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Differential patterning of neuronal, glial and neural progenitor cells on phosphorus-doped and UV irradiated diamond-like carbon

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ABSTRACT

Diamond-like carbon (DLC) is an attractive biomaterial for coating human implantable devices. Our particular research interest is in developing DLC as a coating material for implants and electrical devices for the nervous system. We previously reported that DLC is not toxic to N2a neuroblastoma cells or primary cortical neurons and showed that phosphorus-doped DLC (P:DLC) could be used to produce patterned neuron networks. In the present study we complement and extend these findings by exploring patterning of dorsal root ganglion (DRG) explants, human neural progenitor cells (hNPC) and U-87 astroglioma cells on P:DLC. Further P:DLC data is provided to highlight that P:DLC can be used as an effective coating material for in vitro multi-electrode arrays (MEAs) with potential for patterning groups of neurons on selected electrodes. We also introduce ultraviolet (UV) irradiation as a simple treatment to render DLC neurocompatible. We show that UV:DLC can be used to support patterned and unpatterned cortical neuron growth. These findings strongly support the use of DLC as tailorable and tuneable substrate to study neural cell biology in vitro and in vivo. We conclude that DLC is a well-suited candidate material for coating implantable devices in the human nervous system.

1. Introduction

DLC is an amorphous form of carbon which exhibits many of the properties of natural diamond. Typically DLC consists of an amorphous network of sp^3 (diamond-like) and sp^2 (graphite-like) hybridised carbon atoms. The sp^3:sp^2 ratio within the DLC matrix mainly determines the properties of the material, the higher the sp^3:sp^2 carbon atom ratio the more "diamond-like" the material is. High sp^3 fraction DLC is also known as tetrahedral amorphous carbon (or *ta*-C), and has excellent mechanical, frictional and tribological properties. DLC is employed in a number of industries, including automotive, computing and high-speed tooling. The biomedical applications of DLC to date have been largely centred on its wear resistance, lubrication and low friction properties. These features make it a material of choice to coat implantable devices such as artificial joints; cardiovascular stents, valves and pumps; surgical instruments and screws; surgical guidewires and artificial discs [1]. In addition to its tribological qualities DLC is also chemically inert, transparent over a wide range of wavelengths (visible and IR), has a low surface roughness and a high refractive index, each of which enhances its utility as a biomaterial or biosensor material.

In terms of its suitability as a coating for nervous system implants and neuron:electrode interfaces, DLC has three other very appealing features. The first of these is that the physical characteristics of DLC can be readily tuned by doping or alloying with different elements to enhance its biocompatibility and control its conductivity. Numerous elements have been used as dopants for DLC (including, silicon, nitrogen, boron, fluorine, nickel, silver, hydrogen and phosphorus) to enhance the performance of DLC coatings for a variety of purposes. We reported previously that neither DLC, phosphorus-doped (P:DLC) nor surface-oxidised DLC (O:DLC) were toxic to N2a neuroblastoma cells or primary cortical neurons [2]. Cortical neurons did however adhere significantly better to P:DLC than O:DLC and DLC. We have been able to take advantage of the poor adhesion of neurons to DLC to create distinct neural networks on P:DLC tracks using micropatterning techniques. The second characteristic is that the surface properties of DLC can be modified. While doping and alloying often alter the surface properties of DLC,

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several other approaches can also be used to functionalise the DLC or doped DLC surfaces. Such modifications include surface oxidization by acid treatment, ion beam bombardment and attachment of functional groups [2]. The final attractive feature of DLC is that it can be deposited as a thin film (from 1 to 100 nm thickness) onto existing components or devices at room temperature [2,3].

The present study complements and extends our previous P:DLC/DLC work and introduces ultraviolet (UV) irradiation of undoped-DLC as a means to promote selective adherence of primary cortical neurons and PC12 cells. We describe patterned growth and migration of dorsal root ganglion (DRG) explants and neural progenitor cells on P:DLC channels and show how this system can also be used to limit adherence of an astroglial cell line, which could provide a strategy for increasing the longevity of implants by reducing glial scarring.

2. Materials and methods

2.1. Preparation of diamond-like carbon substrates

For preparation of the DLC samples a pulsed laser deposition (PLD) set-up was used. The output of an Argon Fluoride (ArF) excimer laser (Lambda Physik, Compex 201, laser wavelength 193 nm) was focused (20 cm focal length lens, 45° angle of incidence) on a target located in a stainless steel vacuum chamber maintained at $\sim 10^{-6}$ Torr. The targets comprised of either a graphite disk (Poco Graphite Inc., DFP-3-2 grade), or compressed disks of graphite and red phosphorus (20 at %) powder mixtures.

The laser fluence used was 12 J/cm^2 for ablation of pure graphite, while the P:graphite targets were ablated at a fluence of 3 J/cm^2. Thin films (1200 laser shots, ~20 nm in thickness) of DLC and P:DLC were deposited onto glass coverslips (13 mm in diameter) at a target-to-substrate distance of 5 cm at room temperature. To produce coated micro-electrode arrays, a thinner film of P:DLC (200 laser shots) was deposited onto the array area of a 59-channel MEAs (Multi Channel Systems, Reutlingen, Germany). The deposition process produces a roughly stoichiometric transfer, and thus results in 20 at % P in the DLC film [4–6].

Patterned P:DLC substrates were prepared by first coating glass coverslips with P:DLC for 600 laser shots. Copper transmission electron microscope (TEM) grids (AGAR, 200 Mesh grids, 28 μm bar width, 90 μm grid squares and PL200 parallel lines, 50 μm spacing) were used as shadow masks and the substrates were coated with DLC for 600 laser shots. Coverslips were subjected to 20 min sonication in acetone and methanol, before being washed in deionised water overnight. Samples were then air-dried and stored in an airtight container until use. UV:DLC was prepared by coating glass coverslips with 1200 laser shots of DLC and then exposing them to 18 h of UV light (125 W medium pressure mercury lamp, Photochemical reactors Ltd., distance = 2–5 cm). To create patterned UV:DLC tracks TEM grids were also used as a photomask.

2.2. Analysis of DLC films

The properties of the deposited DLC and UV:DLC films were analysed as previously described for P:DLC and O:DLC [2]. The films were positioned under the microscope objective (50× magnification) of a Renishaw 2000 Raman system with an excitation wavelength of 514.5 nm. Per sample at least three measurements were taken on three different positions. Typically, these measurements revealed quasi-identical Raman spectra, indicating spatially coherent thin films. A Kruss DSA10-Mk2, with deionised water (18 MΩ resistance) was used to measure the sessile drop contact angle measurements. The ~2 mm diameter water droplets were manually dispensed via a syringe from ~5 mm height. The contact angle measurements were performed on dry, pristine DLC thin films on glass slides/coverslips and were repeated at least three times. The droplet shape was analysed via droplet shape analysis software. The reported measurements are the averaged values of the measured results. We also analysed the surface properties of DLC, P:DLC and UV:DLC using X-ray photoelectron spectroscopy (XPS). XPS was performed on films deposited on a Si(100) substrate using a Fisons Instruments VG Escascope equipped with a Mg Kα (1253.6 eV) source and an analyser energy resolution of approximately 0.9 eV. The wide scan spectra were dominated by the C1s peak at 285 eV. Additionally an O1s peak at 531 eV was observed in all spectra. Given the insulating behaviour of DLC, the XPS spectra suffered from charging and were overall shifted by a few eV, and the O1s peak was used to offset the energy scale of the XPS spectrum and correct for the charging effect. The XPS spectra were fitted by XPS peak 4.1 software to Voigt profiles (80% Lorentzian and 20% Gaussian) to obtain quantitative information about the bonding.

2.3. Primary cell cultures

2.3.1. Cortical neurons

Cortical neurons were cultured on substrates using standard tissue culture protocols. Briefly, substrates were sterilised in ethanol and coated with poly-D-lysine (70,000–150,000 M_w). Neuron-rich cultures were produced by isolating the cerebral cortices from the pups of embryonic day 18 (E18) Wistar rats. The meninges were carefully removed and the cortical tissue dissociated by protease digestion using 10% (v/v) trypsin for 20 min. The tissue was triturated using a fire-polished Pasteur pipette to produce a homogeneous, single cell suspension. Cells were diluted in Neurobasal medium™ containing 2% B-27, L-Glutamine, Glutamic acid, Penicillin, Streptomycin, and plated at a concentration of 1,00,000 cells per well on DLC and tissue culture polystyrene (control) substrates. Lower cell densities of 75,000 cells per well were used for patterned DLC substrates. The cell cultures were placed in an incubator at 37 °C/5% CO_2 and fed at 3 day intervals by replacement of half of the medium (minus Glutamic acid).

2.3.2. Dorsal root ganglia (DRG) explants

DRGs were dissected from embryonic day 18 (E18) Wistar rat pups. Explants were placed onto micropatterned DLC substrates with 200 μl Neurobasal media™ containing 2% B-27, L-Glutamine, Penicillin, Streptomycin and NGF (1 ng ml^{-1}). The media was topped up to 500 μl after 1 day in vitro (DIV), once explants had adhered. Explants ($n = 12$ from three separate cultures) were fixed at 3 DIV with paraformaldehyde (4%) in PBS for 20 min.

2.3.3. Human neural progenitor neurosphere culture

Human neural progenitor cells (hNPCs) from the developing cortex were kindly provided by Clive Svendsen (University of Wisconsin at Madison). Cells were grown and passaged as neurospheres as previously described (see Svendsen et al. 1998 for details) [8]. In brief, cells were cultured in DMEM/HAMS F12 (3:1) medium containing PSF (1%), N2 (1%), FGF-2 (20 ng ml^{-1}), EGF (20 ng ml^{-1}) and heparin (5 μg ml^{-1}) and routinely passaged every two weeks. Following 10–12 passages individual neurospheres (~300–400 μm in diameter) were collected and placed onto our substrates in the same medium as above but without mitogens (i.e., with no FGF or EGF) or heparin. P:DLC substrates were coated overnight at 4 °C and washed three times with PBS. Warmed Laminin (37 °C, 10 μg ml^{-1}) was then spotted onto the grid pattern and placed in an incubator for 30–45 min. The Laminin was removed and the well flooded with medium prior to individual neurospheres being placed at the edge of our P:DLC grids under a dissecting microscope and being placed back into the tissue incubator. Neurospheres were allowed to settle for 24-h and observed each day for 7-days.

2.4. Cell line cultures

2.4.1. U-87 astroglioma cell line

Human U-87 cells were grown in 'complete' Dulbecco's modified Eagle's medium (DMEM) media containing 10% foetal calf serum (FCS; Gibco, USA), 0.1 mg ml^{-1} streptomycin (Sigma) and 100 U ml^{-1} penicillin in a humidified incubator at 37 °C/5% CO_2. Cells were removed from the culture flasks using Trypsin–ethylenediaminetetraacetic acid (EDTA) solution (500 μg ml^{-1} trypsin and 200 μg ml^{-1} EDTA in PBS; Sigma) and plated in DMEM medium containing 10% FCS.

2.4.2. N2a neuroblastoma cell line

Mouse N2a cells were grown in 'complete' Dulbecco's modified Eagle's medium (DMEM) media containing 10% foetal calf serum (FCS; Gibco, USA), 0.1 mg ml^{-1} streptomycin (Sigma) and 100 U ml^{-1} penicillin in a humidified incubator at 37 °C/5% CO_2. Cells were removed from the culture flasks using Trypsin–ethylenediaminetetraacetic acid (EDTA) solution (500 μg ml^{-1} trypsin and 200 μg ml^{-1} EDTA in PBS; Sigma) and resuspended in DMEM medium containing 1% FCS. Cells were then plated using 500 μl of the DMEM (1% FCS) medium at a concentration of 10,000 cells per well. At 4 days in vitro (DIV), cells were either fixed using paraformaldehyde (4%) and photographed or subjected to a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) cytotoxicity/proliferation assay ($n = 12$ from 3 separate cell passages).

2.4.3. Phaeochromocytoma (PC12) cell line

Rat PC12 cells were cultured in DMEM containing 5% (v/v) FCS and 5% (v/v) horse serum under 5% CO2 in air at 37 °C. For patterned PC12 attachment, micropatterned P:DLC/DLC substrates were incubated in a solution of Type VII collagen (30 mg ml^{-1}) for 4 h and washed once in PBS. The cells were plated at densities of 40,000 cells/well and fixed after one day with 4% paraformaldehyde in PBS for 20 min ($n = 12$ from 3 separate cell passages).

2.5. Methyl-thiazolyldiphenyl-tetrazolium bromide (MTT) assay

The MTT assay was used to assess the viability of cultured cells – producing a readout of mitochondrial function. In short, 50 μl of MTT (5 mg ml^{-1}, Sigma) was added directly to the culture media and incubated for 2 h (37 °C/5% CO_2). After this incubation period all media were removed and 120 μl of acidified isopropanol was added to the cells. Colour dissolution was assisted by gentle agitation and colour intensity at 550 nm was determined using a Dynex MRX^{TC} revelation 4.22 plate reader (Dynex technologies, Virginia, USA).