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PLASMA DEPOSITED DIAMOND-LIKE CARBON FILMS FOR LARGE NEUTRAL ARRAYS*

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To understand how large systems of neurons communicate, we need to develop methods for growing patterned networks of large numbers of neurons. We have found that diamond-like carbon thin films formed by energetic deposition from a filtered vacuum arc carbon plasma can serve as "neuron friendly" substrates for the growth of large neural arrays. Lithographic masks can be used to form patterns of diamond-like carbon, and regions of selective neuronal attachment can form patterned neural arrays. In the work described here, we used glass microscope slides as substrates on which diamond-like carbon was deposited. PC-12 rat neurons were then cultured on the treated substrates and cell growth monitored. Neuron growth showed excellent contrast, with prolific growth on the treated surfaces and very low growth on the untreated surfaces. Here we describe the vacuum arc plasma deposition technique employed, and summarize results demonstrating that the approach can be used to form large patterns of neurons.
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1. INTRODUCTION

The study of the functional unit of the nervous system, the neuron, has been an active field of investigation both at the single-cell level and at the level of large numbers of interconnected neurons, for example within the human brain [1]. The behaviour of individual neurons has been studied using microelectrodes to monitor the electrical signals ("action potentials") generated within the neuron and along its dendrites (the branch-like arms that carry signals toward the neuron cell body) and axons (the long "tail" that carries the neuron output signal to other cells). One can think of the single-cell electrical behaviour as the performance at the "device level" [2], and at this level much is known. At the "system level", however, much less is known – we know very little about how large numbers of neurons communicate among themselves. There has been good progress made in the growth of neuron cultures *in vitro*. The neurons grow, extend dendrites and axons, form synapses, and create neural networks [2]. In order to explore the electrical characteristics of large numbers of associating neurons, however, we need first to develop techniques for forming 2-dimensional patterned arrays of neurons. The pattern parameters should all be under control of the experimenter, including geometry of the pattern, line width, and pattern size (number and density of neurons). Several approaches to patterning have been explored [3], including mechanical fabrication of troughs and ridges [4], laser micromachining [5], surface photochemical methods [6], photoresist methods, among others. These methods work and have been used to grow neural arrays. Here we describe some exploratory work investigating the suitability of vacuum-arc-plasma based methods of surface modification as a tool for forming large patterned neuronal arrays.

2. EXPERIMENTAL METHOD

Plasma deposition was done using a filtered vacuum arc system that has been described in detail elsewhere [7,8,9]. The vacuum arc is a high current discharge between two electrodes in vacuum [9]. Metal (or carbon) plasma is produced in abundance from the cathode material, and it is this plasma that carries the arc current. For the work described here, a repetitively pulsed vacuum arc plasma source was used; the pulse length was 5 ms and the repetition rate was 1 pulse per second. A 90^{o} magnetic filter was used to remove the 'macroparticle' flux (tiny droplets of cathode material) from the plasma stream [10]. The plasma stream exiting the magnetic duct was allowed to deposit onto a 1" x 3" glass substrate mounted on a grounded holder positioned about 10 cm from the duct exit. A simplified schematic of the filtered vacuum arc plasma deposition system is shown in Fig. 1, and a photograph of the plasma stream in Fig. 2.

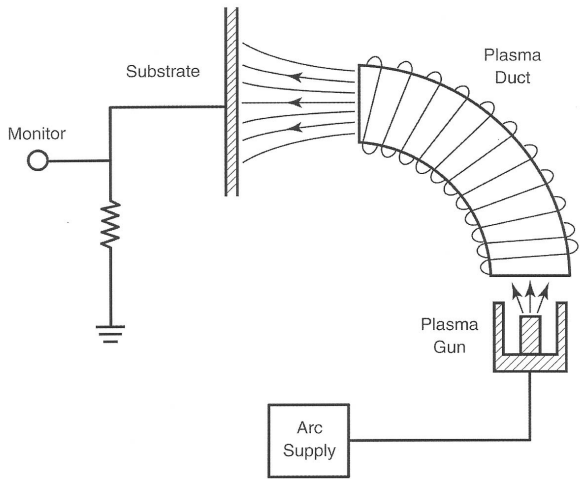

Fig. 1. Simplified schematic of the filtered vacuum arc plasma deposition set-up

Films of thickness in the approximate range 30–1000 Å were formed on glass microscope slides. We investigated neuron growth on films formed from a wide range of materials, including C, Mg, Ti, Pd, Ta, Ir, Pt and Au; and by depositing at a somewhat elevated background pressure we also formed and explored a number of metal oxides, e.g. aluminum oxide, titanium oxide and tantalum oxide were also deposited.

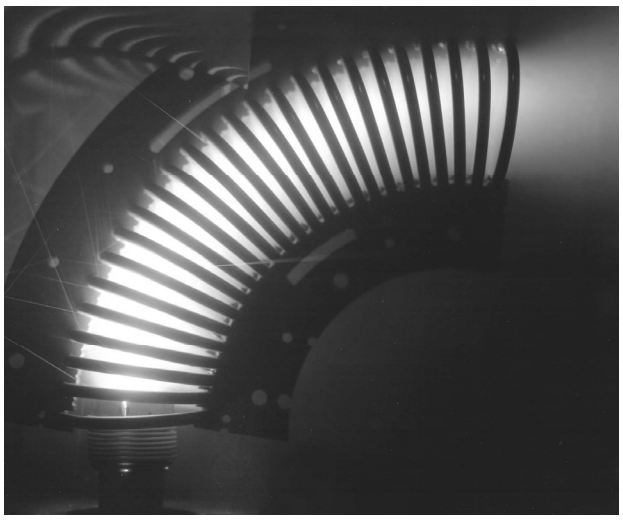

Fig. 2. Plasma produced by the vacuum arc plasma gun at the lower left streams through the magnetic filter duct and is deposited onto a substrate at the upper right

A characteristic feature of vacuum-arc-produced plasmas is the relatively high directed energy of the ions, in the range 20–150 eV depending on the ion species [11]. The film deposition is thus an energetic deposition (even for the case of zero substrate bias), and for the case of carbon this results in the film material formed being a high quality, hydrogen-free, diamond-like carbon (DLC) [12,13], as opposed to amorphous carbon or graphite. As described below, we found that the carbon films were particularly advantageous for enhanced neuron growth. All of the neuron growth work described in the following was done with DLC.

We used PC-12 neurons derived from a transplantable rat pheochromocytoma from the adrenal gland. The cells are grown in RPMI 1640 media with 2 gm/L glucose (Invitrogen), 10% heat-inactivated horse serum (Invitrogen), 5% fetal bovine serum (HyClone), 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, pen strep at 37^0C, 7.5% CO_2 on Type I Collagen coated Biocoat^{TM} (Becton Dickinson) plastic 100 mm petri plates. Stock cultures were fed every three days with 2/3rds fresh media, and subcultured every 9 days with a 1:4 cell split ratio. Nerve Growth Factor (NGF) 2.5S (Invitrogen) was added to cell media at concentrations of 50 ng/ml. On a collagen-coated substrate, neurite elongation proceeds at an average rate of ~50 μm/day for at least 10 days. After 2 weeks of NGF exposure, the cultures generated a dense mat of neuritic processes.

PC-12 cells were inoculated at 1 x 10^5 cells/ml onto sterile glass slides that were pre-cleaned, then coated by plasma deposition with DLC, and then coated with Type I collagen. Cells were allowed to adhere to the slide in a 7.5% CO_2 incubator at 37 ^{o}C, for 3 hours, and then gently flooded with growth media. Cell growth was monitored by phase light microscopy. After 3-6 days of cell growth, NGF was added to the media at 50 ng/ml. After the addition of NGF, cell division stops and differentiation begins. PC-12 cells double every 96 hours. Cultures were visually monitored daily and images captured every other day, up to 1.5 months after initiation of the cultures.

3. RESULTS

3.1. DLC CHARACTERISTICS

The quality of a DLC sample can be quantified in a number of ways depending on the particular application for which the material will be used, but one way that is general useful is to specify the ratio of diamond-bonded carbon atoms to graphitically-bonded carbon atoms in the material. This is called the sp^3:sp^2 ratio, referring to the coupling between carbon atoms; alternatively the sp^3 fraction can be stated, this being a measure of the fraction of diamond-bonded carbon atoms in the sample. A second important characteristic of a particular DLC sample is whether or not it contains hydrogen. DLC films can be deposited in a variety of ways. A common method of forming DLC is to use a hydrocarbon precursor gas, and in this case the material is hydrogenated; the hydrogen content can be as high as several tens of percent.

The kind of DLC that is formed by a carbon vacuum arc is of high quality in both of these respects, since the precursor material is purely carbon, and the energetic deposition (high ion streaming energy) leads to high sp^3 fraction. Fig. 3 shows the sp^3 content as measured by an EELS (electron energy loss spectrometry) technique taken for the case of deposition onto a metallic substrate, for which case the substrate can be pulse-biased so as to control the ion deposition energy. The diamond-bonded fraction maximizes at about 85% for a carbon ion energy of about 100–150 eV; this result has been confirmed by a number of groups around the world. For the case of a glass (insulating) substrate, it is not possible to bias the substrate, and the ion deposition energy for a carbon plasma is about 20 eV, the directed energy with which the vacuum arc carbon plasma is formed. Importantly, we see from Fig. 3 that even for this case (zero bias), the sp^3 fraction is very high, about 80%.

We conclude that the DLC formed by our technique and used in this work for neuron growth experiments is of high quality – high diamond-bonded fraction (about 80%) and hydrogen-free (about <1%).

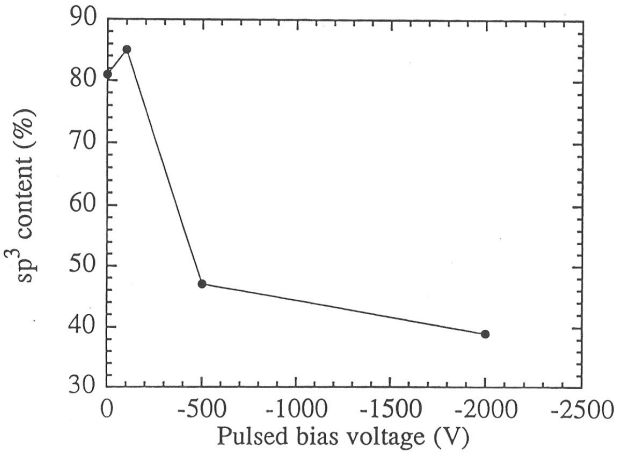

Fig. 3. sp^3 content of DLC films deposited by filtered vacuum arc plasma deposition as a function of energy of the carbon ion flux arriving at the substrate

3.2. NEURON GROWTH

Neurons grew on all the processed substrates, but there was a wide variation observed in the total number of attached cells and their morphology. We found that the metals provided a generally positive growth enhancement and that all of the metal oxides were generally negative in their effect. The single film material that stood out as providing vastly enhanced growth was carbon, which as described above is deposited in the form of hydrogen-free diamond-like carbon, or DLC. We therefore chose to investigate neuron growth on diamond-like carbon surfaces in more detail. Variation of DLC film thickness indicated that about 100–300 Å was near optimum. With thinner films, the neuron "contrast ratio" – ratio of neuron growth density on the DLC-coated region to density on the non-DLC-coated region – was less, and thicker films tended to delaminate from the substrate. Fig. 4 shows the effect of DLC on neuron growth. Fig. 4(a) shows neurons grown on a glass substrate, and Fig. 4(b) shows the growth for the same period of time for a DLC substrate. Clearly the DLC provides superbly enhanced growth.

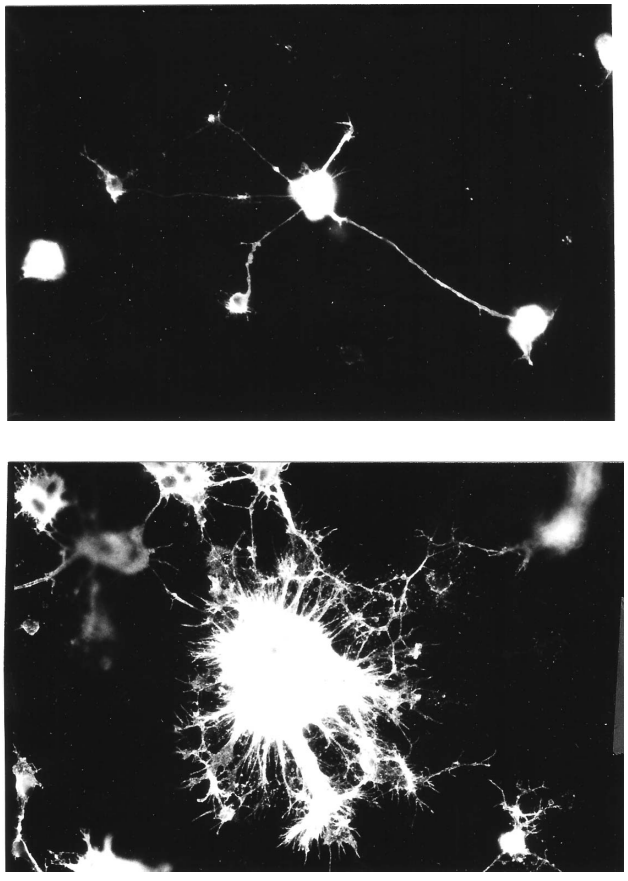

Fig. 4. Neuron growth under similar conditions for growth on a glass substrate (upper), and growth on a DLC-coated substrate (lower)

The photograph in Fig. 5 shows clearly how a kind of "fence" is provided by a DLC/non-DLC boundary. One can see that (i) neuron growth is healthy on the upper DLC-coated region, with virtually no growth on the lower uncoated region, (ii) in the DLC region, neurons grow extended processes (axons and neurons), (iii) the neuron extensions show a pronounced tendency to confine their growth to the DLC region.

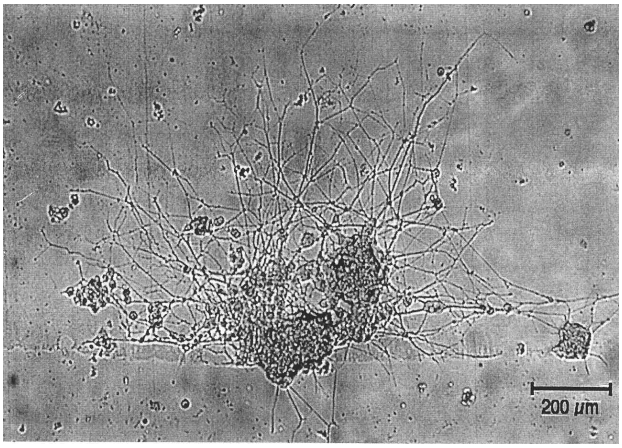

Fig. 5. Selective neuron growth on DLC-coated substrate. Neuron growth after 15 days on a glass slide onto which a 100 Å thick film of DLC was deposited. The DLC region can be seen as a slightly darker region occupying the upper 80% of the photograph; there was no DLC coating on the lower part of the image. The whole slide was coated with Type I Collagen.

The results of another growth experiment are shown in Fig. 6. Here the neuron density is prolific, much greater than would be chosen for a controlled experiment. But the point is made clear that the growth is limited to only the DLC-coated region. Neuron growth is on a glass substrate processed by plasma deposition of ~100 Å coating of diamond-like carbon (DLC) film. The plasma deposition was such that the lower part of each photo is the DLC-treated region, and the upper part is not DLC-treated. The entire substrate was collagen coated, and the neurons were seeded over the entire surface. The upper photo shows the delicate neurite growth that develops on the DLC-treated region; the lower photo shows that the neuron growth in the DLC-treated region continues to a dense and prolific neuron density. These results indicate that neurons grow selectively on the lower DLC-treated regions and not on the upper untreated regions. The contrast (ratio of neuron density in the treated region to neuron density in the untreated region) is very high, and neuron growth in the treated region is healthy.

The results of our first attempt at neuron patterning are shown in Fig. 7. Neuron growth is on a glass substrate processed by plasma deposition of ~150Å diamond-like carbon (DLC) film. Prior to deposition, "LBNL" was written on the glass slide using a fine-point marker pen, and then the DLC was deposited. After DLC deposition, the ink was removed with alcohol, thus leaving "LBNL" patterned in negative in the DLC film. The slide was then coated with Type I Collagen and seeded with PC-12 rat neurons. The neurons were allowed to grow for 3 days, at which point NGF (Nerve Growth Factor) was added. The micrographs shown in Fig. 7 were taken after a growth period of 6 days after initiation of the cultures.

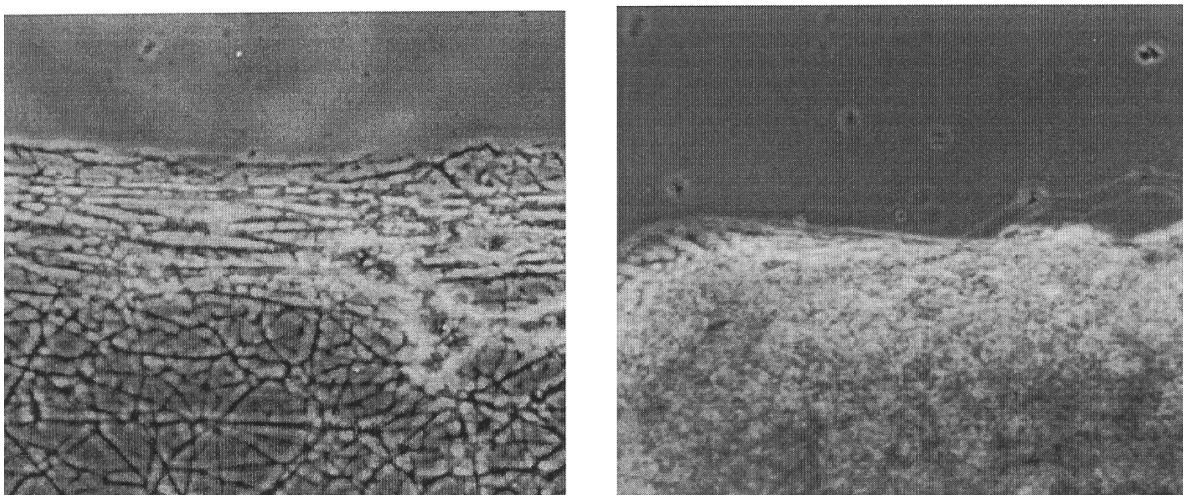

Fig. 6. Selective neuron growth on DLC coated surface. The lower part of each photograph shown was DLC coated and the upper part not coated; the entire substrate was then collagen coated, and neurons were then seeded over the entire surface. A delicate neurite growth develops on the DLC-treated region (left photo), which subsequently develops into a dense and prolific neuron field (right photo). (Scale: the width of each photograph is several hundred microns).

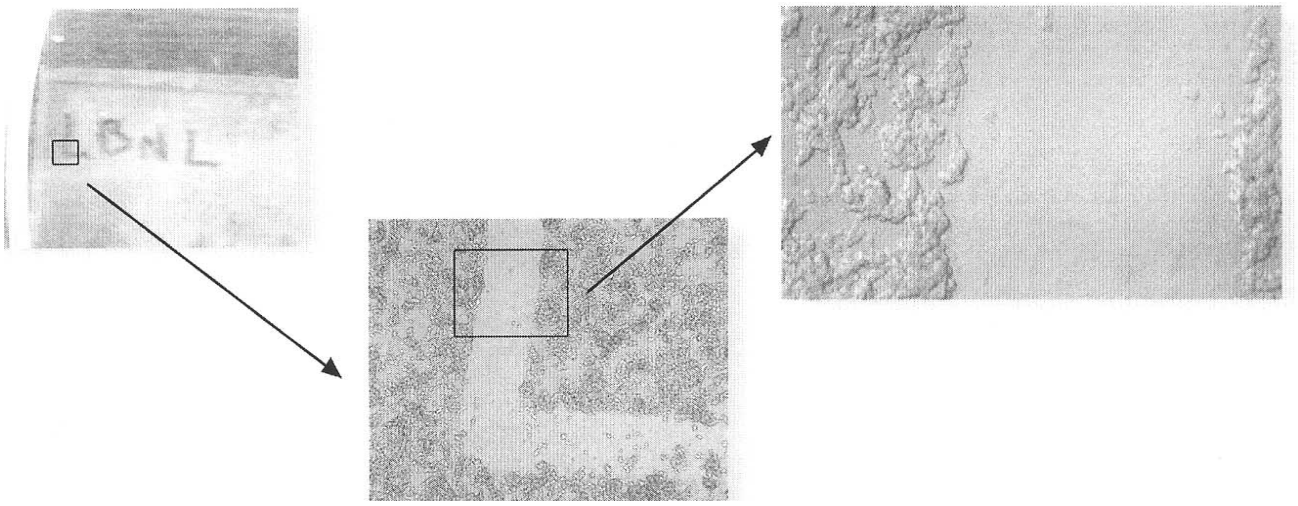

Fig. 7. Patterned growth of neurons to form "LBNL" (in negative).

4. DISCUSSION AND CONCLUSION

As discussed, the DLC film is deposited first onto the glass substrate, subsequent to which a collagen film is added; we estimate the collagen to be at least several hundred Angstroms thick. Then the neurons are cultured on the collagen. The precise mechanism that promotes preferential growth in the presence of DLC is not known, but we suggest some mechanisms that may play a role.

Collagen has a fibrous structure that is not impervious to cell growth. The intertwined fibrils of the collagen film do not form a solid barrier between the neurons and the DLC, but present an open fibril matrix that is conducive to cell growth. The biocompatibility of DLC is known from a sizeable body of prior work that has been reported in the literature having to do with DLC coating of various kinds of prostheses and devices implanted into the body [14-16], and in this sense our results are consistent with the larger body of reported work and not unexpected.

Another possible explanation for the observed beneficial effect of DLC resides in the effect of the DLC on a likely ordering of the long collagen molecules on the substrate. The DLC surface consists of carbon atoms and dangling bonds [17]. We speculate that this surface may promote ordering of the collagen molecules parallel to the glass surface, and that this molecular ordering may favour neuron growth as opposed to a direct interaction between neuron and DLC.

The work described here demonstrates the suitability of filtered-vacuum-arc deposition of diamond-like carbon for forming patterned arrays of large numbers of live neurons. We have shown that energetic plasma deposition of carbon to form an ultra-thin layer of DLC on the substrate surface provides a means for selective neuron attachment, growth, and differentiation on that surface. The neuron growth contrast ratio (ratio of neuron density on plasma-treated regions to neuron density on untreated regions) can be very high, adequate for the fabrication of large patterned arrays of neurons.

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