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TECHNICAL NOTE

Improvement of neuronal cell adhesiveness on parylene with oxygen plasma treatment

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We improved adhesiveness of a neuron-like cell, PC12, on a Parylene-C surface by O_2 plasma treatment which changes the surface from hydrophobic to hydrophilic. Neural cell adhesiveness on the plasma-treated Parylene-C was more than twenty times better compared to non-treated Parylene-C and it was close to that on a conventional polystyrene tissue-culture dish.

[**Key words:** Parylene; Neural cell adhesiveness; Surface modification; Neural device; Plasma treatment; PC12]

In the past few decades, poly(chloro-para-xylylene) (Parylene-C) has become a representative coating material for insulating neural electrodes (1–5). The chemical vapor deposition of Parylene-C provides a thin, conformal, and pinhole-free coating on virtually any substrate, and the coating protects the substrates from moisture, chemicals, and accumulating an electric charge around implant sites (5–8), while being compatible with micromachining processes (2–5). On the other hand, the usage of Parylene-C is limited for other neural devices, particularly, regeneration-type nerve electrodes for interfacing with the peripheral nervous system and multi-electrode array systems for in vitro electrophysiology. These neural devices require cellular growth and repopulation for stable recording and stimulation, but it is extremely difficult for Parylene-C to support and promote growth of neuronal cells because its hydrophobic characteristic leads to extremely low adhesiveness with neuronal cells.

Seong et al. reported that an oxygen plasma treatment changes a Parylene-C surface from hydrophobic to hydrophilic (9), and Chang et al. reported that increasing the hydrophilicity of Parylene-C by plasma treatment stabilizes growth of cell cultures of fibroblast and hepatocytes (10). However, the improvement of neuronal cell adhesiveness cannot be directly inferred from these reports because the cells used in their studies are generally known to have better cell adhesiveness than neuronal cells (11). Thus, neuronal cell adhesiveness on the plasma-treated Parylene-C surface remains unknown. Here, in this work, we investigated how the plasma-treated Parylene-C surface affects the degree of neuronal cell adhesiveness by using neuron-like cells, i.e., the rat adrenal pheochromocytoma cell line (PC12 cells), while following these concepts to change the Parylene-C surface from hydrophobic to hydrophilic by using short-time oxygen reactive ion etching (RIE).

Glass substrates (Micro Slide Glass, Matsunami Glass Ind., Ltd., Kishiwada, Osaka, Japan) were diced into 2.5 × 2.5 cm rectangles and washed with ethanol and distilled water. Next, a 2-μm layer of Parylene-C was deposited (PDS2010 LABCOTER 2, Specialty Coating Systems, Indianapolis, IN, USA) on the diced glass substrates. Finally, the surface of the Parylene-C coating on the glass substrates was modified by using an O_2 RIE system with a 4 Pa etching chamber pressure and a 13.56 MHz radiofrequency (ULVAC Japan, Ltd., Chigasaki, Kanagawa, Japan) under the following O_2 flow rate conditions: 10 cm^3/min at 150 W for 5 min (specimen 1); 20 cm^3/min at 150 W for 5 min (specimen 2); 10 cm^3/min at 200 W for 5 min (specimen 3); 20 cm^3/min at 200 W for 5 min (specimen 4). We also prepared a control substrate (specimen 5) with no surface modification by RIE (i.e., no plasma treatment) and a tissue-culture grade polystyrene (PS) substrate (specimen 6) (see Table S1). All of the specimens were sterilized with 70% ethyl alcohol and rinsed with distilled water. We note that prior to this study, etching rate of our RIE system was examined by observing electrical resistance around the center of a specimen which was coated with Parylene-C after an Au layer was deposited on the glass substrate. The resulting etching rate was about 0.17 μm/min under the RIE conditions of O_2 flow rate of 10 cm^3/min at 200 W. Thus, we chose etching duration of 5 min because it was long enough to roughen the Parylene-C surface, but not to reach the Si substrate under our experimental conditions.

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The surface modifications of Parylene-C for specimens 1 and 5 were evaluated by X-ray photoelectron spectroscopy (XPS) and energy-dispersive X-ray spectroscopy (EDX). The XPS measurements were carried out in a conventional photoelectron spectroscopy apparatus, and its measuring spot diameter was more than 5 mm. The X-ray source was the Mg Kα line ($h\nu = 1253.6$ eV), which was incident at 45° with respect to the surface normal. The base pressure in the chamber was 2×10^{-8} Pa. The EDX measurements were carried out in a scanning electron microscope with the EDX function (SEM: S-2250N, Hitachi, Ltd., Chiyoda-ku, Tokyo, Japan, EDX: super dry, Kevex Instruments, San Carlos, CA, USA) using a 10 keV electron beam, and its measurement area was less than 0.1×0.1 mm^2.

PC12 cells (RCB-0009, Riken cell bank, Tsukuba, Ibaraki, Japan), which serve as a standard model for neurons (12,13), were maintained in neuro basal medium (21103, Gibco-Invitrogen, Carlsbad, CA, USA) containing 5% horse serum and 10% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO_2. PC12 cells were cultured on the specimens immersed for 72 h in 10-cm-diameter tissue-culture grade PS dishes (3020-100, IWAKI, AGC Techno Glass Co., Ltd., Funabashi, Chiba, Japan). Their morphologies were observed with an inverse phase contrast microscope (IX-70, Olympus, Shinjuku-ku, Tokyo, Japan), and the numbers of neuronal cells adhering to the specimens were counted at 24 and 72 h. The areas around the edges of the specimens were excluded from cell observation and EDX analysis.

Fig. 1a shows the XPS band of the Parylene-C surface for specimens 1 (plasma treatment) and 5 (no plasma treatment). Specimen 5 had Cl and C peaks that were detected and large enough to measure the intensity, but only small Si and O peaks were observed. In contrast, for specimen 1, the Cl, O, C, and Si peaks were all readily detected. After the plasma treatment, the Cl peaks decreased and the O and Si peaks increased. Fig. 1b shows the results of the EDX analysis. The Cl, C, and Al peaks were detected for specimens 1 and 5. After the plasma treatment, the Cl peak decreased and the O peak appeared.

Fig. 2 shows the PC12 cell morphologies for all conditions at 72 h. Without plasma treatment, Parylene-C had extremely low adhesiveness to PC 12 cells in all periods of culturing. PC12 cells did not adhere on this surface. Instead, they floated in the culturing medium or a few cells settled on the surface in round-shaped deposit. In contrast, PC12 cells adhered to the surface of the plasma-treated Parylene-C. Notably, there were no differences in the morphologies of the PC12 cells that had adhered to the plasma-treated surfaces and the tissue-culture grade PS dishes at 72 h.

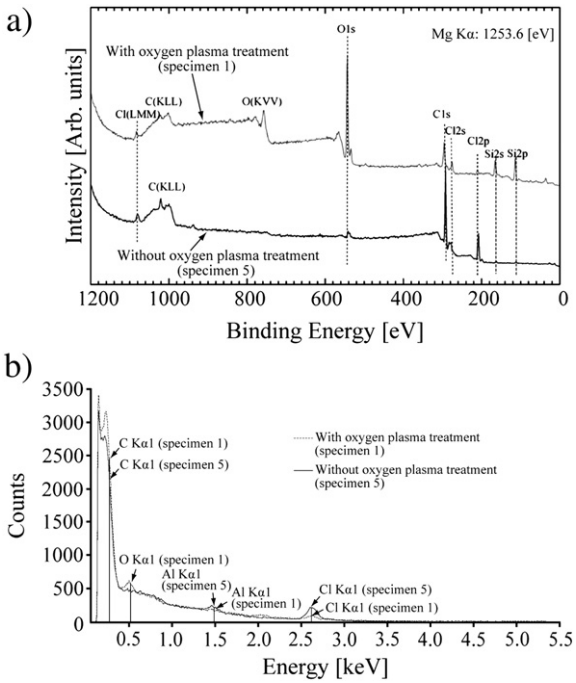

FIG. 1. Results of (a) XPS and (b) EDX analyses of specimens 1 (O_2 RIE with 10 cm^3/min of O_2 flow rate at 150-W for 5 min) and 5 (no-plasma treatment).

Fig. 3 shows the average density of adhering PC12 cells for all specimens. The error bars show the standard deviations. Significantly larger numbers of cells adhered to the Parylene-C surfaces with oxygen plasma treatment ($p < 0.001$ with Student's t-test) than to the Parylene-C surface without plasma treatment. There was no significant difference in the number of adhering cells among the treated specimens at 24 and 72 h ($p > 0.01$ with Tukey–Kramer test). On specimen 5, there were hardly any PC12 cells even at 72 h. However, PC12 cells adhered to the oxygen-plasma-treated Parylene-C surfaces at 24 h and increments of adhesiveness of PC12 cells were observed between 24 and 72 h. At 72 h, the number of cells adhering to all plasma-treated Parylene-C surfaces was almost equivalent to that at 72 h ($p > 0.1$; Student's t-test, Fig. 3), although the adherence on the plasma-treated Parylene-C was significantly less ($p < 0.01$; Student's t-test, Fig. 3) than that on the tissue-culture grade PS dishes at 24 h.

As shown in Fig. 2, neuronal cell adhesiveness to Parylene-C was increased by oxygen plasma treatment and was equivalent to that on the tissue-culture grade PS dish. The Parylene-C surface became hydrophilic after exposure to oxygen plasma. Seong et al. (9) and Mitchell et al. (14) reported that the contact angle is decreased by plasma treatment and that the decrease is related to the exposure period. We detected oxygen in the Parylene-C surface coating after plasma treatment by the XPS and EDX analyses. These results indicate that hydrophilic groups containing oxygen, such as a hydroxyl group and a carboxyl group, appear on the surface due to oxygen bombardment of the Parylene-C surface, which is similar in hydrophilicity to the surface of the PS dishes (14–16). Dekker et al. (17) and Mitchell et al. (14) reported that these groups can induce increased adsorption of adhering molecules of the culturing medium. This hydrophilization technique has been commonly applied to tissue-culturing grade PS dishes (14,15,18). In the results of the XPS analysis, the increment of the Si peaks between specimens 1 and 5 is attributed to a Si contamination due to a tweezers scratch on Parylene-C surface that exposed the substrate glass in the area of the X-ray beam. The Al peak in the EDX results is attributed to contamination of the EDX detector, because the chamber and microstage are made of aluminum alloy and the Al peaks in the spectrum of specimens 1 and 5 are detected. Since no effect on cell adhesiveness was evinced by specimens with the observed Si and Al peaks and the possibly exposed area of the glass substrate of the specimens around the edges was excluded from cell observation, we did not take into account the effect of these peaks in this examination. Although the number of neuronal cells adhering in Fig. 2 shows that the adhesiveness of the plasma-treated Parylene-C surface is lower than that of the conventional tissue-culture grade PS dish in the early period of culturing, its adhesiveness increased and became close to that on the tissue-culture grade PS dish in later periods. The lower adhesiveness on the surface-modified Parylene-C in the early period implies that the adsorbability of serum proteins on the surface-modified Parylene-C is slightly lower than that on the commercial tissue-culture grade PS dish. No significant relationship between the plasma conditions and cellular adhesiveness was found in our results. This indicates that high adhesiveness was achieved at all plasma conditions. Thus, plasma treatment of Parylene-C surface is applicable for improving neuronal cell adhesiveness as a practical method in microfabrication processes. This promises a potentially useful coating material for neural devices.

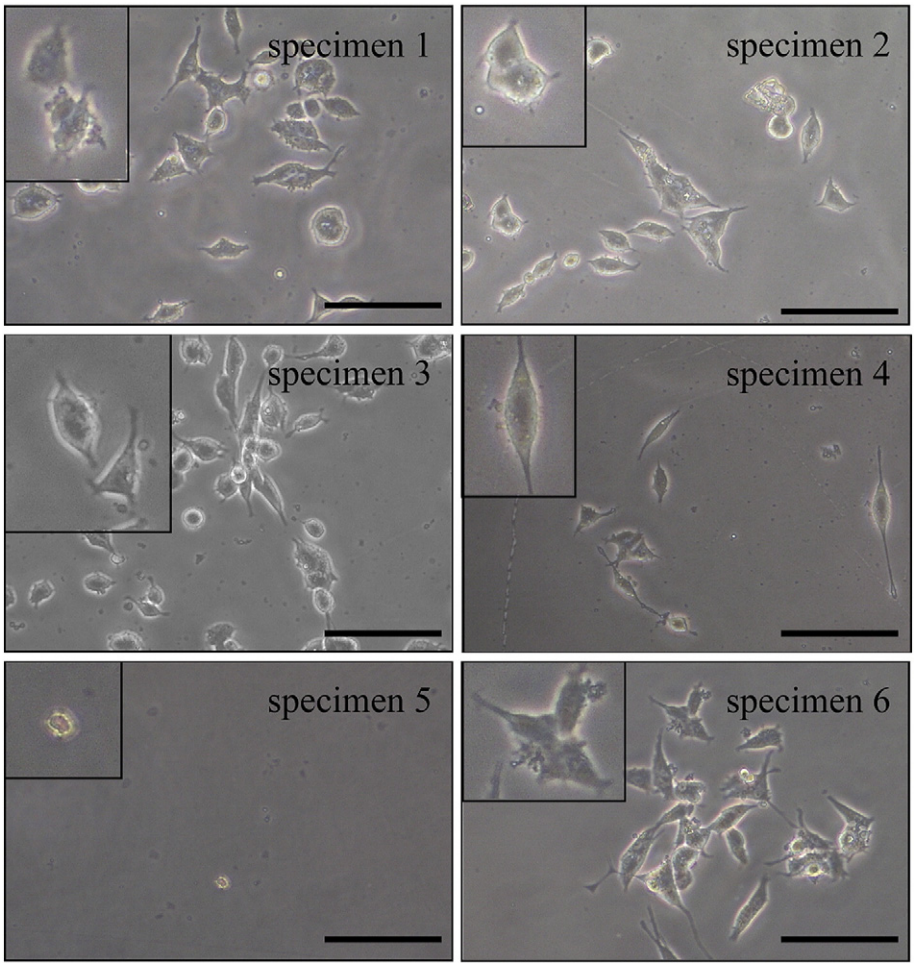

FIG. 2. Morphologies of PC12 cells on substrates at 72 h. Scale bar is 100 μm. Each top-left image is a 2× magnification.

Supplementary materials related to this article can be found online at doi:10.1016/j.jbiosc.2011.11.003.

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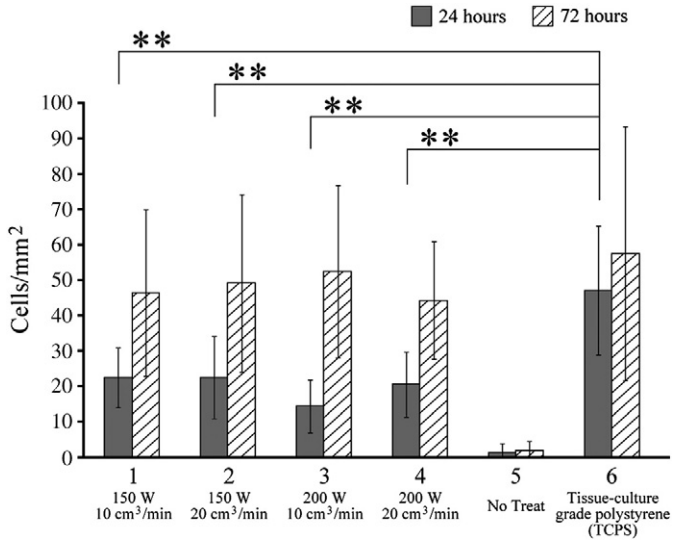

FIG. 3. Experimental condition dependence of neuronal cell adhesiveness (Student's *t*-test; $^{**}p<0.01$). Error bars show the standard deviations.

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