 10.8 ± 2.4, and 18.4 ± 5.6% at 6, 8, and 10 wt% gelatin, respectively.
Figure 9.4 Sensor resonant frequency (a), phase angle (b), and quality factor (c) responses associated with thermoreversible gelation of gelatin solutions (data shown for 8 wt% gelatin solutions). Total changes in sensor frequency (d), phase angle (e), and quality factor (f) upon gelation of 6, 8, and 10 wt% gelatin solutions ($n = 3$ repeated studies per concentration). Sensor resonant frequency (g), phase angle (h), and quality factor (i) responses associated with chemical gelation of alginate solutions (data shown for 0.25 wt% alginate solutions). Total changes in sensor frequency (j), phase angle (k), and quality factor (l) upon gelation of 0.25, 0.5, and 0.75 wt% alginate solutions ($n = 3$ repeats per concentration). Sensor time
series data (i.e., panels a-c and g-i) is presented using a 5-point moving median filtering (red line indicates a 25-point moving average).

Similar to the thermoreversible gelation of gelatin solutions, the chemical gelation of alginate solutions caused exponential decreases in various sensor signals (see Figures 9.4g-i). For example, the phase angle and quality factor changed exponentially at all concentrations examined (0.25, 0.5, and 0.75 wt%; see Figures 9.4h-i). The chemical gelation of 0.5 wt% alginate solutions did not cause a significant change in the resonant frequency, but led to an increase in the sensor noise level (see Figure 9.4g). A summary of the resonant frequency, phase angle, and quality factor changes caused by the chemical gelation of alginate solutions are summarized in Figures 9.4j-l. Interestingly, while only the quality factor changes correlated with the hydrogel concentration and low-frequency storage modulus across the 6 - 10 wt% concentration range for the gelatin system (see Figures 9.4d-f), both phase angle and quality factor changes correlated with the hydrogel concentration and low-frequency storage modulus across the 0.25 - 0.75 wt% concentration range for the alginate system. For example, the phase angle decreased by 0.3 ± 0.1, 0.6 ± 0.1 and 0.9 ± 0.1% as a result of hydrogel crosslinking for 0.25, 0.5, and 0.75 wt% alginate, respectively (see Figure 9.4h). As shown in Figure 9.4i, the quality factor decreased by 9.1 ± 1.4, 22.6 ± 3.7, and 34.2 ± 4.3% during the crosslinking process for 0.25, 0.5, and 0.75 wt% alginate, respectively. The total resonant frequency changes were not significant relative to the signal noise level at all concentrations.

These collective results from the gelatin and alginate hydrogel systems show that in addition to enabling real-time monitoring of the gelation process using resonant frequency,
phase angle, and quality factor responses, the sensor responses (specifically, the phase angle and quality factor) enabled the quantification of hydrogel polymer content (i.e., concentration) and low-frequency viscoelastic properties across a wide dynamic range through a calibration approach (i.e., a set of offline DMA measurements taken on experimental standards). While not the focus of this study, sensor data associated with real-time monitoring of photo-gelation processes is provided in Figure S9.2 of Supplementary Information for the PEGDA system. The correlations between phase angle and quality factor changes with hydrogel composition and low-frequency viscoelastic properties found in the gelatin, alginate, and PEGDA hydrogels suggest that millimeter cantilever sensors provide useful signals for real-time monitoring of gelation processes and rheological characterization of sol-gel systems.

9.4.4 Quantification of Hydrogel High-frequency Viscoelastic Moduli using Sensor Data

Having established a sensor-based approach for characterization of sol-gel phase transitions and low-frequency hydrogel viscoelastic properties based on benchmarking (i.e., calibration) of total changes in sensor signals with offline DMA data acquired using traditional techniques, we next used a cantilever fluid-structure interaction model for viscoelastic materials (Equations (1) – (4)) to examine the behavior of the high-frequency storage and loss moduli obtained at the resonant frequency ($G'_r$ and $G''_r$, respectively) and understand their correlation with low-frequency moduli ($E'$ and $E''$). In other words, it was our goal to understand the relationship between low-frequency (1 Hz) viscoelastic moduli obtained using traditional characterization platforms (e.g., DMA) and high-frequency viscoelastic moduli (~35 kHz) obtained using PEMC sensors. Figures 9.5a and b show the
representative trends in $G'_f$ and $G''_f$ during gelation for both the gelatin and alginate systems (data shown for 8 and 0.5 wt%, respectively). Both $G'_f$ and $G''_f$ increased throughout the gelation process, as was observed with low-frequency viscoelastic moduli.\textsuperscript{53} However, there was not a crossover point between $G'_f$ and $G''_f$, which is typically associated with low-frequency gelation rheology and has been previously reported for gelation of gelatin\textsuperscript{54} and alginate hydrogels\textsuperscript{53}. Regarding the relative magnitudes of $G'_f$ and $G''_f$, it is not unreasonable for $G'_f$ to be greater than $G''_f$ at high frequencies, even in the solution phase due to the relatively slow relaxation time of long biopolymer solutions.\textsuperscript{55}
Figure 9.5 Real-time monitoring of high-frequency shear moduli at the resonant frequency (~35 kHz) based on sensor resonant frequency and quality factor responses using the cantilever viscoelastic material-structure interaction model throughout gelation of 8 wt% gelatin (a) and 0.5 wt% alginate (b) solutions (green and blue lines show 25-point moving averages associated with the storage and loss moduli response, respectively). High-
frequency shear moduli obtained at the resonant frequency of hydrogels formed from 6, 8, and 10 wt% gelatin (c) and 0.25, 0.5, and 0.75 wt% alginate (d) solutions (n = 3 experiments for each concentration). Sensor transfer functions associated with quality factor (Q) change vs. $G'_f$ and $E'$ with linear regressions shown (panels e and f, respectively).

As shown in Figure 9.5c, the shear storage moduli of gelatin hydrogels at the resonant frequency ($G'_f$) were $15 \pm 8$, $25 \pm 0.4$, and $31 \pm 7$ kPa and the shear loss moduli ($G''_f$) were $14 \pm 4$, $20 \pm 0.7$, and $28 \pm 4$ kPa for 6, 8, and 10 wt%, respectively. These values are of the same order of magnitude as previously reported values obtained using traditional low-frequency rheological techniques (0.1 – 10 Hz).[56] For the case of alginate hydrogels, $G'_f$ were $47 \pm 3$, $75 \pm 2$, and $80 \pm 3$ kPa and $G''_f$ were $35 \pm 3$, $62 \pm 9$, and $77 \pm 3$ kPa for 0.25, 0.5, and 0.75 wt%, respectively (see Figure 5d). Similar to the characterization of gelatin hydrogels, these values are of the same order of magnitude as previously reported values obtained using traditional low-frequency rheological techniques.[57] The fact that $G'_f$ and $G''_f$ obtained from sensor data were larger than $E'$ and $E''$ obtained from DMA is consistent with the frequency response of dynamic moduli, which typically increase with increasing frequency.[24] We note that while storage modulus of a stable system increases with frequency, this is not the case for loss modulus. Thus, the relative increase in moduli is not unexpected when considering the magnitude of the resonant frequency of the sensor (~35 kHz; see Figure 2). The limit of detection for changes of $G'_f$ in gelatin and alginate hydrogels based on sensor quality factor response was 13.2 and 11.4 kPa, respectively (see the associated $Q$-$G'_f$ sensor transfer function in Figure 9.5e). The limit of detection for changes of $E'$ in gelatin and alginate hydrogels based on sensor quality factor response was 1.9 and 7.1 kPa (see Table 9.1 and Figure 9.5f). A discussion of detection limit calculations is provided in Supporting
Information. As shown in Figures 9.6a and b, the shear moduli obtained at resonance ($G'_f$ and $G''_f$) exhibited a positive correlation with the low-frequency storage modulus ($E'$) acquired with DMA. While $G''_f$ exhibited a positive correlation with for alginate hydrogels, the gelatin hydrogel system exhibited limited correlation. This relationship is likely dependent on the hydrogel’s Poisson’s ratio and material property frequency dependence.\cite{24} These results suggest that real-time monitoring of high-frequency viscoelastic moduli using sensor-based approaches provides a promising technique for characterization of gelation dynamics and quantification of hydrogel viscoelastic properties over a wide frequency range (Hz – kHz).
Figure 9. 6 Comparison of storage (a) and loss (b) moduli at the resonant frequency obtained from sensor data with low-frequency viscoelastic moduli obtained via traditional DMA of gelatin and alginate hydrogels (error bars represent the standard deviation of $n = 3$ repeated experiments). c) Comparison of temporal responses of the high- and low-frequency
shear moduli obtained from sensor data and traditional rheology (1 Hz), respectively, through the thermoreversible gelation of 8 wt% gelatin solutions.

In addition to correlation between high- and low-frequency viscoelastic properties in cured hydrogels obtained using sensor-based approaches and traditional platforms, we also found that the high- and low-frequency shear moduli exhibited similar temporal responses throughout the gelation process (see Figure 9.6c). The time at which the temperature of the gelatin solution reached room temperature after quenching from the solution phase at 40 °C was taken as the time $t = 0$ in study. The time scales were normalized by the time at which $G'$ reached 95% of the total change. It should be noted that the traditional rheology data exhibits a crossover in $G'$ and $G''$ at $t = 0.08$, while the $G'$ was greater than $G''$ for the duration of the experiment. While there is no crossover point to indicate a specific gelation time in the PEMC data, it is apparent that the increase in moduli occurred at a relatively later time than in the traditional rheology data. This could be due to the documented effect of increasing shear rate slowing gelatin gelation.[58] As shown by de Carvalho et al., increasing the shear rate from 1 to 1000 Hz not only delayed the onset of moduli increase (often referred to as phase II of gelation) but also depressed the slope of the increase in moduli,[58] similar to the data collected using the PEMC sensors. Strain magnitude may also contribute to changes in gelation processes relative to those occurring in the presence of static solid boundaries. For reference, the PEMC sensor data shown here were collected at the cantilever resonant frequency.
9.4.5 Modeling of Gelation Process Dynamics using Sensor Data

Given the previous sections establish that PEMC sensors enable characterization of low- and high-frequency viscoelastic properties and real-time monitoring of gelation processes, we next examined if sensor data could be leveraged to model the dynamics of gelation processes. Sensor responses to chemical gelation of alginate solutions were analyzed using a modified Hill equation based on its previous use in modeling of gelation processes that were characterized using traditional rheological techniques and is given as:\textsuperscript{[59, 60]}

\[ \hat{G}'(t) = \frac{t^n}{t^n + \theta^n} \tag{5} \]

where \( \hat{G}' \) is the normalized storage modulus, \( t \) is time, \( n \) is the Hill coefficient, and \( \theta \) is the half-gelation time determined by the time at which \( G' \), and thus, \( \hat{G}' \), has reached 50% of the total change. The modified Hill equation can also be used to calculate a characteristic gelation rate (\( P \)) as:

\[ P = \frac{nG'_\text{gel}}{4\theta} \tag{6} \]

where \( G'_\text{gel} \) is the storage modulus of the final gel. As shown in Figure 9.7a, the modified Hill model exhibited a reasonable fit to the sensor data (shown for chemical gelation of 0.75 wt% alginate solutions). Additional analyses of 0.25, 0.5, and 0.75 wt% alginate hydrogels are shown in Figure S9.3 of Supplementary Information. The initial time point \( (t = 0) \) was taken as the time at which the crosslinking agent was applied to the alginate solution. The dependence of the half-gelation time on hydrogel concentration is shown in Figure 9.7b. Chemical gelation of the 0.25 wt% alginate solution exhibited the longest half-gelation time.
of 210 ± 11 s compared to 93 ± 20 and 104 ± 27 s for 0.5 and 0.75 wt% alginate, respectively. There was no significant difference in the half-gelation time between 0.5 and 0.75 wt% alginate. As shown in Figure 9.7c, the Hill coefficient and the characteristic gelation rate increased with increasing alginate concentration. The characteristic gelation rates were 564 ± 114, 1844 ± 934, and 3516 ± 944 Pa/s for 0.25, 0.5, and 0.75 wt% alginate, respectively. The characteristic gelation rate obtained via sensor responses during alginate gelation was higher than previously reported values.\textsuperscript{[61, 62]} Junior et al. found a characteristic rate of $P = 46.8$ Pa/s for the chemical gelation of 2 wt% alginate hydrogels.\textsuperscript{[62]} The significant increase in the $P$ values extracted here may be largely attributed to the significantly higher final storage modulus measured using resonant PEMC sensors in this study. For example, multiplying the characteristic rate determined using sensor data for 0.75 wt% alginate ($P = 3516$ Pa/s) by the ratio of the low-frequency modulus measured by Junior et al. to the high-frequency modulus measured here ($G'/G'_f = 0.92$ kPa/80 kPa) yields a calibrated characteristic rate of 40.4 Pa/s, which agrees well with the results of previous studies.\textsuperscript{[62]} These results indicate that in addition to providing quantitative characterization of hydrogel viscoelastic moduli and real-time monitoring of gelation processes, the sensor responses associated with gelation processes can be used for quantitative characterization of gelation process dynamics.
Figure 9. 7 a) Fit of a modified Hill model to normalized sensor-derived storage modulus responses associated with the chemical gelatin of alginate solutions (sensor data presented as a 5-point moving average). Dependence of half-gelation time, $\theta$ (b) and Hill coefficient, $n$ (c) for 0.25, and characteristic rate, $P$ (d) for chemical gelation 0.25, 0.5, and 0.75 wt% alginate solutions (error bars represent the standard deviation for $n = 3$ repeated studies).

9.4.6 Real-time Monitoring of Hydrogel Dissolution Processes

To further evaluate the utility of the sensor for future applications in viscoelastic characterization of hydrogels and continuous monitoring of gelation processes, we next examined the real-time monitoring of hydrogel dissolution processes. As shown in Figure
9.8, the application of 3 mL of 1M EDTA chelating solution following chemical gelation of alginate solutions led to a recovery in the phase angle and quality factor, which is consistent with hydrogel dissolution. EDTA is a well-established chelating agent that is capable of dissolving alginate hydrogels based on its affinity for calcium cations that cause the chemical gelation of alginate solutions.\textsuperscript{[63]} Interestingly, the application of the dissolving agent resulted in a decrease in resonant frequency (see Figure 9.8a; \( n = 3 \) repeated studies), which could be attributed to a mass-change response associated with EDTA uptake by the surrounding material. Previous studies that examined the adsorption of metal-EDTA complexes on various surfaces also suggest that EDTA and calcium-EDTA complexes may also adsorb to the sensor surface.\textsuperscript{[64]} While the phase angle returned to the original value after the dissolution process (see Figure 9.8b), the quality factor did not fully recover (see Figure 9.8c), which may be attributed to a mass-damping effect associated with the observed resonant frequency decreases or differences in the rheological properties of the initial and final solutions. These results support the fact that cantilever sensors can assess a range of sol-gel transition processes, which are of use for characterizing hydrogels across a wide range of applications. The observed change in resonant frequency also suggests that considerations of chemical binding to the sensors during rheological studies, such as binding of polymer or crosslinking agents, may be important for accurate quantification of rheological properties based on sensor data.
Figure 9.8 Sensor resonant frequency (a), phase angle (b), and quality factor (c) responses corresponding to the dissolution of alginate hydrogels. Alginate hydrogels formed by chemical gelation of alginate solutions using saturated CaCl$_2$ (applied at 400 s) were dissolved by application of a dissolving agent (1 M EDTA solution; applied at 700 s; sensor responses presented as a 5-point moving median).

9.5. Conclusions

In this paper, we report that resonance in cantilever sensors persists in hydrogels. This result was shown to enable the characterization of low- and high-frequency hydrogel viscoelastic properties and the real-time monitoring of sol-gel phase transitions (i.e., gelation processes). Studies were performed on various hydrogel systems that underwent thermoreversible-, chemical-, and photo-gelation processes. Changes in the sensor phase angle, quality factor, and high-frequency shear moduli obtained at the resonant frequency ($G'_f$ and $G''_f$) correlated with low-frequency moduli obtained using traditional DMA and rheology platforms. These results suggest that real-time monitoring of high-frequency viscoelastic moduli using sensor-based approaches provides a promising technique for characterization of gelation dynamics and quantification of viscoelastic properties of hydrogels over a wide frequency range (Hz – kHz). This work also suggests that cantilever sensors could provide a promising platform for sensor-based characterization of hydrogels.
that may lead to future breakthroughs in process control and high-throughput characterization. In addition, resonance and quality factor tracking in millimeter-scale cantilever sensors appears to provide an attractive integrated characterization and bioanalytical platform for gel-based biomanufactured products, such as molded or 3D bioprinted hydrogel-based tissues, via real-time detection of rheological property changes, chemical sensing, and bio-sensing.
9.6 References


9.7 Supplementary Information

This supporting information contains: impedance spectra associated with the phase angle data shown in Figure 2 of the main text, data associated with real-time monitoring of PEGDA photo-gelation processes, discussion of limit of detection analysis, and results associated with modified Hill model fitting to experimental data for all alginate concentrations examined.
Figure S9.1 Impedance magnitude spectra in air, solution phase, and gel phase for 6 wt% gelatin (a), 0.25 wt% alginate (b), and 10 wt% PEGDA (c).
Figure S9.2 Real-time monitoring of sensor signals during UV curing of a 10 wt% PEGDA shown in terms of the resonant frequency (a), phase angle (b), and quality factor (c) responses. Exposure to a negative control (light on with UV blocked) at 200 s and exposure to UV light begins at 600 s.
**Determination of Limit of Detection**

Given that the limit-of-detection (LOD) is a function of the sensitivity (m) and noise level of the sensor (N), one can use Figure 9.5 to estimate the sensor’s LOD. The following expression is commonly used to quantify LOD and assumes a signal of a factor of 3.3 times the noise level provides the ability to resolve an associated change in stimulus with sufficient measurement confidence:

\[
LOD = \frac{3.3 \times N}{m}
\]  

(1)

Sensitivity of the sensor for rheological changes were shown in Figure 9.5. Time series data from the main text shows that the noise level associated with the quality factor response for signals obtained in alginate and gelatin hydrogels was \( N = 3.195 \) and \( 2.269 \), respectively (we remind the reader that the quality factor is a dimensionless quantity).
Figure S9. 3 Predicted gelation kinetic for chemical gelation of 0.25, 0.5, and 0.75 wt% alginate solutions based on the modified Hill modeling of associated sensor responses ($n = 3$ experiments for each concentration).
Chapter Ten: Conclusions

10.1. Conclusions

This thesis described research and development of process, material, and sensing capabilities for neural microphysiological systems with applications to disease modeling, drug discovery, and fundamental organ science. Materials were formulated and synthesized to tune both the rheological properties for 3D printing and the mechanical and biological properties for neural tissue culture. 3D bioprinting processes were improved through augmentation with pick-and-place capabilities and mixing manifolds to integrate prefabricated components and control spatial concentration of dilute species. Sensing capabilities to quantify biological quality parameters such as cell viability and type during extrusion, and for characterizing viscoelastic properties both during manufacturing and long-term cell culture were described. The specific conclusions of this thesis are:

10.1. 3D printing enabled controlled distribution of plasmonic nanoparticles in hydrogel constructs

3D printing enabled the fabrication of novel functionally graded 3D plasmonic constructs with distinct classes of graded material properties through the additive assembly and dynamic mixing of multiple plasmonic inks. Flexible utility with both crosslinking and non-crosslinking systems was successfully realized. This work opens the door for creating novel plasmonic materials and 3D printed functional materials as well as paves the way for new applications of plasmonic materials and devices such as plasmonic sensor functionalized microphysiological systems.
10.1.2. Controlled drug release could be programmed through the tool path of a 3D printer

A systematic study of the effect of programmed concentration profile on temporal release profile in Pluronic F-127 pills was reported. Programmed concentration profiles of varying spatial distribution were examined in this work, including core-shell, multilayer, and gradient structures. A combination of experimental studies and finite element models revealed programmed concentration profiles in 3D printed pills are dynamic throughout both the 3D printing (processing) and solidification (post-processing) intervals. A novel finite element model revealed that the experimentally observed dynamics can be described by the diffusion of active species within the pill under driving forces imposed by the concentration gradient associated with a given spatial distribution. Temporal release studies showed that programmed concentration profiles with core-shell, multilayer, and gradient structures can be used to produce delayed, pulsed, and constant temporal release profiles, respectively.

10.1.3. Pick and place capabilities enabled seamless integration of prefabricated components into 3D printed constructs

Additive manufacturing processes composed of robotic pick-and-place functionality with multi-material 3D printing enabled the fabrication of microfluidic-based 3D MEMS devices. Piezoelectric transducers were embedded with 3D configurations consisting of orthogonal in-plane and out-of-plane configurations using a combination of vacuum- and adhesion-based embedding techniques, and these transducers exhibited multiple resonant modes over the 0 – 20 MHz frequency range. Additive manufacturing was also used to interweave microchannels between the 3D transducer configurations.
10.1.4. Hybrid bioinks containing synthetic polaxamers and natural macromolecules provided the properties needed for 3D printing and neural tissue culture

Novel biomimetic bioinks for 3D bioprinting of free-standing soft neural tissues were created based on the combination of natural polymers for ECM biomimicry (gelatin and hyaluronic acid) with a synthetic polymer (Pluronic F-127) that endows desirable rheological properties for 3D bioprinting. Slight crosslinking based on thiol – catechol chemistry resulted in thermally gelling inks with Herschel-Bulkley type rheological behavior and enabled the fabrication of soft, free-standing neural tissues. The bioinks could be cured through chelating and photocuring pathways, resulting in hydrogels with similar moduli to native neural tissue ECM. High viability was observed among Schwann, glioma, and neuronal cells over seven days in culture.

10.1.5. A 3D printed assay for analysis of chemotactic response to multiple attractant sources provided insight into glioblastoma migration toward vasculature

A user-friendly, multiplexed migration assay enabled a study of the effect of spatially-opposing gradients of the peptide bradykinin (BK) on the migration response of glioblastoma cells toward epidermal growth factor (EGF) sources was reported. Competitive migration assays involving spatially-opposing gradients of EGF and BK at their respective optimal levels determined from single chemoattractant assays showed that glioblastoma cells exhibited a relatively increased migration velocity and extent of migration toward EGF sources. However, a substantial fraction of cells migrated toward the BK source, which was the relatively ‘weaker’ chemoattractant. The migration velocities toward spatially-
distributed sources in the presence of spatially-opposing EGF and BK gradients were reduced relative to those found in the absence of the competing chemoattractant.

10.1.6. Multifunctional hollow fibers provided in-line impedimetric sensing for real-time quantification of bioink quality during 3D bioprinting

Monitoring of fiber electrical impedance was shown to enable sensing of bioink compositional characteristics, such as cell viability, type, and extent of stem cell differentiation. This work shows that microextrusion 3D bioprinting using hollow multifunctional fiber sensors provides a potential path to improving process monitoring capability of 3D bioprinting processes, particularly regarding in-line monitoring of deposited cell viability, type, stemness, and extent of differentiation. This work also suggests that fiber-based sensors may provide useful platforms for controlled delivery of cell therapies, such as injectable stem cell therapies.

10.1.7. Dynamic mode milli-scale piezoelectric cantilevers enabled real-time characterization of viscoelastic properties of polymer solutions and gels

Cantilever resonance was found to persist in the gel phase. This result was shown to enable the characterization of low- and high-frequency hydrogel viscoelastic properties and the real-time monitoring of sol-gel phase transitions. Changes in the sensor phase angle, quality factor, and high-frequency shear moduli obtained at the resonant frequency ($G'$ and $G''$) correlated with low-frequency moduli obtained using traditional DMA and rheology platforms. Resonance and quality factor tracking in millimeter-scale cantilever sensors appears to provide an attractive integrated characterization platform for gel-based biomanufactured products, such as molded or 3D bioprinted hydrogel-based tissues.