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UV-irradiated parylene surfaces for proliferation and differentiation of PC-12 cells

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ABSTRACT

PC-12 cells originate from neuroblastic cells, which have an ability to differentiate into neuronlike cells. In this work, the purpose was to estimate the influence of microenvironments on cell attachment and neuritogenesis capacity of PC-12 cells on parylene-*N* and parylene-*C* films with and without ultraviolet (UV) light treatment. The estimate of total cell number after incubation for 72 h, the ratio of adherent to suspended cells, counting of neurite outgrowths on parylene-*N* or parylene-*C* films after UV exposure suggested that these films were suitable for proliferation as well as differentiation of PC-12 cells. The differences in surface properties of parylene-*N* and parylene-*C* films with and without UV exposure were analyzed by contact angle measurement, Fourier-transform infrared spectrometry, and X-ray photoelectron spectroscopy. According to these analyses, introduction of oxygen-related chemical functional groups was presumed to result in increased hydrophilicity and efficiency of protein immobilization on parylene-*N* and parylene-*C* films after UV treatment. According to fluorescent staining, western blotting, and cell cycle analysis, UV-treated parylene-*C* and parylene-*N* films appear to effectively facilitate simultaneous proliferation and differentiation of PC-12 cells with neurite outgrowth.

1. Introduction

Neuronlike PC-12 cells originate from neuroblastic cells, which have an ability to differentiate into sympathetic-neuron-like cells and thus have been regarded as a tool for studies of molecular processes associated with neuronal differentiation and morphogenesis [1,2]. Neuronal differentiation often involves the outgrowth of neurites, which are fated to become axons and dendrites that form functional connections with other neurons [3,4]. Common approaches to promotion of neuronal differentiation in PC-12 cells are supplementation with nerve growth factor (NGF), serum starvation, and addition of either laminin- or collagen IV-rich substrates for cellular attachment [5–7]. After such treatments for neuronal differentiation of PC-12 cells, there are morphological changes with the outgrowth of neurites and a reduction in the proliferation rate of PC-12 cells due to accumulation of cells in the G1 phase of the cell cycle [8,9]. With further treatment with NGF, PC-12 cells are arrested in the G1 phase, and the proportion of the G2/M phase is also increased [10]. After long-term exposure to NGF, terminal differentiation is induced, and PC-12 cells acquire a number of properties characteristic of sympathetic neurons including multiple neurites outgrown [3].

Recently, Kato et al. reported that PC-12 cells show high adhesiveness on the surface of parylene-H and parylene-AM films, and the outgrowth of neurites was also observed without the treatment with NGF. Parylene-H and the parylene-AM are classified as specially functionalized parylene films that have chemically functional formyl and aminomethyl groups on the aromatic ring of a parylene precursor. Although parylene-*C* film was estimated to have low adhesiveness, the adhesiveness of parylene-*C* film was reported to increase more than 20-fold after the treatment of oxygen plasma [11,12]. Generally, parylene films are polymers of *p*-xylene; parylene-*C* (with chloride in the aromatic group of parylene precursor) and parylene-*N* (without any functional group in the aromatic group of the parylene precursor) films have been widely used for structural purposes. Because both of these parylene films are chemically inert, nonbiodegradable, biocompatible, pinhole free, and weakly water swelling, these polymers have been extensively used as coating for insulating implantable biomedi-

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cal devices, such as stents, neural electrodes, cardiac pacemakers, intra-oral magnets [13–15], and for high-fidelity micropatterning of biomolecules [16,17]. Usually, these parylene films can be vapor-deposited onto substrates to generate uniform coating even on a complicated three-dimensional structure, and these films can be subsequently dry-etched using oxygen plasma to obtain microscale features and patterns that are ideal for culturing of cells [18]. In various studies, the plasma and ultraviolet (UV) light treatment have been reported to change the surface of parylene-*N* and parylene-*C* films so that they become hydrophilic, and these parylene substrates are known to retain hydrophilic properties for relatively longer periods in comparison with other polymers, such as polydimethylsiloxane (PDMS) and polystyrene (PS) [19–23]. In particular, UV treatment of parylene films was found to increase the hydrophilicity of both parylene films and efficiency of immobilization of proteins on these films. Because cell attachment is known to be possible on the artificial surfaces of polymers after immobilization of proteins, UV treatment of parylene films is believed to improve the attachment of cells through the increased hydrophilicity.

In this work, the aim was to analyze proliferation and differentiation characteristics of PC-12 cells on parylene-*N* and parylene-*C* films with and without UV treatment. As the first step, proliferation and outgrowth of neurites were examined by morphological analysis, counting of adherent and suspended cells. The changes in the chemical environment of parylene-*N* and parylene-*C* films with and without UV treatment were analyzed by contact angle measurement, Fourier transform infrared (FT-IR) spectrometry, and X-ray photoelectron spectroscopy (XPS). Finally, biomarkers of proliferation and differentiation of PC-12 cells were separately analyzed by a fluorescence staining test and western blotting, and then by cell cycle analysis of PC-12 cells cultured on the parylene-*N* and parylene-*C* films with and without UV treatment.

2. Experimental

2.1. Materials and methods

Ham's F–12 K (Kaighn's) medium and horseradish peroxidase (HRP) were purchased from Sigma-Aldrich Korea (Yongin-si, Gyeonggi-do, Korea). Horse serum (HS), fetal bovine serum (FBS), a trypsin/EDTA solution, and trypan blue solution were acquired from Thermo Fisher Scientific (Waltham, MA, USA). Penicillin, streptomycin, and paraformaldehyde were also purchased from Sigma-Aldrich Korea (Yongin-si, Gyeonggi-do, Korea). Dimers of parylene-*N* and parylene-*C* were acquired from Femto Science Co. (Suwon Si, Gyeonggi-Do, Korea). Viable cell counting kit (CCK-8) was purchased from Dojindo Laboratories (Kumamoto, Japan). Primary anti-neurofilament heavy chain (NF-H) antibodies were acquired from Cell Signal (Tokyo, Japan), and an anti-Ki67 antibody (clone: SP6), anti-cyclin D1 antibody, anti-α-actin antibody, and all of the secondary antibodies were purchased from Abcam (Seoul, Korea). Propidium iodide (PI) was purchased from Thermo Fisher Scientific. Chemiluminescent substrates and a bicinchoninic acid (BCA) assay kit were purchased from Thermo Fisher Scientific Korea (Seongnam-si, Gyeonggi-Do, Korea).

2.2. Preparation of parylene-coated plates

Commercially available parylene deposition system (Kisco, Japan) was used to prepare parylene coated plates. Dimers of parylene-*N* and parylene-*C* were first evaporated at 160 °C, followed by pyrolysis at 650 °C where the parylene dimers were converted into highly reactive free radicals. These highly reactive free radicals were then polymerized and deposited on substrates. The process was carried out in vacuum at 25 °C. Thicknesses of the parylene films was controlled by the initial amount of parylene dimers. The surface of the parylene films was treated with UV irradiation using a UV-lithography system at fixed power of 23 mW/cm^2 and wavelength 254 nm (Seoul, Korea) [22].

2.3. Surface analysis of the parylene films

Surface properties of the parylene films were analyzed with and without UV irradiation. An automated contact angle measurement system (Smartdrop from FEMTOFAB Co., Pohang-si, Gyeongsangbuk-do, Korea) was used to measure the contact angle. For measurement of contact angles, a 5-μL droplet was initially placed on the surface of parylene-*N* (n = 3) [20]. Functional groups of the parylene films were analyzed by FT-IR spectrometry (Spectrum-100, PerkinElmer Co., Waltham, MA, USA) with a specular reflectance accessory (VEEMAX III, PIKE Technology, Madison, WI, USA) and a liquid-nitrogen-cooled mercury–cadmium–telluride (MCT) detector. For FT-IR spectrometry, 100 nm of parylene-*N* was deposited on gold coating of the sample holder [20].

2.4. Cell culture and the proliferation test

Neuronlike PC-12 cells were purchased from the Korean Cell Line Bank (KCLB) and cultured in the Nutrient Mixture F-12 Ham medium containing 15% HS, 2.5% FBS, and an antibiotic solution: penicillin (100 U/mL) and streptomycin (100 μg/mL). All the cells were handled only on a clean bench and incubated at 37 °C in a humidified atmosphere containing 5% of CO_2. When the cells reached confluence of approximately 90%, they were subcultured for the intended use. Cell images were captured using an inverted microscope (Eclipse TS100, Nikon, Tokyo, Japan) 24 and 48 h after seeding of PC-12 cells (20,000 cells).

2.5. Immunostaining and western blotting

To characterize differentiation and proliferation of PC-12 cells, immunofluorescence staining was performed for neurofilament heavy chain (NF-H) and Ki67 protein. PC-12 cells were seeded in parylene-coated 96-well plates, and after 24 h of incubation, the cells were washed with PBS and fixed by means of 2% paraformaldehyde for 30 min. After that, PC-12 cells were permeabilized with 0.1% Triton X-100 and blocked with 10% goat serum in PBS for 30 min. Anti-NF-H (dilution 1:100 in the 5% bovine serum albumin in PBS) and anti-Ki67 (dilution 1:300 in the 5% bovine serum albumin in PBS) primary antibodies were reacted with the cell samples overnight at 4 °C. After washing with PBS, PC-12 cells were separately incubated with two kinds of secondary antibodies (Fluorescein isothiocyanate (FITC) –conjugated anti-rabbit IgG antibody and CY3-conjugated anti-mouse IgG antibody). After incubation for 1 h at room temperature, PC-12 cells were washed three times and analyzed under a fluorescence microscope (model: IX71/IX81) from Olympus Co. (Tokyo, Japan).

For protein extraction, PC-12 cells were washed two times in PBS and then lysed in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl pH 8, NP-40 1%, sodium deoxycholate 0.5%, and SDS 1%) with *in situ* prepared protease inhibitors. Protein concentration was assayed by using the BCA kit. Total protein extracts were subjected to SDS-PAGE.

For western blotting of marker proteins, the transfer process of protein bands was carried out overnight at 4 °C. Nonspecific binding was blocked with 5% (w/v) skimmed milk in TBS-T for 1 h with gentle agitation at room temperature. After three washes in TBS-T, the diluted primary antibody (NF-H 1:100 and alpha-actin 1:5000) solutions were incubated with the samples overnight at