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Early Onset of Electrical Activity in Developing Neurons Cultured on Carbon Nanotube Immobilized Microelectrodes

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Abstract— **In this study, we test the hypothesis that increased surface roughness due to carbon nanotubes (CNTs) enhances neuronal adhesion and consequently electrical excitability of single neurons. Neurons are grown on CNT modified microelectrode arrays (MEAs). Multi-unit activity was seen as early as 4 days after seeding compared to 7 days in control cultures on microelectrodes without CNTs. The results overall, show earlier onset and higher level of electrical activity in neurons seeded on CNT modified MEAs compared to non-modified control MEAs. We conclude that CNTs on microelectrodes enhance electrical excitability of single neurons in culture.**

I. Introduction

Carbon nanotubes are cylindrical carbon molecules whose diameter and length are on the order of nanometers. CNTs exist as a single sheet and are referred to as single wall carbon nanotubes (SWCNT) or as an array of sheets wrapped around each other referred to as multi walled carbon nanotubes (MWCNTs). Due to their unique mechanical, electrical, chemical and surface properties, nanomaterials have potentially exciting applications in biomedical research [1-3].

In particular, CNTs have been shown to be potentially useful in improving neuronal stimulation and recording capabilities of neuronal prostheses. This is in large part due to improved interfacial electrical impedance characteristics of microelectrodes modified with CNT.

Recently, several groups have investigated the effect of CNT modified substrate on neuronal adhesion. Work done by Ben-Jacob and Hanein demonstrated the potential for using CNT patterned islands for guiding neuronal growth [4]. In addition, work done by Webster et al showed that that neurons and astrocytes have a very different adhesion response to CNT modified substrates which can aid in creating neuron specific artificial substrates ideal for use in enhancing the neural prosthesis [2]. This specificity has been linked to changes in the substrate topography brought on by CNT modification, in particular surface roughness.

Generally, neurons have been shown to exhibit preference to rough surfaces (roughness around 20-70 nm) over flat ones regardless of the surface chemistry. Increased surface area due to the increase in surface roughness seems to allow for more favorable adhesion conditions [5]. Proper neuronal adhesion governs survival and has an effect on the ability of neurons to spread and form new connections. Adhesion is typical mediated through heterodimeric receptor complexes referred to as integrins which recognize and bind to adhesion proteins in the cellular environment called ligands. Grouping of integrin/ligands leads to the formation of protein complexes that leads to changes in the cytoskeleton and subsequently cell morphology. The formation of these adhesion complexes is affected by the topography of the adhesion substrat [6]. Furthermore, several prior studies have implicated integrf[ffin activation with causing changes in the electrophysiology of neurons. This has been attributed to integrin-gated and cytoskeleton anchored ion channels.

In this study, we attempt to assess the effect of modifying the substrate with CNTs on the electrical excitability of developing rat primary hippocampal neurons in culture. In this model, brain slice is enzymaticaly digested and neurons are separated from the slice via mechanical titration. The neurons are then seeded on a silicon substrate embedded with an array of metal microelectrodes. Hippocampal neurons were seeded on CNT modified 16-channel microelectrode arrays and their single neuronal electrical activity was monitored after 4 days in vitro.

II. Materials and Methods

A. Silicon MEA fabrication

The microelectrode arrays (MEA) are based on a silicon substrate with patterned gold microelectrodes (25 μm diameter). The MEA featured 16 microelectrodes arranged in a 4 x 4 with a 200 μm pitch. The reference and ground electrodes were fabricated on the MEA (500 μm x 200 μm). A 1 μm thick isolation layer of Si_3N_4 was deposited using plasma enhanced chemical vapor deposition (PECVD). Using thermal evaporation deposition a 20 nm chrome layer was deposited as an adhesion layer followed by a 250 nm gold layer. A wet etch technique using photolithography was used to create the MEA pattern. After the metal layer was patterned a 300 nm insulation layer of Si_3N_4 was deposited using PECVD. Photoresist was used as the masking material during the etching of the Si_3N_4. Reactive ion etch (RIE) was used to etch the Si_3N_4 layer in order to open the bond pads, ground electrodes, and microelectrodes.

B. CNT modification

Single walled carbon nano tubes (SWNT) were purchased from Sigma Aldrich. The procedure of depositing SWNT was as previously reported [7]. Briefly, the SWNT were made into a black suspension by mixing 10 mg of pure SWNT with 10 ml of dimethyl formamide (DMF). The solution was placed under ultrasonic agitation for 50 minutes. Several drops were placed on the microelectrodes

typically 5-10 µl and allowed to evaporate at 100°C. After the solution evaporated the MEA was rinsed with DI water wiped with clean room wipes. The steps were repeated 4-5 times until the microelectrodes appeared black. After mechanical polishing the carbon nanotubes (CNT) stayed only on the gold microelectrodes.

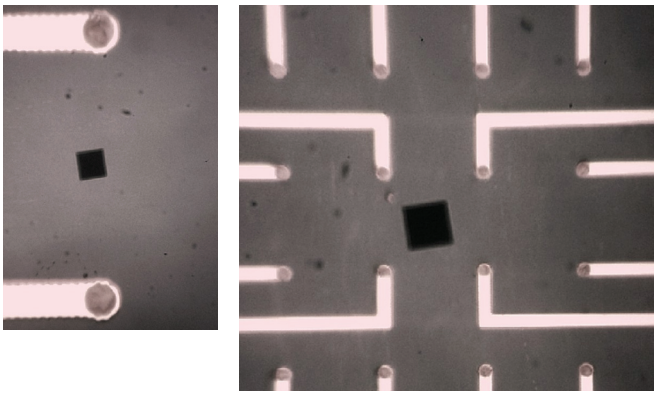

Figure 1: Micrograph of the 16 channel silicon MEA coated with CNT (seen as dark circular spots at the tip of the leads).

C. *Neuronal culture protocol*

Embryonic, day 18, Sprague/Dawley rat hippocampus slices obtained from Brainbits were used to prepare neuron cultures. Tissue was digested using papain and neurons where seeded on the MEA surface coated with laminin and PEI (seeding density 60,000/mm^2) [8].

D. *Recording network activity*

A multichannel data acquisition system (Plexon Inc., Dallas, TX) fitted with a custom recording stage was used for recording multi-unit activity from neurons. Sort client and offline sorter software (Plexon Inc, Dallas, TX) were used to analyze the data. A threshold of 4 times standard deviation of the noise was set for the spike activity. The recorded spikes passing threshold are then fitted to a preloaded templates matching features of neuronal spike activity. Data was collected simultaneously from all 16 microelectrodes. On occasion, microelectrodes picked up the activity of more than one neuron (multiple units). Cultures were removed from the incubator on day 4, 5, and 7 for recording. Recording sessions were fixed at 3 minutes each and the cultures were returned to the incubator.

E. *Impedance measurement*

The impedance measurements of both types of microelectrodes (n=10) (gold and gold with CNT) were tested by using a 3-electrode electrochemical workstation (CH instruments Inc., Austin, TX). A glass well was bonded to the MEA using biocompatible non-conducting epoxy. The glass well was filled with 1 M phosphate buffer saline (PBS). A platinum wire acted as the counter electrode while a Ag/AgCl was used as a reference electrode. Voltage amplitude of 5 mV and a frequency between 100 Hz-100 kHz was sampled.

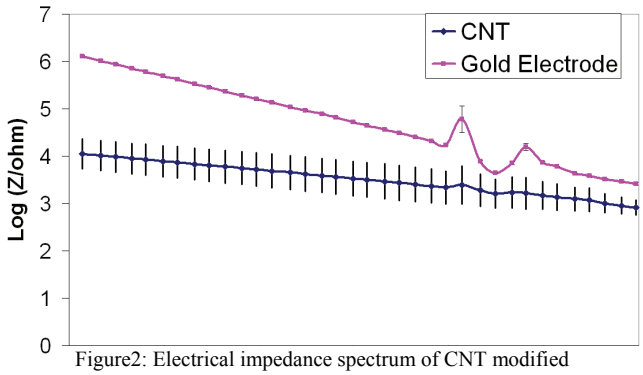

Figure2: Electrical impedance spectrum of CNT modified microelectrode shows lower impedance compared to bare gold microelectrode

III. Results

CNT were deposited on gold microelectrodes using simple post-processing procedures that have been previously described [6]. The presence of CNT is confirmed by the black appearance of the gold microelectrode (Fig. 1). Impedance data shown in Fig. 2 shows an order of magnitude drop measured at 1 kHz indicating increase of conductivity of the gold electrodes.

Neurons were cultured on MEAs and activity was recorded on day 4, 5 and 7 after seeding. The results shown in Figures 3-7 show that the neural activity was detected earlier in the modified CNT electrodes compared to bare gold electrodes (control). The electrical activity of the primary neurons were recorded as early as 4 days after seeding in the modified CNT electrodes, while the bare electrodes did not show activity until day 7. As shown in Fig. 3, the spike rate increased over the period of observation in the CNT immobilized microelectrodes.

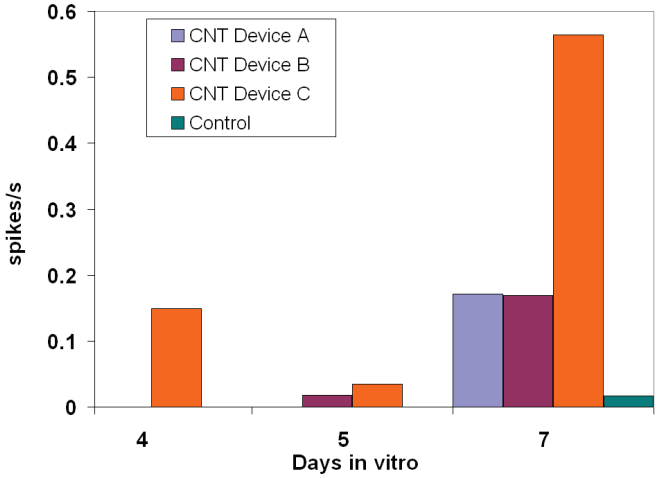

Figure 3: Average spike rate per device at three different time points after seeding.

The average number of units detected per microelectrode increased throughout the duration of the experiment as shown in Fig. 4. All of the devices with CNTs showed an increase of more than three times in the number of units detected per microelectrode from day 4 to day 7. The signal levels (or peak-to-peak amplitudes) showed marginal increase in amplitude from day 4 to day 7 as shown in Fig. 5.

However, on day 7 all of the devices had approximately the same peak-to-peak amplitude levels, which is also dependent on the distance of the neuron from the electrodes. The percentage of functional electrodes per device showed a slight increase each day as shown in Fig. 6. Typical multi-unit activity that was detected on day 7 is shown in Fig. 7.

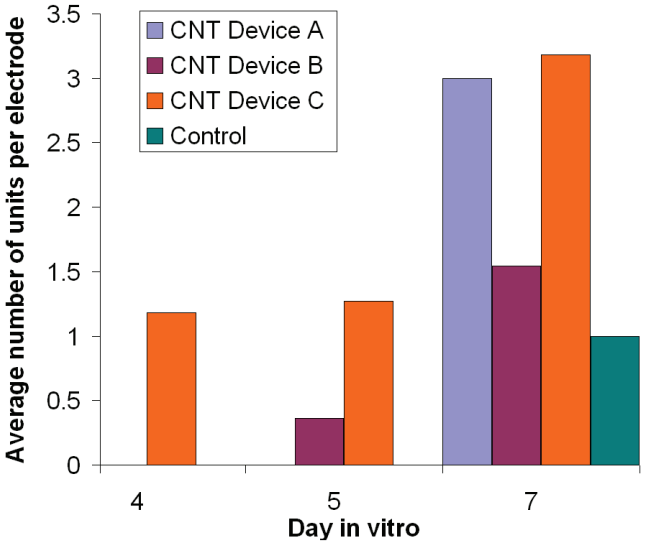

Figure 4: Average number of units per microelectrode at different time points after seeding

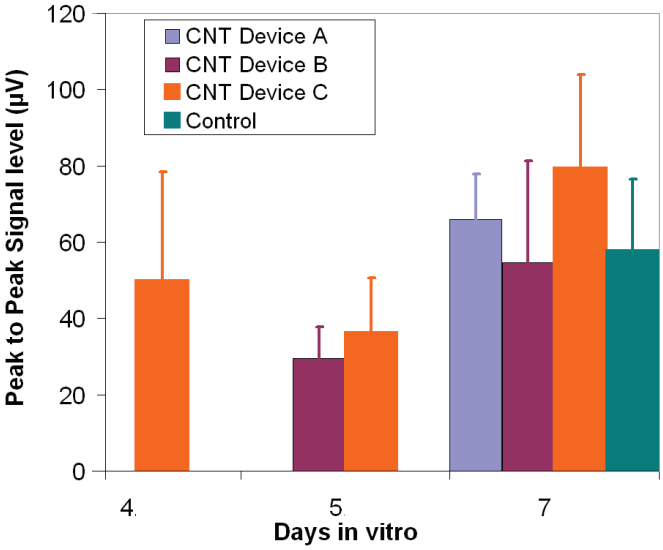

Figure 5: Average *peak* to *peak* amplitude of single units per active microelectrode at different time points after seeding.

IV. Discussion

Our results in Fig. 2 confirmed the results of earlier studies that showed CNT immobilization on the surface of microelectrodes lowers the electrical impedance of microelectrode [4,7,9].

Multi-unit recordings from developing neuronal cultures indicate that neurons grown on CNT modified microelectrodes exhibit earlier onset of electrical activity as shown in Fig. 3. The activity observed in neurons on CNT immobilized microelectrodes seems to progressively increase until day 7 both in average spike rate per electrode and number of units recorded. In contrast, control neuronal

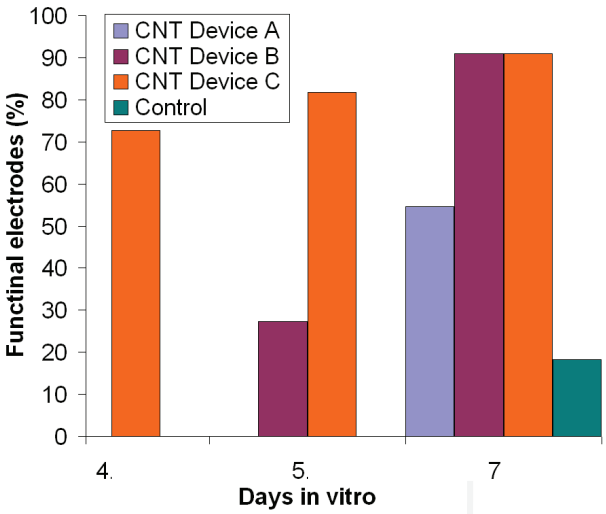

Figure 6: Percentage of microelectrodes picking multiunit activity at different time points after seeding

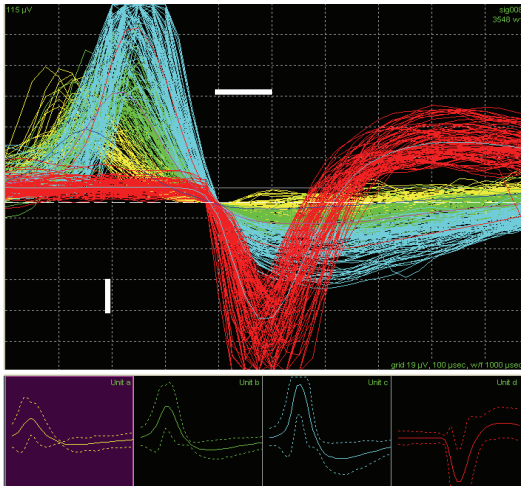

Figure 7: Voltage spike activity recorded from a single electrode. The figure above shows 4 units (each represented by a different color). Vertical scale bar 38 µV, horizontal scale bar 100 µsec.

cultures on microelectrodes with no CNTs showed no electrical activity until day 7 in vitro. Typically, neurons exhibit electrical activity around day 5 to day 7 in vitro after seeding at a high density (>70,000 neurons/mm^2). The mechanical titration used to separate neurons from brain slice for the purposes of growing them in culture leads to the loss of all the neuronal process. When neurons are seeded after harvesting they adhere and start to spread and from new processes to reestablish connections and return to functioning as a network [8].

We hypothesize that the increase in surface roughness in CNT immobilized microelectrodes provides cells with a larger surface area to adhere leading to an increase in the activation of adhesion integrins. Taking in account the role integrins play in neuronal development by modulating the neuronal cytoskeleton and activation of transmembrane ion channels, it is possible that the early onset of activity in neurons is mediated by an increase in integrin activation [10-14]. The same reasoning may explain the higher number of

electrically active neurons on CNT modified MEAs (Fig.3). Overall, we find that Figs. 1-3 show that neurons grown on CNT modified MEAs exhibit a distinct increase in the level of activity compared to the control. Since the peak-to-peak amplitude of action potentials recorded from single neurons depends on the distance of the neurons from the microelectrode, it is hard to draw firm conclusions on the level of activity based on just Fig. 4.

V. Conclusion

In this study, we have demonstrated that surface immobilized CNTs have a measurable effect on the electrical excitability of developing neurons in culture. Results demonstrated that neurons became electrically active earlier and in larger numbers than control cultures that were grown on microelectrodes without any CNTs. Future studies will have to confirm whether changes in integrin activation induced by the nanotopography is responsible in this distinct change in neuronal function.

VI. Acknowledgement

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VII. Bibliography