Biomaterials 29 (2008) 2573–2580

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Patterned growth of neuronal cells on modified diamond-like carbon substrates

Stephen Kelly [a,1], Edward M. Regan [a,b,1], James B. Uney [a], Andrew D. Dick [b], Joseph P. McGeehan [c], The Bristol Biochip Group[2], Eric J. Mayer [b,**], Frederik Claeyssens [d,*]

[a] Henry Wellcome L.I.N.E., Clinical Sciences South Bristol, University of Bristol, UK
[b] Bristol Eye Hospital, University of Bristol, Lower Maudlin Street, Bristol BS1 2LX, UK
[c] Department of Electrical & Electronic Engineering, University of Bristol, UK
[d] School of Chemistry, University of Bristol, Bristol BS8 1TS, UK

ARTICLE INFO

Article history:
Received 14 December 2007
Accepted 4 March 2008
Available online 21 March 2008

Keywords:
Laser ablation
Neural cell
Carbon
Diamond
Micropatterning

ABSTRACT

Diamond-like carbon (DLC) has been explored as a biomaterial with potential use for coating implantable devices and surgical instruments. In this study the interaction of DLC with mammalian neuronal cells has been studied along with its modifications to improve its function as a biomaterial. We describe the use of DLC, oxidised DLC and phosphorus-doped DLC to support the growth and survival of primary central nervous system neurones and neuroblastoma cells. None of these substrates were cytotoxic and primary neurones adhered better to phosphorus-doped DLC than unmodified DLC. This property was used to culture cortical neurones in a predetermined micropattern. This raises the potential of DLC as a biomaterial for central nervous system (CNS) implantation. Furthermore, patterned DLC and phosphorus-doped DLC can direct neuronal growth, generating a powerful tool to study neuronal networks in a spatially distinct way. This study reports the generation of nerve cell patterns via patterned deposition of DLC.

1. Introduction

Diamond-like carbon (DLC) is emerging as a promising material for biomedical applications for various reasons: it is chemically inert, wear/corrosion-resistant and bio/haemo-compatible [1,2]. Further important properties of DLC include its high electrical resistivity, infrared transparency, low surface roughness and high refractive index. Additionally, the physical characteristics of DLC can be tuned via doping or alloying with different elements. Thus DLC is a versatile biomaterial that can be modified for different purposes and applications as required. The material has similar properties to diamond, another biomaterial based on carbon, which shows significant promise as a biosensor platform [3]. The deposition of pure diamond requires processing temperatures of 400–800 °C [4], while high-quality DLC can be deposited at room temperature (by pulsed laser deposition or cathodic arc deposition). These low temperature processing conditions make it possible to coat glass and plastics with DLC.

DLC has been used with some success to coat surgical instruments, cardiovascular stents, surgical guidewires and replacement joints [5–11]. Traditionally, these items were made of metals (including stainless steel, titanium oxide/nitride and cobalt–chromium alloy) which wear and corrode, releasing metal ions which may be cytotoxic. For example stainless steel may release Cr and Ni [7] which can cause allergic reactions and may promote tumours [12]. DLC-coated surfaces improve haemocompatability (by reducing activation of the coagulation cascade, platelets and inflammatory cells) [2,13–15]. Further improvements in haemocompatibility occur when DLC is doped with P and Si [16–23]. Results depend on the method of DLC deposition and the form of DLC used [24–26]. The coating of surgical implants with DLC aims to reduce the wear and corrosion, improving their function and reducing tissue destruction and debris at the implant site.

Multiple cell types including osteoblasts, fibroblasts, retinal pericytes, endothelial cells and glial-like cell lines have been demonstrated to grow on DLC in tissue culture, without significant changes in cellular metabolism [17,21,27–29]. The growth of central nervous system (CNS) cells on DLC has been reported by Ignatius et al. [30] who described the growth of embryonic day 10 chick

* Corresponding author. Tel.: +44 117 954 6863; fax: +44 117 925 0612.
** Corresponding author.
E-mail addresses: e.mayer@bristol.ac.uk (E.J. Mayer), f.claeyssens@bristol.ac.uk (F. Claeyssens).
[1] SK and EMR contributed equally to this work.
[2] The Bristol Biochip Group is a cross-faculty research consortium at the University of Bristol consisting of scientists from the Clinical Sciences, Electrical & Electronic Engineering Departments and the School of Chemistry.

0142-9612/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2008.03.001

CNS tissue on DLC-coated glass coverslips in the presence of poly-D-lysine (PDL) and PDL/laminin up to 3 days. This tissue was grown in media containing foetal calf serum, an enhancer of adherent glial cell growth. This study demonstrated the potential of DLC as a substrate for CNS tissue growth, although the test was not carried out with mammalian tissue.

We describe the growth and survival of an N2a neuroblastoma cell line and primary cortical neurones (up to 14 days *in vitro*) on three types of DLC film – DLC, surface oxidised DLC (O:DLC) and phosphorus-doped DLC (P:DLC). We also describe a means by which DLC and P:DLC can be combined to pattern cortical neurones in a spatially controlled manner. This is the first time that micropatterning of functionalised DLC has been used to pattern mammalian nerve cells. The ability to direct neurones in such a way lends itself to the study of neuronal networks (down to the single cell level) and has extensive potential ramifications for spatially controlled tissue engineering.

2. Materials and methods

2.1. Preparation of diamond-like carbon substrates

For preparation of the DLC samples a typical 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) on a target located in a stainless steel vacuum chamber. The pressure in the vacuum chamber was maintained at high vacuum ($\sim 10^{-6}$ Torr) by a turbomolecular pump backed by a rotary pump. Targets used for ablation comprised of (i) a graphite disk (Poco Graphite Inc., DFP-3-2 grade) and (ii) hydraulically pressed 20 mm disks (maximum force of 560 kN) made from graphite and red phosphorus (20 at.%) powder mixtures. The disks were mounted in the vacuum chamber on a rotation stage ($\sim$1 rotation per minute) at a 45° angle between the incident laser beam and the target surface normal. 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, $\sim$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. From former studies, under similar deposition conditions at room temperature we can infer that the 20 at.% P:graphite mixing ratio coincides with a roughly stoichiometric transfer, and thus results in 20 at.% P in the DLC film [31–33]. A number of the undoped DLC samples were oxidised after DLC deposition by placing the coverslips into a 9:1 mixture of sulphuric acid and nitric acid for a period of 72 h. The same procedure has been used for oxidation of diamond and results in carboxylation of the surface groups [34]. Samples were stored in water before sterilisation took place.

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 and 90 μm grid squares) were placed onto substrates and held in place using a spot of silver dag. The exposed areas of the grid were then coated with DLC for 600 laser shots and the coverslips were stored in water.

2.2. Analysis of the P:DLC thin films

The properties of the deposited DLC, O:DLC and P:DLC films were analysed by (i) laser Raman spectroscopy (LRS) employing a Renishaw 2000 system and an excitation wavelength of 514.5 nm, (ii) sessile drop contact angle measurement and drop shape analysis (Kruss DSA10-Mk2, with deionised water (18 MΩ resistance)) and (iii) field emission gun scanning electron microscopy (JEOL JSM 6330F SEM).

2.3. Neuroblastoma (N2a) cell lines

Mouse neuroblastoma (N2a) cells were grown in 'complete' Dulbecco's modified Eagle's medium (DMEM) 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) media at a concentration of 10,000 cells/well. At 3-day *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) assay.

2.4. Primary cortical neurone culture

Cortical neurones 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 MW). Neurone-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 homologous, single cell suspension. Cells were diluted in Neurobasal media™ containing 2% B-27, L-glutamine, glutamic acid, penicillin, streptomycin, and plated at a concentration of 100 000 cells/well on DLC and tissue culture polystyrene (control) substrates (Note: lower cell densities of 75,000 cells/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 media (minus Glutamic acid).

2.5. Semi-quantitative cell adhesion count

Neurone growth and adhesion was observed throughout the experimental period using light microscopy. At set times neurones were prepared for microscopic analysis by fixation with paraformaldehyde (4%, pH 7.2). Under normal culture conditions (tissue culture plastic) cortical neurones are distributed fairly evenly in a monolayer and they send out processes to neighbouring cells. Samples were semi-quantitatively scored for total cell coverage, cell density and degree of monolayer growth/clumping characteristics (Fig. 1A–C).

For cell density scores, eyepiece graticule units were used to sample areas for cell counting within an area of 100 μm^2. Ten sample areas were selected and the mode average number of cells in each square was used as the scoring value. As homologous densities of cells were not always presumed across the coverslip, the percentage area of the coverslip with cells adherent (reflected by the density score – previously derived) was scored between 0–10 (a score of '10' equalled 100% coverage). Scoring for the degree of monolayer growth allowed an estimation

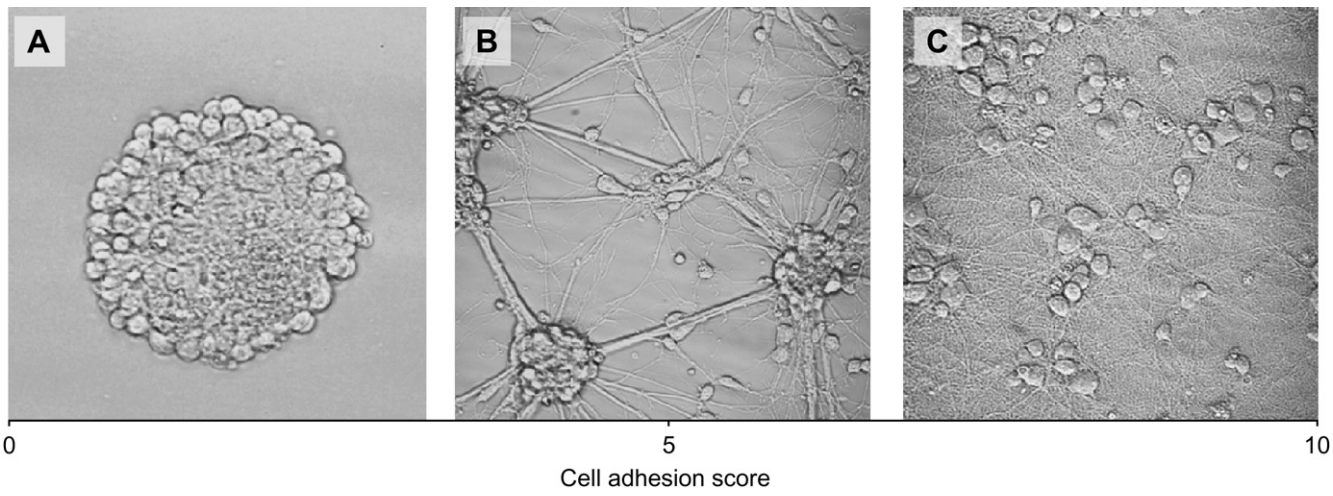

Fig. 1. The clumping characteristics of neurones were assessed using a scoring method. The degree of neuronal monolayer growth was scored between 0 and 10. (A) A score of 0 represented only neurosphere growth. (B) A score of 5 was given to growth of predominantly clumped up balls of cells (termed 'nacelles' by Gross and Kowalski [46]). (C) A score of 10 was given to a primary monolayer neuronal growth pattern.