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Neuronal excitability and network formation on optically transparent electrode materials

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Abstract

With the advent of genetically-encoded optical tools to trigger or report neuronal activity, new designs for multielectrode arrays (MEAs) used in neural interfacing incorporate both optical and electrical modes of stimulating or recording neural activity. Likewise, the need to improve upon the biocompatibility of implanted MEAs has moved the field towards the use of softer, more compliant materials in device fabrication. However, there is limited available information on the impact of the materials used in MEAs on the function of interfaced individual neurons and neuronal networks. We assessed the responses of rat cortical neurons on optically transparent materials commonly used in the construction of “next-generation” devices: indium tin oxide (ITO), parylene-C, and polydimethylsiloxane (PDMS). We found that neuronal network formation and spiking responses to electrical stimulation were enhanced in neurons cultured on ITO. We observed reduced excitability and synaptic connectivity between neurons cultured on PDMS. We hypothesize that the superior conductivity of ITO and suboptimal neuronal attachment to PDMS contributed to our results.

I. Introduction

The research and clinical applications of implanted microelectrode arrays (MEAs) are rapidly expanding. Devices are becoming increasingly sophisticated to accommodate multi-modal sensing and stimulation capabilities (electrical, optical, chemical), enabling more precise and targeted interrogation of neural circuits [1]. Likewise, “next-generation” MEAs are being designed to improve biointegration with the brain, where high-density arrays with smaller site sizes and softer, more biocompatible material interfaces are used to increase the

longevity and spatial resolution of neuronal sampling[2]–[5]. In recent years, MEA designs with integrated optoelectronics fabricated from polymer substrates have been reported[1]. These devices combine the advantages of improved biocompatibility with advanced interfacing modalities.

As the development of innovative MEAs progresses, there is a need to understand the influence of design features on effective neuronal sampling. Studies characterizing the effects of device architecture/dimensions, material stiffness, and surface chemistry on tissue-device integration are necessary to identify guiding principles for design[6]. In this study, we explored the influence of ITO, Parylene-C, and PDMS on the excitability and network formation of cultured primary cortical neurons over a three week period. These materials are all optically transparent and have been used in MEA construction; therefore, they are amenable to the development of devices with integrated optoelectronics. We found that ITO accelerated the adoption of mature electrophysiological characteristics and promoted synaptic integration of neurons cultured on its surface. Conversely, PDMS exhibited diminished cellular attachment, network formation, and excitability. Parylene-C produced neuronal responses which were indistinguishable from tissue culture-treated plastic coverslips (“control”). Substrate stiffness, surface chemistry, and conductivity are known to influence neuronal responses[6], and we propose that ITO has a superior blend of traits that favors the formation of active neuronal networks.

II. Materials and Methods

A. Neuronal culture

Primary cultures of rat embryonic cortical neurons (E18) were seeded and maintained according to the supplier’s instructions (ThermoFisher). Three discrete cultures of four substrate conditions were explored: (1) ITO coated PET (polyethylene terephthalate) (639303-5EA, Sigma-Aldrich), (2) parylene-C, (3) PDMS, and (4) control tissue culture plastic (polyolefin Thermanox coverslips, ThermoFisher). Five-micron parylene-C was deposited on untreated coverslips using a chemical vapor deposition (CVD) system (PDS2010 Parylene Coater, Specialty Coating System). PDMS prepolymer was prepared by mixing the elastomer base and curing agent in a ratio of 10:1 (Sylgard 184, Dow Corning). The PDMS prepolymer was poured on a petridish, baked at 40°C for 4 hours, and cut into small disks with the same size as the coverslips. All substrates were coated with poly-D-lysine to facilitate neuronal attachment.

B. Quantification of synaptic connectivity

Neurons were fixed at 7, 14, and 21 day time points in 4% paraformaldehyde. Cells were rinsed in PBS, blocked in 20% normal goat serum (NGS), and incubated in primary antibody solution overnight at 4°C. Primary antibody solution contained synaptophysin (1:1000, Abcam) and PSD-95 (1:500, Abcam) in a solution of 5% NGS and 0.3% triton in PBS. Cells were rinsed before applying a secondary antibody solution containing Alexafluor goat anti-rabbit 594 (1:200, ThermoFisher) and Alexafluor anti-mouse 488 (1:200, ThermoFisher) in a solution of 5% NGS and 0.3% triton in PBS. Cells were rinsed and mounted with ProLong Antifade medium (ThermoFisher). Synaptic puncta were imaged on

an Olympus Fluoview confocal microscope. Acquired images were then analyzed using the puncta analyzer plugin for ImageJ 1.29 written by Barry Wark to quantify somatic synaptic puncta density per cell imaged[7].

C. Electrophysiology

Cells are placed in carboxygenated physiological external cellular saline solution containing (in mM): 126 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 2 CaCl₂ 2H₂O, 2 MgSO₄ 7H₂O, 26 NaHCO₃, and 10 Glucose (pH=7.4). Borosilicate glass pipettes were filled with internal solution (in mM) of 135 K Gluconate, 7 NaCl, 10 HEPES, 2 MgCl₂, 2 Na₂ATP, and 0.3 Na₂GTP. Pipette resistances were ~5-10 MΩ. Cortical neurons were sealed to a resistance >1 GΩ. Following break-in and verification of access resistances (<50 MΩ), cells were stimulated with step injections of current (0.1 nA) from a holding potential of ~-70-80 mV. To assess excitability, recordings of ~10-15 cells per condition per time point were analyzed for the maximum number of action potentials elicited in any single trace ("APmax"), the amplitude of the first spike, and the average overall number of spikes per trace ("average spikes").

D. Statistical analysis

Results were analyzed with a mixed-model ANOVA and post-hoc Fisher's Least Significant Differences test (significance defined at p<0.05 level).

III. Results

A. Neural network formation is increased in developing neurons cultured on ITO and decreased on PDMS at later time points.

Neurons cultured on ITO had robust neurite outgrowth and extension across the substrate surface, while PDMS showed limited attachment and clustered cellular growth within "islands." Parylene-C exhibited cell growth that was indistinguishable from neurons cultured on control plastic coverslips. These observations were supported by quantitative analysis of synaptic puncta staining (Fig. 2). By the 21 day time point, ITO had significantly enhanced synaptic connectivity in comparison to all other conditions (p<0.05). For the duration of the study, PDMS had significantly reduced synaptic formation in comparison to the other conditions tested (7 days: less than ITO and parylene; 14 days: less than all other conditions, p<0.05; 21 days: less than all other conditions, p<0.001).

B. Regenerative spiking is increased in developing neurons cultured on ITO and decreased on PDMS at early time points.

ITO favored enhanced excitability at the 7 day time point in culture in comparison to PDMS (increased APmax, Fig. 3-4A, p<0.05; increased average spikes, p<0.05, not shown). However, at later time points (14 and 21 days), there was no effect of substrate condition on excitability. Likewise, there was no difference in the amplitude of the spikes between any substrates condition at any time point (Fig. 4B); this implies that intrinsic excitability of individual neurons was unaffected by the substrate used. However, spike amplitude increased in culture over time for all conditions (Fig. 4B), which is an expected consequence of neuronal maturation[8].

IV. Discussion

Parylene-C, PDMS and ITO are all transparent, biocompatible candidate materials for the fabrication of “next-generation” electrode arrays[9]–[12]. Each substrate offers unique advantages in addition to their superior optical properties. Parylene-C is commonly used as an encapsulation material as it is an electrical insulator which is also biologically and chemically inert [9], [10]. As such, it is a useful barrier to protect electrical components from biological environments while isolating tissue from the potentially toxic products of electrode degradation[10]. PDMS is an extremely compliant biomaterial with a Young’s modulus much closer to brain tissue than MEA materials such as silicon, polyimide or parylene[12]. ITO has the distinctive characteristic of excellent conductivity [11], and our study revealed additional benefits of ITO on the synaptic development and electrophysiology of interfaced neurons.

We found that neurons plated on ITO displayed increased synaptic connectivity in long-term culture (Fig. 1, 2) which followed observations of enhanced excitability recorded during the first week *in vitro* (Fig. 3, 4). These observations may be linked: enhanced spiking activity can promote and reinforce neuronal connectivity [13]. ITO is conductive, whereas the other tested materials are not (resistivity: $\sim 10^{16} \Omega\text{-mm}$ for PDMS and parylene-C; $\sim 10^{-3} \Omega\text{-mm}$ for ITO). Neurons cultured on conductive carbon nanotube substrates develop enhanced synaptic connections, higher instances of spontaneous firing, and longer neurite extensions[14]. It was suggested that conductive nanotubes potentiate dendrosomatic electrotonic coupling in attached neurons, effectively ‘shorting’ synaptic inputs to the somatic compartment and increasing regenerative spiking[11]. While the authors found that conductivity in the absence of nanotube topography (as for ITO substrates) could not reproduce the results of the conductive nanotubes, they performed their assessment in the 8-14 day time frame[11]. We found that enhanced repetitive spiking on ITO substrates was evident at 7 days but absent afterward. It is possible that the conductivity of ITO accelerates adoption of repetitive spiking characteristics during maturation, but that all substrates reach a “plateau” of mature excitability in long-term culture. Additionally, increasing the conductivity of ITO produces spiking characteristics which were closer to nanotube substrates[11]; it may be that neuronal excitability can be tuned by altering the resistivity of the substrate.

Our data indicate suboptimal network formation and electrophysiological characteristics of neurons cultured on PDMS in comparison to the other materials tested. PDMS is a softer substrate in comparison to the remaining conditions (Young’s moduli: $\sim 10^{-1}$ MPa for PDMS, $\sim 10^3$ MPa for parylene, and $\sim 10^3$ MPa for PET substrate for ITO surface). Substrate stiffness can be sensed by translating external forces from focal adhesion points into intracellular biochemical signals[15]. For neurons, stiffer substrates favor increased excitability and network formation[16], [17]. A proposed underlying mechanism is that stiffer substrates facilitate calcium influx and augment synaptic transmission as a consequence. Our observation of relatively reduced network formation and excitability on PDMS may be due to a related mechanism. Since ITO has a similar stiffness to parylene-C and control plastic coverslips, the accelerated electrophysiological maturation followed by

enhanced connectivity on ITO is likely due to a combination of its superior blend of electrical and mechanical characteristics.

V. Conclusion

Our study provides a direct comparison of the influence of optically transparent candidate electrode materials on the function of interfaced neurons and neural networks. Our work suggests that conductive substrates accelerate electrophysiological maturation and soft substrates impede it. These findings are applicable to the design of “next-generation” neuroprostheses as well as the choice of substrate materials used in the culture of developing neurons. Future work is aimed at observing candidate materials within the *in vivo* environment, which has greater complexity than *in vitro* conditions.

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VII. References

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Figure 1.

Representative images of synaptic puncta staining on transparent electrode materials and control tissue culture plastic (red = PSD-95, green = synaptophysin, yellow = colocalized staining indicative of synapses). Neurons cultured on ITO had increased expression of colocalized puncta after 21 days in culture. Scale = 50 microns.

Figure 2.

Quantification of synaptic puncta reveals reduced network formation on PDMS substrates at all time points and enhanced synaptic connectivity on ITO at the 21 day time point. # = less than parylene and ITO at the same time point, $p < 0.05$; * = significantly different from all other conditions at the same time point, $p < 0.05$; ** significantly different from all other conditions at the same time point, $p < 0.001$. $n = 10-30$ per condition per time point.

Figure 3.

Representative spike trains for substrate materials (based on APmax). ITO displayed enhanced spiking at the earliest time point (7 days).

Figure 4.

A) Quantification of spiking responses reveals increased APmax in ITO and control versus PDMS samples, * = significantly different within the same time point, $p < 0.05$. B) No differences were observed in spike amplitude (a reflection of intrinsic excitability). $n = 10-15$ cells per condition per time point.