Published in final edited form as:

Adv Funct Mater. 2021 April 1; 31(14): . doi:10.1002/adfm.202010246.

3D Particle Free Printing of Biocompatible Conductive Hydrogel Platforms for Neuron Growth and Electrophysiological Recording

Chen Wang,

Frederick Seitz Materials Research Laboratory and Department of Materials Science and Engineering, University of Illinois at Urbana-Champaign, 1304 West Green Street, Urbana, IL 61801, USA

Stanislav S. Rubakhin,

Beckman Institute, MC-251, University of Illinois, Urbana, Illinois 61801, USA

Michael J. Enright,

Department of Chemistry, University of Illinois at Urbana Champaign, 600 South Mathews Avenue, Urbana, IL 61801

Jonathan V. Sweedler,

Department of Chemistry, University of Illinois, 71 RAL, Box 63-5, 600 South Mathews Avenue, Urbana, IL 61801

Ralph G. Nuzzo

Department of Chemistry, University of Illinois at Urbana Champaign, 600 South Mathews Avenue, Urbana, IL 61801

Abstract

Electrically conductive 3D periodic microscallops are fabricated using a particle-free direct ink writing approach for use as neuronal growth and electrophysiological recording platforms. A poly (2-hydroxyethyl methacrylate) (pHEMA)/pyrrole ink, followed by chemical *in situ* polymerization of pyrrole, enables hydrogel printing through nozzles as small as 1 μm . These conductive hydrogels can pattern complex 2D and 3D structures and have good biocompatibility with test cell cultures (~94.5% viability after 7 days). Hydrogel arrays promote extensive neurite outgrowth of cultured *Aplysia californica* pedal ganglion neurons. This platform allows extracellular electrophysiological recording of steady-state and stimulated electrical neuronal activities. In summation, this 3D conductive ink printing process enables preparation of biocompatible and micron-sized structures to create customized *in vitro* electrophysiological recording platforms.

Keywords

3D printing; conductive hydrogel; microfabricated neuron recording; *Aplysia californica*

1. Introduction

Ever since the groundbreaking experiments on bioelectricity by Galvani in the 18th century, [1] there has existed an enduring fascination with the roles of electric charges in living tissues. Research in this area has helped promote understandings of a variety of phenomena including the working mechanisms of the brain and development of new tools for disease diagnosis and therapy. Bioelectronics provide important tools and capabilities for such investigations and forms a bridge between the generally hard, rigid world of electronic materials and the soft, flexible forms of matter that comprise biological systems. From classic blood glucose biosensors and cardiac pacemakers to modern brain-machine interfaces and deep-brain stimulators, [2–4] the rapidly evolving field of bioelectronics has led to progress in fabricating important new classes of biomedical devices.

Despite remarkable achievements over the past decades, the physiochemical and geometric mismatches of man-made, hard electronics to soft tissues and cells have posed significant restrictions for development and application of bioelectronic tools. Specifically, development of high-quality electrical interfaces allowing monitoring of different signals in biological systems remains challenging. Current technologies rely mostly on inorganic materials (e.g. metals or silicon), [5–8] but property mismatches (e.g. chemically inorganic vs. organic, mechanically high vs. low Young's modulus, and physiochemically dry vs. water-rich) lead to compatibility challenges at electronic-tissue interfaces and have greatly hindered development and application of bio-integrated technologies. Existing conventional metal and silicon brain electrodes evoke immune responses that lead to scar formation and limited probe life. [9] Hydrogels have been proposed as an alternative platform for such interfaces. Their biological similarities to tissues in concert with tunable electronic and mechanical functionalities of hydrogel materials provide opportunities for seamless integration of electronic devices and functional biological structures. [10] A number of successful applications have been reported in the past decades including the formation of biocompatible microenvironments, delivery of chemical and electrical stimuli, and recordings of cellular activities. [11–13]

Hydrogels have broad bioelectronics applications. The simplest examples involve coating hard devices with soft hydrogels to improve biocompatibility and minimize interfacial impedance for better transmission of high quality signals in electrophysiological systems. [9,14] Due to inferior electrical properties compared to conventional metal conductors, hydrogel coatings have been limited merely to enhancing surface characteristics rather than enabling more advanced use as stimulation and recording electrodes. To improve electrical conductivity issues without losing biocompatibility, hydrogels with carbon-based nanocomposites have been used successfully to impart electrical conductivity into biomaterial supports that would otherwise be nonconductive. [15,16] It has been shown, however, that inhomogeneous distributions of phases within the nanocomposites can lead to unstable and low mechanical and electrical performances. [17] The biocompatibility of some CNT-based materials have also been questioned since relatively few studies have explored the toxicity of CNT technologies and their results have often been inconclusive or contradictory. [18] While pristine and chemically functionalized CNTs for drug delivery have demonstrated minimal or no cytotoxicity, unrefined CNTs have been shown to have some

degree of toxicity attributed to residual transition metal catalysts. As a result, conductive composites formed using templated hydrogel matrices and intrinsically conductive polymers, such as polypyrrole, polyaniline, and poly (3,4-ethylenedioxythiophene), have been explored as viable alternatives capable of translating between bio- and electronic signals.^[19–21] These hydrogels have found applications as neural probes, chemo sensing devices, drug delivery systems, and have shown early promise for demanding applications in spinal cord repair.^[22–25]

Unfortunately, the microfabrication of complex 3D structures with high spatial resolution using such conductive hydrogels remains a crucial challenge. Hydrogel patterns exhibiting subcellular spatial resolution are a critical prerequisite to allowing single neuron activity discrimination and reliable signal recording from multiple cells.^[26] Signal quality can also be improved by forming better seals between cell membranes and 3D cellular size electrodes, which neurons can engulf to minimize leakage currents.^[27,28] Spatial, physiochemical, and biocompatibility requirements for single cell analysis platforms necessitate further development of approaches to attain complex conductive hydrogel-based 3D-integrated structures. Several methods, including jet printing, stereolithography, electrospinning, and micromolding, have been used to pattern conductive hydrogels.^[29–32] These methods, however, suffer from limitations in 3D microstructural assembly, suitable material options, and spatial resolution during fabrication.^[29,32–34] To address these challenges, direct ink writing, an extrusion-based 3D printing method, has been developed as a powerful and complimentary tool to programmatically pattern complicated 3D microstructures using viscoelastic inks of diverse compositional form.

Here, we demonstrate the use of a new type of particle-free ink suitable for printing biocompatible, electrically conductive 3D hydrogel architectures that can be directly used as neuronal scaffolds and signal recording devices. Individual electrodes form functional junctions at cellular and subcellular dimensions. A new type of 3D printable ink is formed by dissolving physically entangled pHEMA chains in a mixture of pyrrole monomer and solvent. Subsequent post-printing chemical treatment with ferric chloride gives conductive and durable scaffolds. Compared to previously reported conductive 3D printable inks, the current ink affords multifunctional composite structures that are more biocompatible (vide supra) and enables printing through much narrower nozzles to give higher resolution features. This suggests a scope of applicability that includes integration with cells and tissues. These prospects are demonstrated below in an exemplary form in a model study of electrophysiological recording of cultures of excised bag cell neurons from *Aplysia*. As we demonstrate there, the particle-free ink formulation facilitates the printing of functional structures through nozzles as small as 1 μm , comparing well to the several hundred micron limiting feature sizes reported for other DIW inks^[35,36] Using this new ink, we have successfully fabricated and characterized several 3D microscale platforms that allow direct investigations of single-cell properties, including the electrophysiological parameters of individually cultured *Aplysia californica* neurons.

2. Results and Discussion

2.1 Printing and characterization of conductive hydrogels

The new ink for direct ink writing was designed to enable fabrication of electrophysiological gel platforms with feature dimensions on the cellular size scale. This printable material is comprised of a physically entangled polymer network of pHEMA chains of various molecular weights with pyrrole comonomers in an aqueous ethanol solution. This composition and the fabrication sequence to obtain electrically conductive gel scaffolds is illustrated schematically in Figure 1. The pHEMA homopolymer content is optimized to meet both the rheological requirements for direct ink writing and functional gel composites suitable for studies of cellular cultures. The polymer network design confines pyrrole monomer within the hydrogel matrix during printing and subsequent treatment with ferric chloride solutions induces *in-situ* polymerization and doping with the resulting interpenetrating polypyrrole. Polymerization and doping are visibly observable as the printed hydrogel evolves from colorless and transparent to a deep black within minutes after treatment. The printed patterns were rinsed extensively and subsequently immersed in deionized water with four water exchanges every 4 hours to remove unreacted monomer species. Since this microfabrication process separates ink printing from *in situ* conductive particle formation, micron-scale feature widths are attainable. Complex 3D structure formation is enabled by facile, non-clogging extrusion of conductive filaments (as illustrated in Figure 2). This process enables printing of features as small as 1 μm and is as good or better than other state-of-the-art hydrogel printing processes.^[37] Pre-polymerization of the pyrrole within the ink gives a material that is unprintable, being such even with large 100 μm inner diameter tips (shown in Figure S1). In comparison, the particle-free composite ink is easily extruded from much smaller tips, yielding features narrow enough to prepare high performance electrophysiological recording platforms. As shown below, devices require features with limiting design rules of less than 50 μm , which cannot be printed using a pre-polymerized composite ink counterpart.

Rheological properties are important factors that influence 3D microstructure printing. Here, the rheological functions of the ink and examples of printed structures are illustrated in Figure 2. The hydrogel ink was in its hydrated state during rheological characterization and a solvent trap with water and ethanol was used to prevent evaporation during measurement. The elastic and viscous moduli of the pHEMA-pyrrole hydrogel ink was optimized to enable ink filaments to span gaps in underlying layers while simultaneously retaining their shape. Figure 2a shows the linear viscoelastic moduli of the optimized ink with a plateau storage modulus (G') of ~ 340 Pa and loss modulus (G'') of ~ 600 Pa under 1 Hz oscillatory shear. The measured G'' value exceeded G' over all experimentally measured shear stress ranges and the hydrogel ink shows a liquid-like response. The pHEMA-pyrrole ink also shows a low zero-shear viscosity, η_0 , of approximately 238 Pa·s and exhibits shear-thinning behavior above the critical shear rate of 10^{-1} s^{-1} (Figure 2b). This is due to the alignment of physically entangled pHEMA polymer chains in response to applied shear stress. In the shear-thinning regime, the ink viscosity can be fitted to a power law, $\eta \dot{\gamma}^{n-1}$, yielding a shear-thinning exponent, n , of 0.48 and is comparable to other pHEMA-based inks developed previously.^[38,39] Consequently, printed patterns have better shape retention

before post-printing polymerization due to their liquid-like response and shear-thinning behavior. During printing, solvent drying at the nozzle tip could slightly enhance the ink elasticity. Since the ink is a homogeneous particle-free hydrogel, tip clogging during extrusion was not an issue and printing micron scale features using tips smaller than 100 μm was found to be both feasible and facile. The exemplary results achieved for printing of hydrogel filaments through a 1 μm diameter nozzle are shown in Figure 2c. Due to die-swelling effects during printing, the polymerized filament has a slightly larger final diameter than the nozzle size, 2.5 μm , but the ultimate spatial resolution achieved remains superior to most particle-based conductive inks described in the literature.^[40,41] Multi-walled carbon nanotube-based inks, for example, can cause clogging in narrow nozzles due to the formation of large bundles.^[42] With the current material, we found it possible to fabricate complex 2D and 3D features that include: multilayer hydrogel arrays created using a 100 μm nozzle (Figure S2); a 2D “Illinois” pattern written using a 200 μm nozzle (Figure S3); and a larger area 3D pyramid structure printed using a 400 μm nozzle (Figure 2d). Taken together, the data suggest considerable versatility for fabricating composite pHEMA-polypyrrole (pPyrrole) structures with feature sizes around 1 μm and above.

The compositional attributes of the hydrogel thin film before and after the post-printing polymerization step were investigated using FTIR spectroscopy. These data indicate that pPyrrole is present following treatment with Fe (III) (Figure S4). The diagnostic bands of pHEMA are present in spectra both before and after polymerization at 3600–3100, 3000–2850, 1716, 1160, 1076 and 1017 cm^{-1} and correspond to respective O-H, C-H, C=O, CO-O, C-O-H and C-O-C group stretching vibrations. The aromatic C-N vibration peaks at 1277 cm^{-1} are also observed in both spectra. After treatment, a new peak emerged at 1618 cm^{-1} and is assigned to C=C stretching in pPyrrole after the polymerization and doping treatment. These results are in good agreement with prior reports.^[43–45] The pPyrrole formation is further confirmed by UV-Vis absorbance after the post-printing curing step as indicated by the emergence of an absorbance feature at 476 nm and is attributed to the bipolaron transition of pPyrrole in its oxidized state (Figure S5).^[46,47]

SEM analysis made of a printed filamentary (dehydrated) structure (Figure 3a–b) revealed the general morphological features of the pPyrrole formed by the curing step; these micrographs reveal a densely penetrating network of nanoparticles (Figure 3c–d). The pHEMA filamentous networks form a more diffuse matrix that physically confines this dense population of nanoparticles in the composite’s hydrated state. As judged from the high magnification top view image of the structure (Figure 3c), these nanoparticles render a composite with a significantly rougher surface as compared to the much smoother boundaries that form for pure pHEMA thin films and printed structures.^[48,49] A high resolution cross-sectional SEM image of the filament structure demonstrates that the pPyrrole nanoparticles with diameters of ~100 nm are present throughout the interior domain of the filament (Figure 3d).^[50,51] These nanoparticles form a conductive path within the hydrogel matrix that renders it electrically conductive.

The composite hydrogel’s conductivity, measured using an electrochemical workstation, is compared to results from four-point probe measurements (Figure 4a). The conductivity is related to pyrrole volume fraction (Figure S6). Above the concentration used in the

optimized ink, multiphase mixtures result that are not printable. The amount of pHEMA used controls ink's rheological properties and determines its printability. To effect smooth printing, the pHEMA chains must be maintained in an entangled state. The ethanol that helps pyrrole to dissolve, relaxes this entanglement and negatively affects the ink's printability. The composite ink used in this work thus reflects a balance made between printability and conductivity. The post-treatment composite has an intrinsic conductivity of $13.59 \text{ S}\cdot\text{cm}^{-1}$. This compares well to the four-point probe measurement of $12.16 \text{ S}\cdot\text{cm}^{-1}$. We carried out a combination of electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV) measurements in a three-electrode system to evaluate the electrochemical ability and determine the ink's potential in fabrication of neuron recording devices. A Bode graph of magnitude of impedance over frequency range of $1\text{-}10^4 \text{ Hz}$ (Figure 4a) using a thin hydrogel film electrode shows the magnitude of impedance decreases with frequency and gave a normalized impedance of $8.4 \text{ }\Omega\cdot\text{cm}^2$, comparable to those reported for other conductive polymer-based materials.^[48,52,53] The Nyquist plot (Figure S7) is nearly vertical at low frequencies, indicating a capacitive behavior for the hydrogel. The electrochemical stability of the hydrogel was explored by running multiple CV scans in PBS buffer vs. Ag/AgCl, as shown in Figure 4b. In exemplary data, the composite hydrogel thin film was found to be stable for 20 cycles with little variation over a -0.6 to $+0.6 \text{ V}$ window of applied potential. This suggests that printed composite conductive hydrogels will be sufficiently stable to support electrophysiological recordings as are described below. Additionally, two peaks at -0.25 V and $+0.25 \text{ V}$ are observed in the cyclic voltammogram within a single redox cycle and are attributed to the reduction and oxidation of polypyrrole respectively. Signal transmission at the electrode/cell interface of conductive polymer-based devices primarily happens through capacitive currents and Faradic reactions from the doping and dedoping process.^[54] The capacitive behavior and Faradic currents of the composite hydrogel helps to improve charge transfer efficiency and eventually recording signal quality at the device/cell interface. We also evaluated the material's performance as an electrical conductor on the macroscopic level (Figure 4c, d). Here a composite hydrogel mesh was printed and inserted into an electrical circuit with a 4.5 V LED light source and power supply. This demonstration shows the printed pattern can support emission by the LED when powered by a 5 V DC applied bias.

2.2 Evaluation of the Composite Hydrogel for Biocompatibility

Both pHEMA and pPyrrole have no reported cytotoxicity and have been used extensively as active and supporting materials for use in various bioanalytical applications.^[55,56] Presented here are the results of direct biocompatibility tests of the new composite that show this material can be used as a substrate to support normal patterns of cell growth in culture and even promote useful forms of cell migration from a glass substrate and attachment onto printed conductive filaments. In these experiments (Figure 5), after device sterilization, MC-3T3-E1 preosteoblasts cells were seeded onto both a conductive hydrogel thin film or glass slide bearing a printed U-shaped composite filament (Figure 5a–d) as detailed in the Experimental Section. Cell viability on thin film supported cultures were evaluated over a seven-day period in culture using a Live/Dead Assay and counting dead (red) and live (green) cells on day 1, 3 and 7 (Figure 5b and e). Cell density did not decrease appreciably during this culture period. After seven days, most of the adhered cells were alive ($94.3\pm0.2\%$

viability on day 7 compared to $96.0 \pm 0.1\%$ on day 1) with only small losses observed, an outcome typical for other standard culture conditions.^[57,58] A pHEMA hydrogel surface usually requires an activating coating (such as by thin-layer proteins) to promote viable degrees of cell attachment.^[38] The conductive ink chemistry of the current work does not require such treatment, and most importantly even permits cell migration onto the U-shaped filament from the glass substrate (a strongly growth compliant surface). Zeta potential measurements (Figure 5f) indicate the *in situ* synthesized polypyrrole nanoparticles counteract the typically negative surface charge of a pHEMA hydrogel matrix immersed in culture media, bringing the overall surface charge (based on Zeta potential measurements) up from -46 mV to $+10$ mV. The growth compliance of compositionally diverse forms of pHEMA as a scaffold material in model 3D cellular microcultures has been deeply studied in our past work.^[38,59–61] Cationic charge affected cell migrations have also been reported by other groups. Lesn'y et al. has demonstrated positively charged HEMA showed significantly higher cell density than negatively charged ones.^[62] De Luca et al. also found similar results as Lesn'y using a different pHEMA material.^[63] Moreover, Metwally et al. also pointed out in their review that surface charge, as well as roughness, is crucial to cell attachment and cell migration.^[64] We believe the surface potential and the rougher interface seen in Figure 3b both contribute to the enhanced affinities seen for cell attachment and growth.^[65] As an additional point of note, nanoindenter results (Figure S6) shows that the hydrogel has a modulus of 445 kPa, a stiffness that is a good match for many living tissues.^[66]

2.3 Conductive Ink-Based Platform for Electrophysiological Recording of Individually Cultured Neurons

The ink's conductivity and biocompatibility allow the fabrication of electrophysiological recording platforms for investigation of the electrical activity of cultured neurons (Figure 6a). Printed conductive arrays were prepared as an extracellular electrophysiological recording platform as described in the experimental section. *Aplysia* pedal ganglia neurons were seeded at a density of 30 count/cm² in artificial sea water onto arrays and cultured for a day at ambient conditions. Corresponding control devices share the same setup but without cells. There instead, cell culture media from recording devices is added to eliminate possible false signals generated from chemical compounds in the media. Concentrated KCl solutions are added to the extracellular media to increase electrical activity of cultured neurons in the stimulation devices. The KCl solution was also added to the corresponding control group with the absence of cells to eliminate possible artificial signals generated from KCl introduction. All devices were connected to the electrophysiological setup through the gold strips (Figure 6a) as detailed in the Experimental Section.

Figure 6b depicts the two types of interactions cells have with printed arrays before recording. These representative data show behaviors for the cellular interactions that prefer maximum contact with the composite hydrogel material of the array: smaller cells tend to rest centered on top of an array filament while larger cells more commonly span across two adjacent array filaments. Like preosteoblast cells, the positively charged surface present in media does not inhibit cell attachment and additional surface treatment is unnecessary. Figure 6c shows electrophysiological recordings acquired from the recording and

stimulation groups along with their corresponding control devices. Both control groups show no action potential-like extracellular activity, indicating compounds in the culture media and KCl interaction with the surface of the arrays does not cause false signals. In stark contrast, signals from the recording and stimulation groups show action potentials, with representative single spikes from recording and stimulation experiments (Figure 6d). The average signal amplitude increases from $6.5 \pm 0.3 \mu\text{V}$ for recording devices to $7.8 \pm 0.4 \mu\text{V}$ for stimulated devices due to the increase of neuronal activities and spikes occur at higher frequencies in the stimulated group. The quality of the signals recorded using these arrays was analyzed by calculating the signal-to-noise ratio (SNR) using methods reported previously.^[67] The current arrays yielded SNRs of 4.6 ± 0.3 (recording group) and 4.2 ± 0.2 (stimulation group) respectively, meaning the signals recorded from the conductive hydrogel platform can be easily distinguished from background noise. Using the classification defined by Martin *et al.*^[68] where good SNRs are higher than 4.0, these arrays are comparable to other previously reported high quality conductive polymer-based recording probes.^[53,65,69] Long-term neuron interaction with the arrays (Figure 6e and Figure S9) highlights the biocompatibility of the conductive hydrogel, where neurons have extensive outgrowth along the filaments of arrays after five days in culture. These results, when taken together, demonstrate that printed arrays are capable of measurement of neuronal electrical activity. These observations also suggest programmable manipulation of spatial neuron arrangement is possible upon these composite hydrogels. By altering printing pattern designs, different conductive hydrogel circuits could be easily fabricated to guide neuron growth along prepared patterns. Moreover, with the high conductivity of hydrogels, the delivery of electrical stimuli may also be exploited to promote neurite outgrowth, eventually with benefits for studies on neuronal regeneration. The strong evidence of neuronal attachment and process outgrowth guidance indicates a potential utility for the use of related multifunctional materials for use in the fabrication of customized spinal cord injury repair scaffolds and provide soft, tissue-like interfaces for neuronal devices that can inhibit scar formation. The latter inferences illustrate areas of opportunity where future studies might be gainfully directed.

3. Conclusions

In summary, a 3D printable, conductive hydrogel ink with excellent biocompatibility and high spatial resolution has been developed and successfully tested. Microperiodic conductive arrays have been fabricated and used as neuronal extracellular signal recording platforms. Capabilities of such arrays to assist detection of neuronal activity in steady state and stimulated states are demonstrated. This programmable conductive hydrogel opens new opportunities to fabricate high spatial resolution platforms that can help studying *in vitro* and *in vivo* activities of different electroactive cells types including neurons.

4. Experimental

Sample preparation and characterization

All chemical reagents are commercially available and were obtained from Sigma Aldrich. Pyrrole monomer is distilled under nitrogen before ink preparation. The conductive ink is

composed of pHEMA chains with two different molecular weight, pyrrole monomer, water and ethanol. The optimal recipe consists of pHEMA (11.5 wt%, average 1000kDa, powder), pHEMA (29.5 wt%, average 300kDa, crystalline), pyrrole monomer (28 wt%, reagent grade, 98%), Milli-Q deionized water (15 wt%, 18.2 M Ω •cm) and ethanol (16 wt%). Each component is added to a vial and homogenized using a Thinky Mixer (model ARE-310, Thinky) at 2000 rpm for 6 min followed by a degassing for 30s until homogeneous. The samples were kept at +4 °C for up to one month until ready to print.

Prepared inks (1.5 g) were loaded into syringes and degassed for 1 min and mounted onto a 3D printer (AGS-1000, Aerotech Inc., Pittsburgh, PA). Inks were extruded onto glass slides through either pre-pulled glass pipette tip printheads (World Precision Instruments Inc.) at 1 and 30 μ m or metal barrel luer-lock nozzles from Nordson EFD at 100, 200 and 330 μ m diameters. After printing, patterns are immersed in a 20% ferric chloride (reagent grade, 97%) aqueous solution for 5 min and washed with water. These treated samples were stored in water with water changes made every four hours for three days. After this initial period, the samples were held in DI water at room temperature until ready for use. For specific physical and spectroscopic characterization measurements (e.g. SEM images and FTIR spectra), samples were dried for 24 hr at 22 C prior to data acquisition. Control experiments demonstrated that structures printed with fine micron scale filament dimensions rapidly rehydrate (~1hr) when reimmersed in water. Rheological behavior was measured using a DHR-3 rotational rheometer (TA instruments) with a 20 mm diameter parallel-plate geometry at room temperature. A solvent trap of water and ethanol at the same concentration used in the ink was used to ensure a saturated atmosphere around the sample and prevent further solvent evaporation during measurements.

FTIR measurements were obtained using a Nicolet Nexus 670 FTIR with ATR accessory over a scan range of 800–4000 cm^{-1} at a 1 $\text{cm} \cdot \text{s}^{-1}$ scan rate. UV-vis measurements were performed on a Varian/Cary 5G spectrophotometers over the wavelength range of 200–1000 nm with a scan rate of 600 nm min^{-1} at ambient conditions. All samples for FTIR and UV-vis measurements were vacuum dried for 4 hours to remove residual water prior to characterization.

Scanning transmission electron imaging of printed patterns were taken using a Hitachi S-4800 high resolution SEM at an accelerating voltage of 5 kV. Other optical images were taken using an Olympus SZX 7 stereoscope, a Nikon D90 camera and an iPhone. Electrochemical performance of printed patterns is measured on a Model 600D electrochemical workstation (CH Instruments). Surface resistance of samples was taken on a Jandel 4-point probe instrument.

Biocompatibility

Preosteoblasts (MC3T3-E1 Subclone 14 ATCC CRL-2594) were cultured in complete media containing alpha minimum essential media (MEM) without calcium or magnesium (ATCC), 10% fetal bovine serum (FBS ATCC 30–2020) and 1% Penicillin-streptomycin (Pen-Strep, Life Technologies). At around 80% confluence, preosteoblasts were incubated in 3mL of Trypsin (0.05% Trypsin-EDTA, Life Technologies) for 10–15 min for effective cell detachment. When cell detachment occurred, solutions were neutralized with 4mL of

complete media and flasks were then rinsed with 3mL PBS to transfer cells into tubes for centrifugation for 10 min. After centrifugation, cell pellets were collected and resuspended in complete medium before seeding. Samples, including ink thin films and filaments, are deposited on plain glass slides as described in the previous section. Before seeding cells, samples are dried and sterilized using 70% ethanol for 10 min followed by 2 h UV sterilization. The samples were then kept sterile until ready to seed. To start cell culture, samples were washed with PBS for 3 times and cell suspension were added to samples at a density of $3.1 \cdot 10^5$ cells $\cdot$ mL⁻¹. The samples are then kept in 37 °C incubator.

Cell survival rate was evaluated using Live/Dead assay kit (for mammalian cells, Life Technologies) on day one, three and seven. 5 μ L of calcein AM “live” stain and 5 μ L of ethidium homodimer “dead” stain were added to 10 mL PBS buffer and all samples were incubated in this solution for at least 30 min before imaging on a Zeiss Axiovert 25 microscope.

Neuron Recording

Samples were kept in artificial sea water cell media for up to two weeks before recording and showed no signs of decreased performance over this time.

Aplysia californica (100–300 g) were supplied by the National Resource for *Aplysia* (Miami, FL) and stored in circulated, aerated seawater at 14°C. Prior to dissection, the animals were anesthetized by the injection of 30–50% body weight of isotonic magnesium chloride solution into the body cavity. Individual *Aplysia* pedal neurons were isolated as described previously.^[70,71] Samples with cells were kept in artificial sea water overnight before connecting to an ac-coupled differential amplifier (model 1700; A-M Systems). Signals were filtered with a 50-Hz high-pass filter and a 1-kHz low-pass filter and data were processed using Clampfit and Origin software.

Printed conductive arrays with extracellular electrophysiological recording platform were prepared as follows. A 20 nm thick gold strip is deposited onto one end of a glass slide. This strip was connected to an extracellular signal amplifier, a digitizer, and a computer for data acquisition. Then, 50 μ m diameter electrode arrays were printed onto the gold modified and bare areas of glass at 50 μ m spacings. *Aplysia* pedal ganglia neurons were used as test cells. Eight individual devices were assembled and divided into four categories with two devices each: the recording group and its control group; the stimulation group and its corresponding control group.

Before signals were recorded, the baseline was allowed to stabilize for 10 min to minimize fluctuation. Cell action potentials of stimulation devices, were recorded after stimulation with the same setup used for the other devices. After a stable baseline was achieved, concentrated 6M KCl solution was added to the culture media to give a final stimulation concentration of 30 mM. The baseline was adjusted again and induced signals were recorded for 5 min. Control devices underwent the same treatment and recording sequence but without cell cultures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

The authors thank Xiyang Wang for help with cell cultures. The authors also thank Gaurav Chaudhary for help with rheology measurements and Yingfeng Yang for assistance with FTIR and UV-Vis studies. The experiments were carried out in part in the Materials Research Laboratory Central Research Facilities, University of Illinois. We gratefully acknowledge funding in the form of an Army Research Office MURI (W911NF-17-1-0351) for their support of this work. Funding to SSR and JVS was provided by the National Institute on Drug Abuse via award no. P30 DA018310. The content is solely the responsibility of the authors and does not necessarily represent the official views of the funding agencies.

References

- [1]. Green RM, A Translation of Luigi Galvani's De Viribus Electricitatis in Motu Musculari Commentarius: Commentary on the Effect of Electricity on Muscular Motion, Elizabeth Licht, Cambridge, MA, USA, 1953.
- [2]. Wang J, Electroanalysis 2001, 13, 983.
- [3]. Benabid AL, Curr. Opin. Neurobiol 2003, 13, 696. [PubMed: 14662371]
- [4]. Donoghue JP, Nat. Neurosci 2002, 5, 1085. [PubMed: 12403992]
- [5]. Rivnay J, Owens RM, Malliaras GG, Chem. Mater 2014, 26, 679.
- [6]. Liao C, Zhang M, Yao MY, Hua T, Li L, Yan F, Adv. Mater 2015, 27, 7493. [PubMed: 25393596]
- [7]. Lacour SP, Courtine G, Guck J, Nat. Rev. Mater 2016, 1, 10.
- [8]. Someya T, Bao Z, Malliaras GG, Nature 2016, 540, 379. [PubMed: 27974769]
- [9]. Spencer KC, Sy JC, Ramadi KB, Graybiel AM, Langer R, Cima MJ, Sci. Rep 2017, 7, 1. [PubMed: 28127051]
- [10]. Seliktar D, 2012, 336, 1124.
- [11]. Willner I, Acc. Chem. Res 2017, 50, 657. [PubMed: 28415844]
- [12]. Zorlutuna P, Jeong JH, Kong H, Bashir R, Adv. Funct. Mater 2011, 21, 3642.
- [13]. Yan J, Sun Y, Zhu H, Marcu L, Revzin A, Biosens. Bioelectron 2009, 24, 2604. [PubMed: 19251408]
- [14]. Lu Y, Wang D, Li T, Zhao X, Cao Y, Yang H, Duan YY, Biomaterials 2009, 30, 4143. [PubMed: 19467702]
- [15]. Cha C, Shin SR, Annabi N, Dokmeci MR, Khademhosseini A, ACS Nano 2013, 7, 2891. [PubMed: 23560817]
- [16]. Goenka S, Sant V, Sant S, J. Control. Release 2014, 173, 75. [PubMed: 24161530]
- [17]. Shi J, Li X, Cheng H, Liu Z, Zhao L, Yang T, Dai Z, Cheng Z, Shi E, Yang L, et al., Adv. Funct. Mater 2016, 26, 2078.
- [18]. Smart SK, Cassady AI, Lu GQ, Martin DJ, Carbon N. Y 2006, 44, 1034.
- [19]. Berggren M, Richter-Dahlfors A, Adv. Mater 2007, 19, 3201.
- [20]. Green R, Abidian MR, Adv. Mater 2015, 27, 7620. [PubMed: 26414302]
- [21]. Rivnay J, Inal S, Salleo A, Owens RM, Berggren M, Malliaras GG, Nat. Rev. Mater 2018, 3, 1.
- [22]. Choi S, Lee H, Ghaffari R, Hyeon T, Kim DH, Adv. Mater 2016, 28, 4203. [PubMed: 26779680]
- [23]. Balint R, Cassidy NJ, Cartmell SH, Acta Biomater 2014, 10, 2341. [PubMed: 24556448]
- [24]. Abidian MR, Martin DC, Adv. Funct. Mater 2009, 19, 573.
- [25]. Abidian MR, Ludwig KA, Marzullo TC, Martin DC, Kipke DR, Adv. Mater 2009, 21, 3764. [PubMed: 26345408]
- [26]. Woepfel K, Yang Q, Cui XT, Curr. Opin. Biomed. Eng 2017, 4, 21. [PubMed: 29423457]
- [27]. Huang WC, Lai HY, Kuo LW, Liao CH, Chang PH, Liu TC, Chen SY, Chen YY, Adv. Mater 2015, 27, 4186. [PubMed: 26074252]

- [28]. Liu Y, McGuire AF, Lou HY, Li TL, Tok JBH, Cui B, Bao Z, Proc. Natl. Acad. Sci. U. S. A 2018, 115, 11718. [PubMed: 30377271]
- [29]. Pan L, Yu G, Zhai D, Lee HR, Zhao W, Liu N, Wang H, Tee BCK, Shi Y, Cui Y, et al., Proc. Natl. Acad. Sci. U. S. A 2012, 109, 9287. [PubMed: 22645374]
- [30]. Heo DN, Lee SJ, Timsina R, Qiu X, Castro NJ, Zhang LG, Mater. Sci. Eng. C 2019, 99, 582.
- [31]. Zampetti E, Pantalei S, Scalese S, Bearzotti A, De Cesare F, Spinella C, Macagnano A, Biosens. Bioelectron 2011, 26, 2460. [PubMed: 21093248]
- [32]. Khosla A, Gray BL, Mater. Lett 2009, 63, 1203.
- [33]. Chan V, Zorlutuna P, Jeong JH, Kong H, Bashir R, Lab Chip 2010, 10, 2062. [PubMed: 20603661]
- [34]. Pataky K, Braschler T, Negro A, Renaud P, Lutolf MP, Brugger J, Adv. Mater 2012, 24, 391. [PubMed: 22161949]
- [35]. Walker SB, Lewis JA, J. Am. Chem. Soc 2012, 134, 1419. [PubMed: 22220580]
- [36]. Fu K, Wang Y, Yan C, Yao Y, Chen Y, Dai J, Lacey S, Wang Y, Wan J, Li T, et al., Adv. Mater 2016, 28, 2587. [PubMed: 26833897]
- [37]. Yuk H, Lu B, Lin S, Qu K, Xu J, Luo J, Zhao X, Nat. Commun 2020, 11, 4. [PubMed: 31911593]
- [38]. McCracken JM, Badea A, Kandel ME, Gladman AS, Wetzel DJ, Popescu G, Lewis JA, Nuzzo RG, Adv. Healthc. Mater 2016, 5, 1025. [PubMed: 26924676]
- [39]. Hanson Shepherd JN, Parker ST, Shepherd RF, Gillette MU, Lewis JA, Nuzzo RG, Adv. Funct. Mater 2011, 21, 47. [PubMed: 21709750]
- [40]. Deng Z, Hu T, Lei Q, He J, Ma PX, Guo B, ACS Appl. Mater. Interfaces 2019, 11, 6796. [PubMed: 30673228]
- [41]. Håkansson KMO, Henriksson IC, de la Peña Vázquez C, Kuzmenko V, Markstedt K, Enoksson P, Gatenholm P, Adv. Mater. Technol 2016, 1, 1.
- [42]. Kamyshny A, Magdassi S, Chem. Soc. Rev 2019, 48, 1712. [PubMed: 30569917]
- [43]. Tian B, Zerbi G, J. Chem. Phys 1990, 92, 3886.
- [44]. Li Q, Chen X, Tang Q, Xu H, He B, Qin Y, J. Mater. Chem. A 2013, 1, 8055.
- [45]. Gambhir A, Gerard M, Jain SK, Malhotra BD, Appl. Biochem. Biotechnol. - Part A Enzym. Eng. Biotechnol 2001, 96, 303.
- [46]. Arjomandi J, Shah AUHA, Bilal S, Van Hoang H, Holze R, Spectrochim. Acta - Part A Mol. Biomol. Spectrosc 2011, 78, 1.
- [47]. Prulliere F, Leem G, Reisinger NC, Lee TR, Curr. Top. Colloid Interface Sci 2006, 7, 19.
- [48]. Arslantunali D, Budak G, Hasirci V, J. Biomed. Mater. Res. - Part A 2014, 102, 828.
- [49]. Sadegh H, Shahryari-Ghoshekandi R, Tyagi I, Agarwal S, Gupta VK, J. Mol. Liq 2015, 207, 21.
- [50]. Wen J, Tian Y, Mei Z, Wu W, Tian Y, RSC Adv. 2017, 7, 53219.
- [51]. Chang LY, Li CT, Li YY, Lee CP, Yeh MH, Ho KC, Lin JJ, Electrochim. Acta 2015, 155, 263.
- [52]. Abidian MR, Corey JM, Kipke DR, Martin DC, Small 2010, 6, 421. [PubMed: 20077424]
- [53]. Yoshida Kozai TD, Langhals NB, Patel PR, Deng X, Zhang H, Smith KL, Lahann J, Kotov NA, Kipke DR, Nat. Mater 2012, 11, 1065. [PubMed: 23142839]
- [54]. Castagnola V, Descamps E, Lecestre A, Dahan L, Remaud J, Nowak LG, Bergaud C, Biosens. Bioelectron 2015, 67, 450. [PubMed: 25256782]
- [55]. Zhang Y, Chu D, Zheng M, Kissel T, Agarwal S, Polym. Chem 2012, 3, 2752.
- [56]. George PM, Lyckman AW, Lavan DA, Hegde A, Leung Y, Avasare R, Testa C, Alexander PM, Langer R, Sur M, Biomaterials 2005, 26, 3511. [PubMed: 15621241]
- [57]. Randall CL, Kalinin YV, Jamal M, Manohar T, Gracias DH, Lab Chip 2011, 11, 127. [PubMed: 21063585]
- [58]. Lin H, Zhang D, Alexander PG, Yang G, Tan J, Cheng AWM, Tuan RS, Biomaterials 2013, 34, 331. [PubMed: 23092861]
- [59]. Badea A, McCracken JM, Tillmaand EG, Kandel ME, Oraham AW, Mevis MB, Rubakhin SS, Popescu G, Sweedler JV, Nuzzo RG, ACS Appl. Mater. Interfaces 2017, 9, 30318. [PubMed: 28813592]

- [60]. McCracken JM, Rauzan BM, Kjellman JCE, Kandel ME, Liu YH, Badea A, Miller LA, Rogers SA, Popescu G, Nuzzo RG, Adv. Healthc. Mater 2019, 8, 1.
- [61]. McCracken JM, Xu S, Badea A, Jang KI, Yan Z, Wetzel DJ, Nan K, Lin Q, Han M, Anderson MA, et al., Adv. Biosyst 2017, 1, 1.
- [62]. Lesný P, Pádný M, Jendelová P, Michálek J, Vacík J, Syková E, J. Mater. Sci. Mater. Med 2006, 17, 829. [PubMed: 16932865]
- [63]. De Luca I, Di Salle A, Alessio N, Margarucci S, Simeone M, Galderisi U, Calarco A, Peluso G, Stem Cell Res 2016, 17, 248. [PubMed: 27591481]
- [64]. Metwally S, Stachewicz U, Mater. Sci. Eng. C 2019, 104, 109883.
- [65]. Elias CN, Oshida Y, Lima JHC, Muller CA, J. Mech. Behav. Biomed. Mater 2008, 1, 234. [PubMed: 19627788]
- [66]. Guimarães CF, Gasperini L, Marques AP, Reis RL, Nat. Rev. Mater 2020, 5, 351.
- [67]. Ludwig KA, Uram JD, Yang J, Martin DC, Kipke DR, J. Neural Eng 2006, 3, 59. [PubMed: 16510943]
- [68]. Kim DH, Wiler JA, Anderson DJ, Kipke DR, Martin DC, Acta Biomater. 2010, 6, 57. [PubMed: 19651250]
- [69]. Harris AR, Allitt BJ, Paolini AG, Analyst 2019, 144, 2973. [PubMed: 30888346]
- [70]. Ye X, Xie F, Romanova EV, Rubakhin SS, Sweedler JV, ACS Chem. Neurosci 2010, 1, 182. [PubMed: 20532188]
- [71]. Wang L, Ota N, Romanova EV, Sweedler JV, J. Biol. Chem 2011, 286, 13765. [PubMed: 21343289]

Figure 1.

A schematic illustration of the formation of conductive particle-free hydrogels. The top row representations depict a macroscopic view of the printed structure while the bottom row illustrates the fine structure of the hydrogel during the three stages of its formation. Green lines in the bottom row pictures represent polyHEMA chains, yellow triangles signify pyrrole monomers, brown dots are pyrrole oligomers, and black dots represent polypyrrole particles.

Figure 2.

Characterization of the hydrogel ink including its rheological behavior and ability to form stable structures. (a) The ink storage modulus (G') and loss modulus (G'') are measured at 1Hz in oscillatory mode at 1Hz frequency and shows ink has liquid-like response. (b) Ink viscosity measured as a function of shear rate and shows shear-thinning property required for 3D printing, black line showing shear-thinning exponent. (c) Transmission light optical microscopy image of patterns printed using 1 μm tip demonstrates feasible printing using small nozzle. Single filament location is denoted by arrows, scale bar 10 μm . (d) Image of a printed three-dimensional pyramid structure shows ink has great 3D buildability, single filament marked by arrows, scale bar 1cm.

Figure 3. Macroscopic and SEM images of a printed out “I” pattern include: (a) a photo of the printed out pattern with a 1 mm scale bar, (b) a top view SEM image of the “I” pattern from the selected area of (a) with a 400 μm scale bar, (c) a magnified image of the highlighted area of a single printed line depicted in (b) with a 100 nm scale bar, and (d) an SEM image of the cross-section of conductive hydrogel pattern with a 500 nm scale bar.

Figure 4.

Electrical and electrochemical characterization of the conductive hydrogel ink. (a) The I-V curve and Bode graph of magnitude of impedance versus frequency for a conductive hydrogel thin film shows an intrinsic conductivity of $13.59 \text{ S}\cdot\text{cm}^{-1}$ and a normalized impedance of $8.4 \text{ }\Omega\cdot\text{cm}^2$. (b) Results of a cyclic voltammetry scan of the conductive hydrogel thin film in PBS buffer vs. Ag/AgCl, 20 cycles, scan rate 20 mV/s show the reduction and oxidation of polypyrrole. (c, d) An LED is illuminated when wired into a circuit with a printed hydrogel mesh as a conductor and a 5V applied voltage. The illumination and darkening of the LED in response to the supplied power demonstrates the electrical conductivity of the hydrogel mesh.

Figure 5.

A biocompatibility evaluation of the conductive hydrogel shows high survival rates and promotes cell migration. (a) A scheme of cell culturing experiments performed on thin hydrogel film with a (b) fluorescence image of preosteoblasts cultured on thin conductive hydrogel film for seven days and stained with live/dead assay. Live cells exhibit green color and dead red. Scale bar 100 μm . (c) Scheme of cultured cells' migration onto printed out U shape conductive pattern with (d) a fluorescence image of cells' migration onto printed filaments after seven days of culture, arrow denotes migration direction, scale bar 100 μm .

(e) Survival rate of preosteoblasts on thin hydrogel film on day 1, 3 and 7. (f) Surface charge measurements of conductive hydrogel and reference in PBS buffer of the pHEMA hydrogel without polypyrrole (black) and the conductive hydrogel (red).

Figure 6.

Extracellular electrophysiological recordings are obtained for *Aplysia* pedal ganglia neurons using printed conductive hydrogel arrays. (a) A schematic of experiment setup depicts glass slides with printed conductive hydrogel arrays are seeded with cells and connected to an electrophysiological setup including an amplifier for signal monitoring and a digitizer. (b) A light microscopy image of *Aplysia* pedal ganglia neurons outgrowing on conductive arrays with a $50 \mu\text{m}$ scale bar. (c) Representative extracellular recordings of electric activity in four types of samples include neuron absent control arrays covered with cell culture media neuron absent control arrays with elevated concentrations of potassium ions in the culture media, steady state activity of neurons cultured on arrays, and electrical activity of neurons during stimulation by elevated extracellular potassium ion concentrations. (d) Examples of an individual spike recorded at two experimental conditions: steady state recording and

stimulated by elevated potassium concentrations. (e) SEM image of *Aplysia* neuron outgrowth after 5 days culture, scale bar 50 μm .