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Low-cost, Flexible Polymer Arrays for Long-Term Neuronal Culture*

N. Catherine Hogan, Giovanni Talei-Franzesi, Omar Abudayyeh, Andrew Taberner, and Ian Hunter

Abstract— **Conducting polymers are promising materials for fabrication of microelectrode arrays for both neural stimulation and recording. Our ability to engineer the morphology and composition of polypyrrole together with its suitability as an electrically addressable tissue/cell substrate have been used to develop an inexpensive, disposable three-dimensional polymeric array for use in neuronal culture and drug discovery. These arrays could be interfaced with a fixed, parallel stimulation and optical imaging system, amenable to automated handling and data analysis.**

I. Introduction

Microelectrode array (MEA) technology has proved useful in both the study of network level information processing in the nervous system and the field of biotechnology [1][2]. Information gleaned from these studies on the effect of topography, functionalization, and time dependent physiological changes has in turn informed the development of neural probes [3].

While MEAs capable of both stimulation and recording are available, they are invariably fabricated from metals and/or inorganic semi-conductors [4]. For *in vivo* applications, the difference in stiffness between the material properties of the brain and those of the microelectronic components can result in a complex inflammatory response at the site of implantation often leading to electrical and mechanical isolation of the implanted probes from the nervous system [5]. Approaches designed to improve the interface between the neuron and implant have included electrochemical deposition of biocompatible polymer with or without biomolecule blends [6-8], covalent immobilization of bioactive peptides and proteins [9-11], and selective localization of protein by microcontact printing [12] and the development of poly(3,4-ethylenedioxythiophene) (PEDOT)-neural cell hybrid electrode coatings and PEDOT-neural cell templated films (*i.e.* biomimetic substrates) [13]. All of these approaches have been shown to improve cell-electrode contact and decrease recording site impedance [13-15]. Other strategies have included using the conducting polymer itself as the electrode [16] and the development of all-polymer MEAs [4].

The high conductivity, low stiffness, ease of processing, scalability, and ability to incorporate bioactive molecules, either as dopants or as surface modifications, make conducting polymers an attractive material for fabrication of MEAs. In this paper, we present an inexpensive method for generating flexible, three dimensional (3D) arrays of polypyrrole (PPy) posts and show that these arrays can support the growth of neuronal cultures. The post configuration permits seeding of a glia cell layer for long term neural growth without impeding neuron-PPy electrode contact. We further discuss interfacing these arrays with a fixed, addressable base array and imaging system, making them eminently suitable for high-throughput drug screening and studies on neural plasticity.

II. Materials and Methods

A. Array Fabrication and Electrodeposition Process

Two 250 µm sheets of implant-grade polydimethylsiloxane (PDMS; SMI) were reversibly bonded and then laser-patterned using a standard $C0_2$ laser to create a bilayered mask. The bulk of the masks used in this study were laser machined using a Trotec Firestar$_{t60}$ series laser engraver in the direct write mode. The physical mechanism underlying laser micromachining results in a slightly (~5°) conical cavity, which helps ensure the mechanical stability of the final electrode array.

Masks were inverted and adhered to 25 mm x 25 mm x 1 mm glassy carbon (GC) electrodes (SPI) using a small volume of ethanol. Each was placed in a custom designed chamber containing a copper counter electrode approximately 3x the surface area of the GC working electrode, filled with deposition solution, degassed, and placed in a temperature controlled chamber. Depositions were conducted galvanostatically at variable temperatures using a current density of 30 A/m^2, high pyrrole monomer (Sigma) concentration (0.1 to 0.2 M), and optimized concentrations of biocompatible dopant ion.

C. Treatment of Arrays Prior to Cell Seeding

PPy arrays were air plasma-treated for 60-120 s to render the PDMS surface hydrophilic. Immediately following treatment, the arrays were placed in 70% ethanol, UV-sterilized under ethanol for 1 h, washed 5x with sterile Millipore water, and coated with cell adhesion and/or proliferation molecules. Plates containing arrays were then

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N. Catherine Hogan is with the BioInstrumentation Laboratory in the Mechanical Engineering Department at Massachusetts Institute of Technology, Cambridge, MA 02139 (phone: 617 324-6051; fax: 617 252-1849; e-mail: hog@mit.edu).

Giovanni Talei-Franzesi is with the Media Lab (Synthetic Neurobiology) at Massachusetts Institute of Technology, Cambridge, MA 02139 (geggio@mit.edu).

Omar Abudayyeh was a UROP student with the BioInstrumentation Lab (omarabu@mit.edu).

Andrew Taberner is with the Auckland Bioengineering Institute and the Department of Engineering Science at The University of Auckland, NZ, 1010 (a.taberner@auckland.ac.nz).

Ian Hunter is Director of the BioInstrumentation Laboratory in the Mechanical Engineering Department at Massachusetts Institute of Technology, Cambridge, MA 02130 (e-mail: ihunter@mit.edu).

incubated at 37 °C for 1 to 12 h after which the coating was carefully removed prior to cell seeding.

D. Hippocampal Cell Culture

For each experiment, hippocampi were dissected from the cortices of 10 wild-type neonatal mice according to a protocol approved by MIT IACUC and in accordance with the NIH Guide for the Use and Care of Laboratory Animals. Briefly, P0 C57B/L6 mouse pups (www.criver.com) were cold anaesthetized, sprayed with 70% ethanol, quickly decapitated and their brains removed and transferred to a single well of a 6 well culture dish containing Hanks Balanced Saline solution (HBSS)(Sigma) pre-equilibrated to 4 °C. Using aseptic technique and in an HBSS slurry mixture, the cortical lobes were removed from each brain, and pooled into a single well containing ice cold HBSS. At this point, each cortex was transferred to a 60 mm culture dish containing HBSS slurry, the hippocampus dissected, and transferred to a 15 mL Falcon tube containing ice cold HBSS. Pooled hippocampi were allowed to settle to the bottom of the tube and excess HBSS removed prior to washing 3x with HBSS. Hippocampi were digested briefly with 1% papain (Worthington) containing 1% DNase (Sigma) in HBSS, followed by 1% DNase in HBSS. The cells were dissociated by trituration with fire polished Pasteur pipets of different bore, pelleted by centrifugation, resuspended in 1.5 mL of Neural Basal Medium containing B27 supplement and 200 mM L-glutamine (Invitrogen) pre-equilibrated to 37°C, and ~100,000 cells plated per array or coverslip (control) by surface tension.

E. Immunocytochemistry

Arrays were washed with 37 °C phosphate buffered saline (PBS) and the cells fixed for 10 minutes at 25 °C by addition of ~1 mL 4% paraformaldehyde in PBS. The fixing solution was removed and the cells washed 4x with PBS. After the final wash, the arrays were examined under the light microscope to ensure that the cells had not lifted off; adherent cells were used as an indicator of cell health. Cells were permeabilized for 5 minutes at 25 °C by addition of 500 µL of 0.25% Triton X-100 in PBS, washed 3x with PBS, and unbound sites blocked in a small volume of blocking buffer (*i.e.* 5% bovine serum albumin, 2% goat serum, 0.05% Triton X-100) for 1 h at 25 °C with gentle agitation. Excess blocking buffer was removed and the arrays covered with primary antibody and incubated at 4 °C overnight. The arrays were then washed 3x with PBS and a small volume of fluorescently labeled secondary antibody added to each followed by incubation for 1 h at room temperature. After a final wash, the arrays were overlaid with water and imaged using a Zeiss confocal microscope.

III. Results and Discussion

A. All Organic 3D Polymer Arrays

Design constraints included suitability for long-term culture, ease and low cost of fabrication, and ability to tolerate sterilization (*e.g.* autoclaving, oxygen plasma treatment, and 70% sterilization). Our experience with the conducting polymer PPy, its demonstrated biocompatibility [17], autoclavability, and capacity to support neural growth [18] and incorporate biologically-relevant molecules [8][15][19] made it the polymer of choice. PDMS, a silicone-based elastomer already used in biotechnology and medicine, provided an inert, self-adhesive, sterilizable, insulating substrate. While the PDMS masks used in these studies were 500 µm thick and contained 6 x 63 electrodes with a bottom hole diameter of 200 µm and an inter-electrode spacing of 500 µm, the feature size can be readily changed by using thinner masks and/or a more powerful laser and the spacing can be changed simply by changing the pattern drawn in the CAD program. The mask once adhered to an electrode (GC yielding more reproducible arrays than indium tin oxide) was placed in a deposition chamber, and pyrrole doped with HA or some other biocompatible dopant ion was electrodeposited through it (Fig. 1).

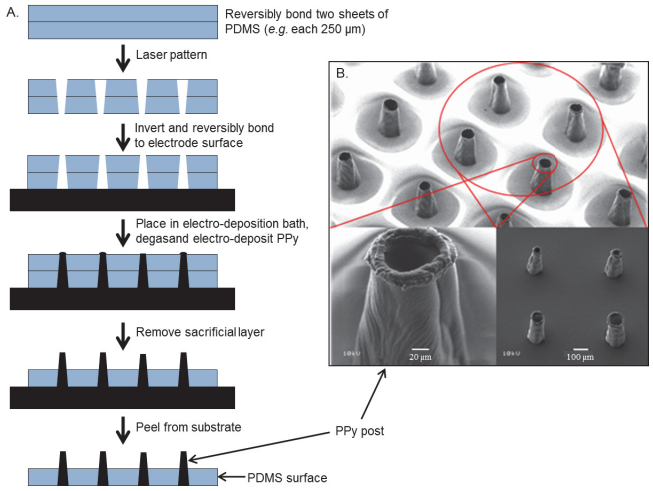

Figure 1. A) Schematic showing technique used to generate 3D polymer arrays. B) SEM images of representative 63-electrode PPy posts generated using a 250+250 µm thick PDMS mask.

Using this fabrication technique, we achieved polymer posts with tip diameters of 75 to 100 µm, comparable to commercially available silicon-based MEAs. The observed variation in tip diameter within an array is a consequence of variation in the diameter of the outermost hole created during laser patterning of the PDMS mask using the CO_2 laser. Thinner masks and/or use of an excimer laser would yield both smaller and more consistent features. Since the height of the polymer posts is dictated by the thickness of the mask, the process could be easily adapted to produce longer posts, more suitable for organotypic slice recordings or for *in vivo* use.

B. Optimization of Deposition Conditions

Given that the polymer conductivity, morphology, and the subsequent growth of cells on the polymer posts are affected by the choice of monomer, dopant ion, deposition temperature, time, and/or inclusion of biomolecules, we evaluated the parameters required to reproducibly generate stable, conductive, aqueous-based PPy films and posts. Firstly, we evaluated the ability to form stable films co-deposited using biocompatible dopant ions at different temperatures and concentrations. Several ions yielded films robust enough to be removed from the electrode for determination of conductivities (data not shown), four of which were used to generate polymer posts: hyaluronic acid (HA), sodium dodecyl benzene sulfonate (NaDBS), sodium

p-toluene sulfonate (ToS), and poly sodium 4-styrenesulfonate (PSS). Although HA, a large anionic, non-sulfated glycosaminoglycan, may not dope the pyrrole as effectively due to steric hindrance, its wide distribution throughout connective, epithelial, and neural tissues make it an interesting candidate dopant. Conductivities along with scanning electron microscope (SEM) images illustrating change in surface morphology due to choice of dopant ion, dopant concentration, and deposition temperature are shown in Fig. 2.

Dopant	Deposition		Posts Measured	Conductivity (S/m)
	Temp (°C)	Time (h)		
HA 0.5mg/mL	4	8	315	0.836
HA 1.0mg/mL	4	8	630	0.722
HA 2.0 mg/mL	4	8	473	2.187
HA 0.5mg/mL	4	12	189	0.082
HA 1.0mg/mL	4	12	252	1.015
HA 2.0mg/mL	4	12	252	6.767
HA 0.5mg/mL	20	6	378	0.151
HA 1.0 mg/mL	20	6	756	0.206
HA 2.0 mg/mL	20	6	756	0.218
HA 0.5mg/mL	20	8	1890	0.185
HA 1.0 mg/mL	20	6	756	0.206
HA 2.0 mg/mL	20	8	1323	0.201
HA 4.0mg/mL	20	8	441	0.902
HA 8.0mg/mL	20	8	443	3.340
HA 12.0mg/mL	20	8	94	3.467
HA 0.5mg/mL	20	12	ND	ND
HA 1.0mg/mL	20	12	ND	ND
HA 2.0 mg/mL	20	12	788	0.090
DBS 0.005M	15	8	630	643.40
DBS 0.05M	15	8	819	2847.99
ToS 0.05M	4	12	693	351.16
ToS 0.1M	4	12	567	843.75
ToS 0.2M	4	12	504	1037.15
ToS 0.05M	25	12	315	160.84
ToS 0.1M	25	12	630	137.27
ToS 0.2M	25	12	284	421.39
PSS 0.05M	4	14	220	2565.07
PSS 0.1M	4	14	95	2897.06
PSS 0.2M	4	14	126	5519.54
PSS 0.05M	25	14	ND	ND
PSS 0.1M	25	14	63	2750.8
PSS 0.2M	25	14	63	1533.42

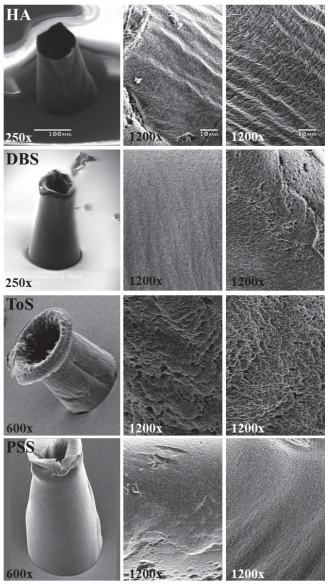

Figure 2. Conductivity measurements and representative SEM images of posts electrodeposited using different dopant ions at different concentrations and temperatures. The resistance of individual posts was determined by first measuring the resistance of six polymer arrays (6 arrays x 63 posts/array comprising a single deposition) and then modeling each post as an individual resistor in parallel. Because the cross sectional area of the posts is non-uniform, resistivity of individual posts (Rs) was determined by integrating the differential area of the outer and inner radius of each post assuming that the thickness is uniform to obtain a constant followed by multiplying the constant by the measured resistance (Rm) and number of posts (N). Conductivity is the reciprocal of resistivity.

A concentration dependent increase in conductivity was observed for each dopant ion when electro-depositions were carried out at 4 °C; NaDBS was an exception as the salt precipitated out of solution at lower temperatures. When doped with HA, conductivities increased with increasing concentration reaching a critical limit of 12 mg/mL due to viscous effects. While HA conductivities are not high relative to those observed with other dopants, they are ~20x higher than those reported in the literature for films [20]. As shown in [20], conductivity could be improved by incorporation of a PPy/HA bilayer.

In general posts electrodeposited at 4 °C appeared to have a more even surface structure; PSS was smoother than NaDBS followed by HA and finally ToS. Wall thickness varied with deposition time while average post height was ~500 µm.

B. Growth of Hippocampal Cells on Polymer Arrays

Cell viability studies are reported for arrays containing PPy/HA and PPy/NaDBS posts. Arrays were seeded with a constant concentration of hippocampal cells following treatment with cell adhesion or proliferation molecules (*e.g.* poly D-lysine [PDL], laminin, human plasma fibronectin [huPF], nerve growth factor [NGF]) to determine the effect, if any, on growth and viability. PDL-, laminin-, and huPF-coated PPy/HA arrays co-deposited with or without laminin all supported cell growth (albeit variably) while neurons did not grow on PPy/HA arrays coated with NGF (not shown).

Neural cells seeded on PPy/0.05 M NaDBS arrays showed signs of neurite outgrowth but died within a week post-seeding probably due to leaching of the salt from the posts. Posts doped with a lower concentration of NaDBS (*e.g.* 0.005 M) may prove to be better substrates.

PPy/HA arrays coated with laminin or huPF consistently supported the growth of healthy neurons for periods of up to 28 days. Furthermore, neurons appeared to extend from the cell bodies around and up the PPy/HA posts (Fig. 3).

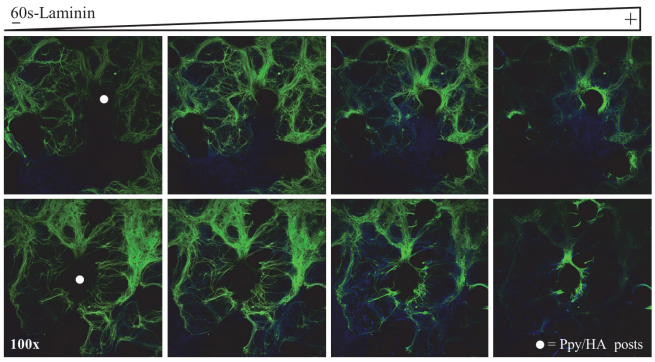

Figure 3. Confocal images of neurons growing around and up PPy/HA posts (24 DIV). Axons and dendrites were identified using a 1:500 dilution of mouse anti-class III β-tubulin followed by Alexa 488-GAM conjugated secondary antibody and a 1:1000 dilution of chicken anti-MAP2 followed by Alexa 633-GAC conjugated secondary antibody respectively. ○ denotes a polymer post. Magnification 100x.

Contact between the dendrites and laminin- or huPF-treated PPy posts is shown in Fig. 4A while the presence of glial cells which are required for long term neuronal culture is shown in Fig. 4B.

The height of the current posts is sufficient to permit growth of a feeder monolayer of glia before seeding neurons while still ensuring direct contact between the neurons and electrodes. Growth of neural cells on PPy/ToS and PSS posts remains to be assessed as does the biostability of the posts over a longer time frame. Inclusion of carbon nanotubes in the PPy/HA posts, co- and/or post-functionalization together with permanent modification of the PDMS surface could be used to improve the conductivity and long term growth of neuronal cultures respectively.

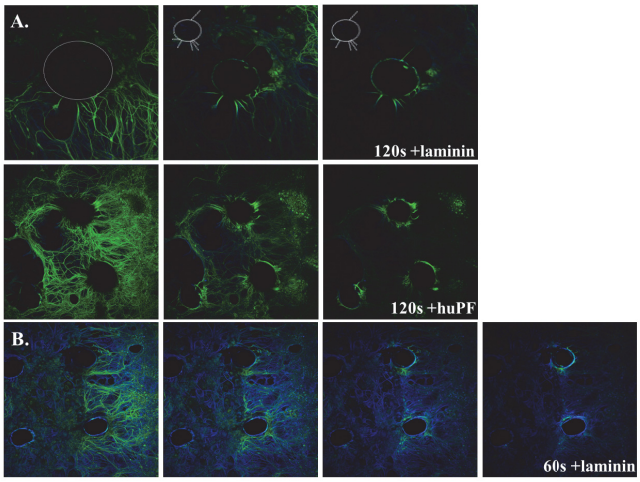

Figure 4. A) Confocal images of 16 DIV neuronal cultures growing around and up PPy/HA posts. Neurons were immunostained with 1:1000 dilutions of each of dendrite specific rabbit anti-MAP2 and astro-glial cell specific chicken anti-GFAP followed by 1:1000 dilutions of Alexa 488-GAR (green) and Alexa 633-GAC (blue) conjugated secondary antibody respectively. B) Confocal images of neural cultures immunostained with anti-MAP2 and anti-GFAP antibodies as discussed in A. Mag 100x.

IV. Conclusion

We have developed an inexpensive, scalable fabrication process that generates 3D polymer arrays capable of supporting neuronal cell growth. The ability to incorporate different dopants and bioactive molecules permits cell specific optimization while the geometry allows for good electrical contact even in the presence of a feeder layer.

Preliminary work on interfacing the arrays with MEAs yielded successful recording of signals from individual posts when PPy/ToS arrays were seated on a commercially available pMEA (www.multichannelsystems.com). Future work would involve improving this interface, demonstrating rapid individual stimulation of each of 64 post electrodes within an array followed by patterned stimulation and imaging of neurons seeded on the arrays using a calcium-sensitive dye.

Finally, the potential to pattern our electrodes in a layer of PDMS to create a flexible, biocompatible PDMS/PDMS polymeric MEA opens the possibility that in addition to their being used in drug discovery and to study synaptic plasticity at the research level, strategies could be devised enabling implantation of these devices into live animals to study neuronal network activity.

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