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In vitro recording of neural activity using carbon nanosheet microelectrodes

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ABSTRACT

The advent of nanotechnology has revolutionised our ability to engineer electrode interfaces. These are particularly attractive to measure biopotentials, and to study the nervous system. In this work, we demonstrate enhanced *in vitro* recording of neuronal activity using electrodes decorated with carbon nanosheets (CNSs). This material comprises of vertically aligned, free standing conductive sheets of only a few graphene layers with a high surface-area-to-volume ratio, which makes them an interesting material for biomedical electrodes. Further, compared to carbon nanotubes, CNSs can be synthesised without the need for metallic catalysts like Ni, Co or Fe, thereby reducing potential cytotoxicity risks. Electrochemical measurements show a five times higher charge storage capacity, and an almost ten times higher double layer capacitance as compared to TiN. *In vitro* experiments were performed by culturing primary hippocampal neurons from mice on micropatterned electrodes. Neurophysiological recordings exhibited high signal-to-noise ratios of 6.4, which is a twofold improvement over standard TiN electrodes under the same conditions.

1. Introduction

Micromachined devices for neural recording and stimulation have emerged as valuable research tools to unravel the complex biophysical aspects of the central nervous system, and even to effectively treat certain neural disorders [1–5]. In this work, we focus on one of the key components of these devices, namely the electrode material that interfaces the neural cells to the electronic read-out. Platinum [3], TiN [6], and metal oxides such as iridium oxide (IrO_x) [7] are currently considered as possible electrode materials. Of these materials, TiN is a promising candidate, as it is both bio- and CMOS-compatible [6], and has excellent thermal stability. In order to stimulate and record cells efficiently, the contact area of the electrode should be as large as possible, leading to studies using highly porous TiN layers. However, the resistance of narrow and deep pores limits the benefits of the increased electrochemical area [4]. Moreover, TiN is susceptible to oxidation, making it less suitable for chronic applications.

Carbon nanomaterials have been proposed as alternative materials for biomedical applications because of their good electrochemical stability and their high surface-area-to-volume ratio. Carbon nanotubes (CNTs) are probably the most extensively studied materials of this class, and have been shown to be beneficial for both electrical recording and as stimulation devices [8–19]. Further, the production cost of these nanomaterials has substantially dropped [20], and low cost printing processes of neural CNT electrodes has been established [21]. While CNTs provide a great increase in surface area, and good cell adhesion, they also suffer from limitations for electrode applications. (i) In our experience, the adhesion of the CNTs to the substrate is typically poor,

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sometimes resulting in delamination of the material. (ii) The synthesis of CNTs relies on catalyst particles, typically transition metals such as Fe, Ni or Co, which can elicit toxic reactions in the cells [22]. (iii) When introduced in liquid environments, which are indispensable for cell growth, the arrangement of nanofilaments can drastically change due to elasto-capillary interactions [15,23,24].

Alternatively, other nanostructured carbon allotropes have recently gained attention as they retain good surface-to-volume ratios and electrochemical stability, while at the same time being more robust, and therefore eliminating adhesion and elasto-capillary challenges [25–28]. In this work, we study the electrochemical behaviour of carbon nanosheets (CNSs) as nanostructured coating materials. Carbon nanosheets consist of several stacked graphene layers oriented vertically on the substrate, with properties similar to multi-layered graphene [29,30]. The as-grown layers can have edges exposed as thin as three graphene layers [29]. Recently, CNSs have been considered for energy storage solutions such as electrochemical supercapacitors [29,31], mainly because of their high conductivity, resistance to oxidation and high surface-to-volume ratio (the surface area of a single graphene sheet is $\sim$2630 $m^2 g^{-1}$). These features make CNSs also an interesting material for biomedical electrodes.

In this work, we demonstrate for the first time CNS-coated TiN electrodes for *in vitro* applications. A fivefold improvement in charge storage capacity and a 10-fold improvement in double layer capacitance was obtained using CNS-coated electrodes over TiN. Primary hippocampal neurons were cultured on the electrode arrays and recorded after 8 days *in vitro* (DIV). Spontaneous electrical activity was recorded with a signal-to-noise ratio as high as 6.4. As opposed to CNTs [9,16], no metallic catalyst like Fe was required for the CNS synthesis, hereby reducing possible cellular toxicity effects [22].

2. Experimental

2.1. Fabrication process

Fig. 1a depicts the fabrication process of individually addressable CNS electrodes. The substrate consists of a 4 inch Si wafer with 200 nm of thermal SiO_2. A 70 nm TiN layer was sputtered from a Ti target in a N_2 atmosphere and patterned using lift-off (step 2 of Fig. 1a). A 200 nm SiO_2 layer was deposited on top of the TiN layer, and after resist coating, exposure, and development, this oxide layer was etched in buffered HF to open the sensing area (step 3 of Fig. 1a). If timed appropriately, this step helps cleaning the TiN surface prior to the CNS deposition.

Next, CNSs were grown by plasma enhanced chemical vapour deposition (PECVD) (NanoCVD – Oxford Instruments plasma technology UK) according to a recent procedure [31]. In brief, samples were pretreated in a H_2 plasma (300 W) for 15 min at 0.5 Torr. Next, CNSs were grown from a CH_4/H_2 (1:10) mixture at 750 °C, 300 W and 0.5 Torr for 30 min. The substrate was removed from the chamber and allowed to cool under vacuum (1×10^{-4} Torr).

This process covers the entire substrate with CNSs as sketched in step 4 of Fig. 1a. For this application, we however only want to retain CNSs on the sensing area, and therefore a third lithography step was carried out to pattern the CNSs (step 5 in Fig. 1a). More precisely, we spin-coated, exposed and developed a photoresist (IX 845), which served as a protective layer to locally protect the CNSs during a subsequent O_2 plasma. As shown in step 6 in Fig. 1a, only the CNSs protected by the photoresist are retained after the plasma treatment. Once the unwanted CNSs are removed, the remaining photoresist is washed off using acetone and isopropanol. Finally, prior to measurement, the surface of the CNSs was modified by a 15 min UV/O_3 treatment to make them hydrophilic (see further).

Overall, the proposed fabrication process enables high throughput as only batch fabrication techniques are used. The use of lithography is, however, only cost-effective for the fabrication of large amounts of devices.

2.2. Characterisation methods

The electrical performance of CNS electrodes was measured using cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS). The *in vitro* experiment setup consisted of a mechanical holder and a printed circuit board (PCB) containing custom electronic circuits for signal amplification and processing [32]. The experiments were carried out using a three-electrode configuration placed inside a Faraday cage, using a commercial double-junction Ag/AgCl (3 M KCl) reference electrode and a large-area Pt counter electrode. Measurements were done using an Autolab PSTAT302N potentiostat with an integrated frequency response analyzer controlled by the NOVA software.

All electrochemical measurements were performed in phosphate buffered saline (PBS, 20 mM, 150 mM NaCl) with a pH 7.4. The potential of the working electrode was swept between −1.0 and 1.1 V at a scan rate of 0.5 V/s and the CSC_c was obtained from the 10th CV cycle. For the electrochemical impedance measurements a 10 mV (RMS) AC signal was applied with a frequency between 1 and 10^5 Hz.

All the recordings were performed with a gain of 1000 and a band-pass filter from 300 Hz to 6 kHz, in order to properly isolate spontaneous action potentials (i.e. rapid de- and repolarisations of the electrical membrane potential) in the cells. Processed signals were sampled at 20 kHz and digitised (12 bits) using a National Instruments USB-6259 data acquisition system. Custom acquisition software was developed to program the electronic circuits, as well as to record data. The Matlab based algorithm Wave_Clus was used to sort neural spikes with similar shapes. Spikes originating from a same neuron (i.e. unit) get separated and sorted in different clusters. This is a well-established method to sort and identify data from neural origin, omitting other interference artefacts [33].

2.3. Cell culture

To make *in vitro* cultures feasible, we packaged the multi-electrode chips on a custom PCB. A glass ring was glued on top of the PCB to contain the cells and the cell medium. Before culturing the cells, the TiN and CNS samples were exposed to 15 min of UV/O_3.

Primary hippocampal neurons were isolated from E18 Friend Virus B-type (FVB) strain mice according to established