Effects of Carbon Nanotube and Conducting Polymer Coated Microelectrodes on Single-unit Recordings *in vitro*

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***Abstract*— Neuronal networks cultured on microelectrode arrays (MEAs) have been utilized as biosensors that can detect all or nothing extracellular action potentials, or spikes. Coating the microelectrodes with carbon nanotubes (CNTs), either pristine or conjugated with a conductive polymer, has been previously reported to improve extracellular recordings presumably via reduction in microelectrode impedance. The goal of this work was to examine the basis of such improvement *in vitro*. Every other microelectrode of *in vitro* MEAs was electrochemically modified with either conducting polymer, poly-3,4-ethylenedioxythiophene (PEDOT) or a blend of CNT and PEDOT. Mouse cortical tissue was dissociated and cultured on the MEAs to form functional neuronal networks. The performance of the modified and unmodified microelectrodes was evaluated by activity measures such as spike rate, spike amplitude, burst duration and burst rate. We observed that the yield, defined as percentage of microelectrodes with neuronal activity, was significantly higher by 55% for modified microelectrodes compared to the unmodified sites. However, the spike rate and burst parameters were similar for modified and unmodified microelectrodes suggesting that neuronal networks were not physiologically altered by presence of PEDOT or PEDOT-CNT. Our observations from immunocytochemistry indicated that neuronal cells were more abundant in proximity to modified microelectrodes.**

I. INTRODUCTION

Planar microelectrode arrays (MEAs) are commonly used to record extracellular action potentials from excitable cells such as neurons and cardiomyocytes *in vitro* [1]–[3]. A typical MEA consists of a glass substrate patterned with a conductor such as gold or indium tin oxide [4], [5]. The recording sites, commonly 10-30 μm in diameter, are exposed while the remaining surface is passivated by an insulator such as polydimethylsiloxane or parylene C [1], [6]. In contrast to single-cell techniques e.g. patch clamp, MEAs are non-invasive and can record activity from multiple neurons simultaneously. Therefore, MEAs are suitable platforms for a variety of applications including pharmacological assessments [7], detecting neuroactive compounds [8], or studying neuronal network dynamics [9].

Although MEAs are intended to quantify network-level activity, the number of well-resolved action potentials from distinct neurons or so-called 'units' is limited compared to the total number of neurons on the substrate [9], [10]. The problem is exacerbated by the fact that: 1) the magnitude of extracellular potentials decays rapidly over distance [11]; and 2) metal microelectrodes can exhibit high impedance resulting in thermal noise which can mask single units by reducing the signal-to-noise ratio [12]. Therefore, approaches which reduce the impedance of microelectrodes offer the promise of improving the signal-to-noise ratio and enhancing the detection of units for neuronal recordings [13].

In recent years, it has been reported that coating the microelectrodes with nanomaterials such as carbon nanotubes (CNTs) and conductive polymers might improve the quality of neuronal recordings, e.g. larger spike amplitudes or higher spike rates both *in vivo* and *in vitro* [14]–[16]. Such nanomaterials produce microelectrodes with a large surface area, high electrical conductivity and robust mechanical properties. Having a large surface area and significant porosity, the coatings enable microelectrodes to behave as super-capacitors which may improve the coupling with electrically active cells [16]. Additionally, the rough surface of the CNTs may provide scaffold-like structure to promote cell adhesion and growth [17].

In this work, we have examined the basis by which electrochemically-deposited PEDOT-CNT and PEDOT microelectrodes affect neuronal recordings *in vitro*. We observed that the yield, defined as percentage of microelectrodes with neuronal activity, was significantly higher for modified microelectrodes. However, activity measures, such as spike rate, were similar for modified and unmodified microelectrodes. The density of neurons and astrocytes surrounding modified and unmodified electrodes was compared using immunocytochemical labeling. There was significantly more neuronal labeling proximal to the modified electrode sites suggesting that an increased density of neurons surrounding coated sites may contribute to increased yield in single unit recording.

II. METHODS

A. Electrochemical Deposition of PEDOT-CNT and PEDOT

An aqueous CNT solution was prepared by ultrasonically dispersing 200 μg/ml carboxylic-functionalized CNTs (Cheap Tubes Inc., Brattleboro, VT) in deionized (DI) water with 0.5% poly(sodium 4-styrenesultanate) (PSS; Sigma-Aldrich, St. Louis, MO) as surfactant. To obtain EDOT-CNT solution for PEDOT-CNT coatings, 10 mM ethylenedioxythiophene (EDOT) monomer was added to the dis-

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persed CNTs prior to the electrodeposition at the ratio of 4:1. In a similar way, EDOT solution for PEDOT-only coatings was prepared by making an aqueous solution containing 10 mM EDOT and 100 mM PSS.

Substrate-integrated MEAs each consisting of an 8×8 grid of 60 μm^2 microelectrodes, were purchased from the University of North Texas (MMEP3; Center for Network Neuroscience, University of North Texas, Denton, TX). A potentiostat/galvanostat Reference 300 (Gamry instruments, Warminster, PA) was used to electrochemically polymerize and deposit either PEDOT-CNT or PEDOT on every other microelectrode of the MEA. Such patterning allowed for comparison between modified and unmodified microelectrodes within the same culture. The deposition was performed under potentiostatic condition by applying 0.9 V for 40-45 seconds using a large platinum wire (Ward Hill, MA) as the reference/counter electrode.

B. Electrochemical and Morphological Characterization

Electrochemical impedance spectroscopy (EIS) was performed on representative subsets of modified and unmodified microelectrodes before and after the deposition. The measurements were done in the presence of 1x phosphate buffer saline (PBS) under a two-electrode configuration. A sinusoidal waveform with 10 mV amplitude was applied over frequencies of 1 Hz to 100 KHz.

The surface morphology of modified and unmodified microelectrodes was examined with a field emission scanning electron microscope (SUPRA-55 VP; Carl Zeiss Microscopy, Thronwood, NY). The SEM was operated at 3 KV.

C. MEA Preparation and Primary Cell Culture

The MEA preparation and primary cortical cell culture methods were similar to that described previously [7], [18]. All animal procedures were approved by the Institutional Animal Care and Use Committee of George Mason University (Fairfax, VA). Briefly, the MEAs were treated with poly-D-lysine (PDL; Sigma-Aldrich) and laminin (Sigma-Aldrich). Cortical tissue from embryonic day 17 mice (CD-1; Charles River, Wilmington, MA) was isolated and then enzymatically dissociated. Cells were seeded at the density of 100,000 in a 50 μL droplet. The cultures were incubated at 37^{o}C with 10% CO_2 and maintained in Dulbecco's minimum essential medium (DMEM; Life Technologies, Grand Island, NY) supplemented with horse serum (Atlanta Biologicals, Lawrenceville, GA), fetal bovine serum, B-27 (Life Technologies), and ascorbic acid (Sigma-Aldrich). After 2-3 days *in vitro*, serum was removed from the culture media and a 50% media change was performed twice a week for at least 21 days.

D. Extracellular Recording

The cultures were allowed to mature for at least three weeks *in vitro*. Extracellular action potentials were recorded using a 64-channel data acquisition system OmniPlex (Plexon Inc., Dallas, TX) with sampling frequency of 40 KHz/channel. The recording sessions were 30-40 minute in duration and the temperature was controlled at 37 ± 1^oC during the session. Spikes were detected if they passed a threshold that was set to be at least 5 times higher than the standard deviation of the background noise. Spikes were sorted into distinct units by scanning K-means algorithm using Offline Sorter V.3 (Plexon Inc., TX). The clustered waveforms were visually inspected and those with relevant physiological shapes were considered as valid units.

E. Immunocytochemistry

The cells were fixed using 4% paraformaldehyde (PFA) followed by permeabilization with 0.1% Triton X-100 (Sigma-Aldrich). The samples were then incubated in the blocking buffer which contained 4% normal goat serum (Sigma-Aldrich) in PBS. Rabbit anti-mouse glial fibrillary acidic protein (GFAP; 1:725, Dako North America Inc., Carpinteria, CA) and mouse anti-rat neuronal nuclei (NeuN; 1:1000, Millipore, Billerica, MA) were used as primary antibodies to detect reactive astrocytes and neurons, respectively. For GFAP and NeuN, goat anti-rabbit IgG Alexa fluor 488 (1:200, Life Technologies) and goat anti-mouse IgG Alexa fluor 546 (1:200, Life Technologies) were used as secondary antibodies, respectively. The samples were then imaged with a fluorescence microscope (Nikon eclipse Ti; Nikon Instruments, Melville, NY).

F. Data Analysis

For extracellular recordings, sorted units with spike rate greater than 0.1 Hz were considered active and networks with more than 10 active units were included in data analysis. A burst was defined as an occurrence of at least three spikes with maximum inter-spike interval (ISI) of 100 ms [19]. For each active unit, signal-to-noise (SNR) was defined as average of peak-to-peak spike amplitudes over the standard deviation of the noise for the corresponding channel. All the calculations for spike rate, spike amplitude, SNR, burst rate and inter-burst interval (IBI) were done in MATLAB (Mathworks, Natick, MA). All the activity measures were normalized to the mean of the same measure for unmodified electrodes.

The fluorescence microscopy images were processed with a custom routine in MATLAB designed to quantify the intensity of fluorescent labeling as a function of distance around modified and unmodified microelectrodes.

Data are reported as mean $\pm$ standard error of the mean. To statistically compare the yield between modified and unmodified electrodes, the test of proportion was utilized. For all the other measures, a non-parametric, two-sample Kolmogrov-Smirnov test (KS test) was utilized to determine whether any given two datasets i.e. modified vs. unmodified had different distributions [20]. For all the statistical tests, $P < 0.05$ was considered significant.

III. RESULTS

A. Electrochemical Characterization and Morphology

Both PEDOT-CNT and PEDOT were successfully electrodeposited on gold microelectrodes. Magnitude and phase

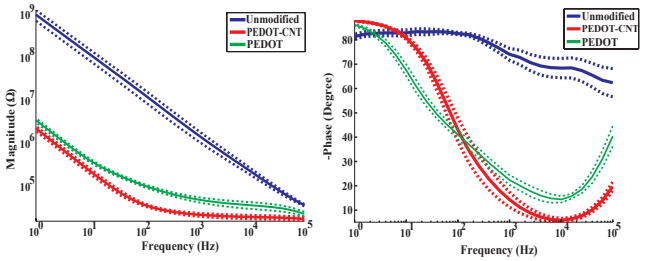

Fig. 1. EIS measurements of typical coated versus uncoated microelectrodes. Reduction in the magnitude (left) and shift in the phase of the EIS (right) suggests an increase in the surface area after deposition of PEDOT and PEDOT-CNT. $N = 9$ microelectrodes for each group. Solid lines are mean and dashed lines show the $\pm$ standard error of the mean.

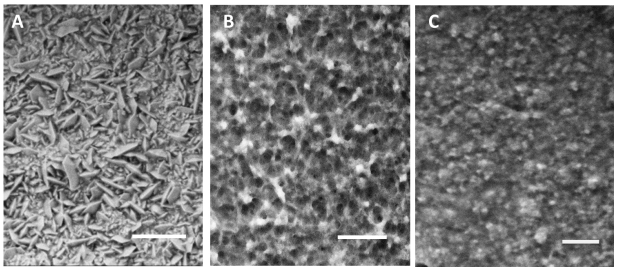

Fig. 2. SEM images of unmodified and modified microelectrodes. The surface contained more nano features and porosity after modification with PEDOT-CNT and PEDOT. A) bare (gold) microelectrode with poly crystalline structure. B) PEDOT-CNT modified surface in which CNTs are embedded inside the polymer C) PEDOT modified microelectrode. The scale is 1 μm.

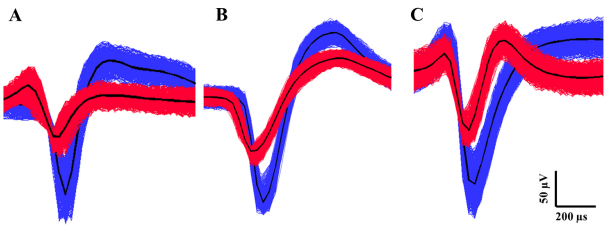

Fig. 3. Represantative well-resolved units detected from an unmodified (A), PEDOT (B), and PEDOT-CNT (C) microelectrode. The spike waveforms from modified electrodes resmebles those from unmodified ones. All the detected spikes were sperated into different clusters using scanning K-means algorithm.

of the impedance for representative modified and unmodified microelectrodes are shown in Fig. 1. A significant reduction in the magnitude between frequencies of 1 to 10 KHz occurred after deposition of PEDOT-CNT and PEDOT. Specifically, the magnitude of the impedance at 1 KHz, the typical frequency associated with extracellular recordings, significantly dropped from 1.7 ± 0.3 $M\Omega$ $(n = 9)$ to 22.9 ± 2.2 $K\Omega$ $(n = 9)$ and to 51.8 ± 6.9 $K\Omega$ $(n = 9)$ for PEDOT-CNT and PEDOT modified microelectrodes, respectively. The shift in the phase of the impedance for modified microelectrodes suggests an increase in effective surface area. Such changes in impedance for both PEDOT-CNT and PEDOT were consistent with previous findings [21], [22].

SEM images of the unmodified and modified microelectrodes are shown in Fig. 2. The microstructures on the surface of the gold microelectrode (Fig. 2A) became smaller and formed nanostructures after the deposition of PEDOT-CNT and PEDOT (Fig. 2B and 2C). The CNTs were embedded inside the polymer (Fig. 2B), which provided a rougher and more porous surface. Such observations are consistent with the features previously reported for PEDOT-CNT and PEDOT modified surfaces [23], [24].

B. Extracellular Recordings

The frontal cortex recording data consist of measurements from 431 total units over 10 networks cultured from 7 different mice. Out of the total 10 MEAs, 5 were modified with PEDOT-CNT and the other 5 had only PEDOT on every other microelectrode.

Spike waveforms recorded from the coated microelectrodes were similar to those from uncoated sites. (Fig. 3). The peak-to-peak (p-p) spike amplitude for the units from unmodified microelectrodes had the range of 40 - 207 μV whereas those from PEDOT-CNT and PEDOT microelectrodes had the range of 25 - 338 μV and 30 - 278 μV, respectively. Although the modified microelectrodes had lower impedance, which should result in lower thermal noise, the RMS noise values in extracellular recordings were similar between modified and unmodified microelectrodes. The RMS noise was 6.7 ± 0.2 μV_{rms} for PEDOT, 6.6 ± 0.1 μV_{rms} for PEDOT-CNT and 7.0 ± 0.3 μV_{rms} for unmodified electrodes.

The PEDOT-CNT and PEDOT modified microelectrodes had yields, i.e. percentage of microelectrode sites showing observable single units, of 53% and 56%, respectively. However, the yield was only 34% for the unmodified microelectrodes which is consistent with prior work [9], [10]. The yield for modified microelectrodes was significantly higher than for unmodified microelectrodes ($P < 0.01$). For those microelectrodes that showed activity, the number of units per microelectrode was 1.53 ± 0.09 for PEDOT-CNT and 1.65 ± 0.12 for PEDOT modified microelectrodes. The unmodified microelectrodes had 1.35 ± 0.1 units per microelectrode. The cumulative distribution of microelectrodes with active units (Fig. 4) shows that comparing to unmodified microelectrodes, it was more likely to have a modified microelectrode with at least one active unit ($P < 0.05$).

The differences between the extracellular recordings from both modified and unmodified microelectrodes were further investigated by examining SNR and burst parameters (Table 1). No significant differences were found for spike rate, SNR and burst parameters, which suggests the electrophysiological properties of neurons around the modified microelectrodes were not affected by the nanomaterials.

C. Immunocytochemistry

Immunostaining for neurons and astrocytes was performed on PEDOT-CNT (n=2 networks) and PEDOT (n=1 network) modified MEAs to assess the proximity of the cells to the microelectrodes. As illustrated in Fig. 5, the mean intensity

TABLE I

NORMALIZED EXTRACELLULAR RECORDING MEASURES FOR MODIFIED MICROELECTRODES. ALL THE VALUES ARE NORMALIZED TO THE MEAN OF THE CORRESPONDING MEASURE FROM UNMODIFIED MICROELECTRODES. IBI IS INTER-BURST INTERVAL.

	Spike Rate	P-P amplitude	RMS noise	SNR	IBI	Burst duration	Burst rate
PEDOT-CNT	1.7 ± 0.5	1.2 ± 0.1	1.0 ± 0.1	1.4 ± 0.2	1.2 ± 0.3	1.6 ± 0.5	1.6 ± 0.4
PEDOT	1.3 ± 0.4	0.9 ± 0.1	0.9 ± 0.1	1.0 ± 0.2	1.2 ± 0.6	1.1 ± 0.2	1.2 ± 0.4

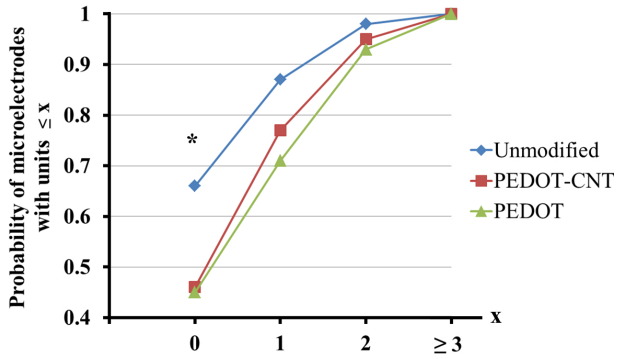

Fig. 4. The cumulative distribution of electrodes with x active units. The x could be 0, 1, 2 or $\geq$ 3. The unmodified microelectrodes had a higher percentage of being non-active ($x = 0$). The modified electrodes were compared against non-modified electrodes. * denotes the significance ($P < 0.05$).

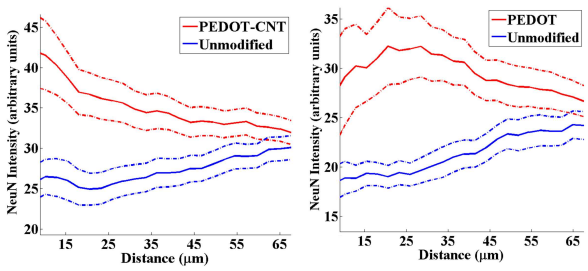

Fig. 5. Quantitative immunohistochemical analysis of neuronal nuclei using NeuN for PEDOT-CNT (left) and PEDOT (right) modified microelectrodes. NeuN intensity suggests there was a higher density of neuronal cell bodies around the coated microelectrodes (between the 10 μm to 40 μm away of the center). The solid line in every figure is the mean and the dashed lines are standard error of the mean. ($P < 0.05$)

of the NeuN marker was higher by 36% and 43% between 10 μm to 40 μm away from the center of the microelectrodes for PEDOT-CNT and PEDOT compared to the unmodified group ($P < 0.05$). These findings suggest that more neuronal cell bodies were localized proximal to the modified microelectrodes than unmodified ones, an observation consistent with the higher yield for modified microelectrodes. In contrast, the immunostaining for GFAP yielded no statistical difference, suggesting that the density of astrocytes was similar for modified and unmodified microelectrodes (data not shown).

IV. DISCUSSION

Our findings suggest that modifying microelectrodes with thin layers of PEDOT-CNT and PEDOT increases the yield in the extracellular activity for *in vitro* neuronal cultures whereas the electrophysiological behavior of the networks might not necessarily be affected. By depositing these nanomaterials on every other microelectrode of a MEA, we were able to study and compare the effect of such coatings on neuronal recordings while the variables such as inter-culture differences were minimized. Furthermore, we observed that the improvement in neuronal recording yield was concurrent with an increased density of neuronal cell bodies around the coated microelectrodes. Considering that the action potentials rapidly decay over distance in extracellular environment [11], [25], a larger yield from modified microelectrodes could be potentially explained by presence of more neurons in proximity of such electrodes, a finding consistent with our immunocytochemistry results.

Although, no significant differences were detected in spike rate and burst parameters between modified and unmodified microelectrodes, some previous studies reported higher spike rate specifically for neurons on CNT-coated substrates [26], [27]. However, those results were in the presence of pristine CNTs, which either had vertically-aligned 3D structures or were deposited on large surface areas e.g. glass coverslips. Our SEM observations indicate that the coatings in the present study had thin mesh-like morphology and were confined to the microelectrode area. The differences in the surface density of the nanomaterials between previous work and the present study may explain the lack of network modulation observed in our experiments.

Considering the large decrease in the impedance after modification of the microelectrodes with PEDOT and PEDOT-CNT, lower RMS noise values on the modified microelectrodes was expected [28]. Surprisingly, the RMS noise was similar for modified and unmodified microelectrodes, which could possibly be due to an increase in the impedance of the coated films over time for modified microelectrodes. As reported in [29], aggregation of proteins in the polymer layer could result in such an increase in the modified microelectrodes. Furthermore, delamination of the coatings, which has been reported to be a limitation for many conducting polymer coatings, might also be a reason that the modified microelectrodes did not sustain their low impedance [30], [31].

Interestingly, our observations from immunocytochemistry suggest a higher density of neuronal cell bodies around modified microelectrodes. It has been reported that nanoscale surface topography of CNT or PEDOT films could provide a scaffold structure which might influence the neuronal adhesion [31]. In addition, it is also possible that adhesion promoters e.g. laminin as well as growth factors in cell culture medium could accumulate in the deposited films and

provide a suitable microenvironment for neuronal attachment and growth [32].

V. CONCLUSIONS

We have investigated the possible effects of CNT and PEDOT-CNT modified microelectrodes on neuronal activity *in vitro*. Our findings suggest that the yield in neuronal recordings was higher for the modified electrodes while the electrophysiological behavior of the network remained similar for both modified and unmodified electrodes. Such improvement *in vitro* may be due to the closer proximity of the neurons to the modified electrodes rather than a reduction in noise. Although the role of CNTs must be further investigated in improving the stability of the coating, we did not find any significant differences in neuronal recordings *in vitro* between PEDOT-CNT and PEDOT modified microelectrodes.

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