Neurite Outgrowth of PC12 Cells on diX (Parylene) Family Materials

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We investigated neuronal cell differentiation, particularly neurite outgrowth, on the surface of diX H and diX AM using an in vitro examination of a neuron-like rat pheochromocytoma cell line, PC12. diX H and diX AM are in the parylene family of diX C (or Parylene-C), which is widely used as a novel coating material to insulate neural electrodes, and they have been recently commercialized; diX H and diX AM offer different features of biocompatibility. Previously, we found that these new parylene materials have high cell adhesiveness to neuronal cells whereas the adhesiveness of diX C is extremely low. However, their cell differentiation remains unknown although neuronal cell differentiation plays a crucial role in their development and regeneration. This study showed that almost all PC12 cells adhering to the surface of diX AM and diX H were differentiated, but the neurite outgrowth was significantly larger on diX H than that on diX AM and a conventional polystyrene culture dish. The result suggests that diX H may be advantageous as a biocompatible coating material for a scaffold, which can be used on virtually any substrate to get various configurations in neural devices.
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Introduction

diX C or Parylene-C [poly(chloro-para-xylylene)] is widely used as a representative coating material for many kind of neural devices,[1–3] because of its attractive physical properties, the ability to form a thin and conformal coating on virtually any substrate,[1–3] and compatibility with a micromachining process.[2,3] However, its uses are limited in supporting and promoting neuronal cell growth due to its hydrophobic characteristic, which leads to extremely low adhesiveness with neuronal cells. Thus, diX C is not suitable as a scaffold for neuronal cells, despite its other attractive characteristics.

Recently, it was reported that treatment on the surface of diX C by immersion and incubation in a horse serum improved neuronal and glial cell adhesiveness.[4–7] This method would be applicable to other studies, but an extra process step after diX C coating is requisite. On the other hand, several functional materials in the diX family have recently been made commercially available, including diX A, diX AM, diX H, diX SR, diX D, and diX CF. Physical characteristics of these materials are similar to those of diX C, but, more importantly, they offer different functionalized features of biocompatibility from diX C. Previously, we examined the surface effect of materials in the diX family on the degree of neuronal cell morphology and adhesiveness and found that diX H [poly(monoaldehyde-para-xylylene)] and diX AM [poly(monoaminomethyl-para-xylylene)] were excellent neuronal cell adhesives.[8] Their cell adhesiveness was almost equivalent to that on the conventional culture polystyrene (PS) dish, although neuronal cells did not settle

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on diX C. However, it remains unclear whether neuronal cells can undergo differentiation on diX H and diX AM after the cells have adhered on the surface of these materials. Cell adhesiveness is the first step in a cascade of events, and it influences differentiation.[9,10] Neuronal cell differentiation plays a crucial role in cell growth and development.[9,10] That is, to apply these materials as a scaffold in a substrate for forming a neural device, we need to confirm whether they can guide neuronal cell growth and development. Therefore, we focused on examining how the surface of diX H and diX AM affects the degree of differentiation of adhered neuronal cells by observing neurite outgrowth. These results were compared with those obtained using the PS dish in an *in vitro* investigation using neuron-like cells, i.e., the rat adrenal pheochromocytoma cell line (PC12 cells).[11,12]

Methods

Details of the sample preparation and cell cultures have been described previously.[8] Briefly, three different coatings were used on a substrate and we cultured cells in four wells of six-well PS culture plates (Falcon 35-3046, Becton Dickinson and Company, Franklin Lakes, NJ) for each of the coating specimen types. The coatings specimen types were diX H (Daisankasei, Tokyo, Japan), diX AM (Daisankasei), and non-diX-coated PS culture plates, which served as the control condition.[13] We did not include diX C in this study since we had not previously observed cells adhering to its surface.[8] The cell line of PC12 cells (RCB-0009, Riken Cell Bank, Tsukuba, Japan) was chosen to study the effect of the diX family materials on neurite extensions because these cells undergo differentiation as a neurite outgrowth when they are exposed to nerve growth factor (NGF).[11,12] Coating specimens were immersed in the PC12-containing medium with the concentration of 2×10^5 cells/mL and the cells were cultured on them for 48 h. The medium was a neuro basal medium (21103, Gibco/Invitrogen, Carlsbad, CA) containing 5% horse serum and 10% fetal bovine serum and maintained at 37°C in a humidified atmosphere containing 5% CO_2. For differentiation, PC12 cells were primed by the addition of 50 ng/mL 2.5S recombinant rat beta-NGF (556-NG-100, R&D Systems, Minneapolis, MN). Their morphologies were observed with an inverse phase contrast microscope (IX-70, Olympus Tokyo, Japan), and more than seven pictures with at least 280 cells for each coating specimen type from all four wells of the six-well PS culture plates were captured. The area of a picture was 0.51 mm^2 around the center of the well and these areas were not overlapped. The number of neurites of the differentiated PC 12 cells, which adhered to each surface after 48 h, was manually counted from the pictures (ImageJ, National Institutes of Health, Bethesda, MD), and the neurite length was measured from the pictures as well. The average number of neurites per cell was estimated by first counting the number of neurites per cell and then averaging the number of neurites per cell by each coating specimen type. The ratio of the number of neurites per cell was calculated as the number of neurites per cell divided by the total number of cells for each coating specimen. To estimate the average length of the neurites per cell, the length of each neurite was measured and the mean length of the neurites per cell was calculated. Then it was sorted according to the number of neurites per cell for each coating specimen. All data presented are expressed as mean ± standard deviation (SD). The Tukey–Kramer test for multiple comparisons was used and the level of statistical significance was defined as $P < 0.05$.

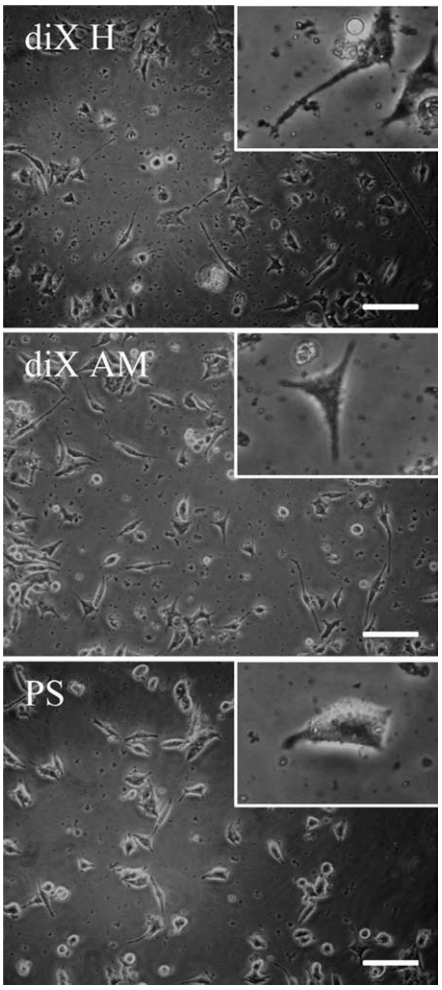

Figure 1. Morphologies of PC12 cells on diX H, diX AM, and PS at 48 h. Each top-right image is a ×40 magnification. Scale bar is 100 μm.

Results and Discussion

The PC12 cell morphologies for all specimens after 48 h are shown in Figure 1. The specimens reached confluence at 72 h. PC12 cells adhered to the surface of the diX H and diX AM with extremely high adhesiveness, although a few PC12 cells floated in the culturing medium or a few cells settled on the surface in round-shaped deposits. Almost all adhered cells were differentiated and observed to grow neurites. Notably, there were no differences in the morphologies of the PC12 cells that had adhered to the non-diX-coated PS culture dishes.

Figure 2a shows the average density of adhering PC12 cells on the surface of diX H, diX AM, and PS at 48 h, and Figure 2b shows the average number of neurites of adhered PC12 cells on the surface of diX H, diX AM, and PS at 48 h. The error bars show the SDs of the means in the images. The average density of adhering cells for all specimens was high and a similar result to our previous study.[8] The average numbers of neurites per cell on diX H, diX AM, and PS were 2.77 ± 1.48, 1.58 ± 0.75, 1.97 ± 0.96, respectively. There was a significant difference in the average number of

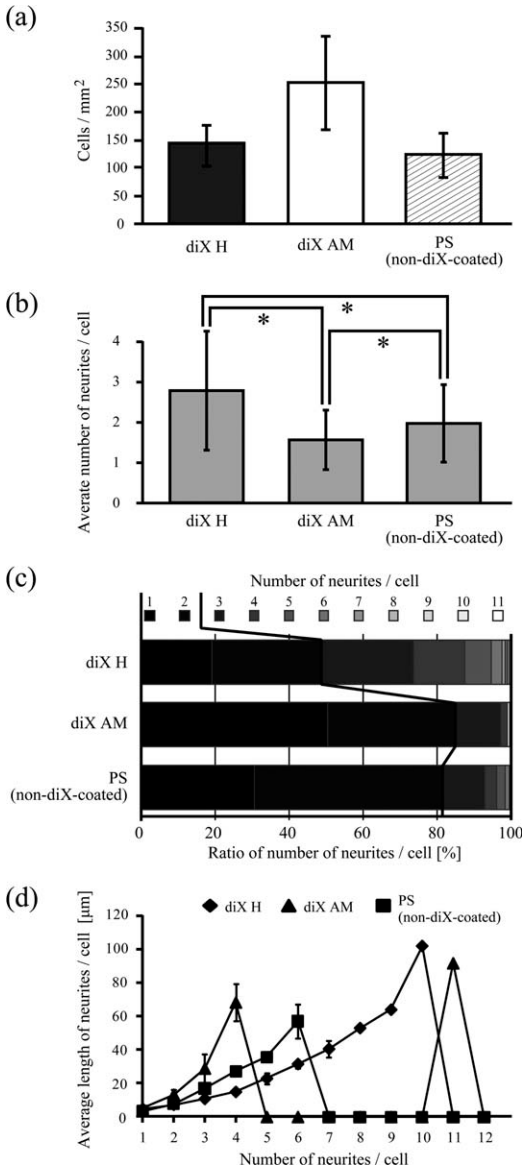

Figure 2. (a) Average density of adhering PC12 cells on the surface of diX H, diX AM, and PS at 48 h; (b) average number of neurites of adhered PC12 cells for all specimens at 48 h (Tukey-Kramer test; * $P < 0.05$); (c) the ratio of the number of neurites per cell for all specimens at 48 h. A ratio of less than three neurites per cell is distinguished by the black bar line; (d) relationship between the number of neurites per cell and the average neurite length of a cell for all specimens at 48 h.

Error bars show the SDs.

neurites per cell among the coating specimens ($P < 0.05$). The maximum number of neurites per cell was 10 on diX H, 11 on diX AM, and six on PS, and their minimum numbers of neurites per cell were all 1, whereas the largest numbers of cells with neurites were two neurites per cell on diX H, one neurite per cell on diX AM, and two neurites per cell on PS. The ratio of the number of neurites per cell is illustrated in the bar graph of Figure 2c. The ratios for less than three neurites per cell are demarcated by the bold black line, and they were 49, 85.4, and 81.9% on diX H, diX AM, and PS, respectively. Eleven neurites per cell on the surface of diX AM was observed at 48 h. But it was 1/384 cells and was statistically more than nine SD away from the mean (1.6 ± 0.88, mean $\pm$ SD). Thus, we assumed that the result of 11 neurites per cell on the surface of diX AM was abnormal behavior of the cell. Figure 2d shows the relationship between the number of neurites per cell and the average neurite length of a cell for all specimens at 48 h. The error bars show the SDs of the means in the images; we note that there was only one specimen each with eight or more neurites per cell so these data points do not have any error bars. This graph shows the trend that the larger the number of neurites per cell was, the longer the neurite length per cell was. This trend became stronger in the order of diX AM, PS, and diX H. The maximum neurite lengths of diX H, diX AM, and PS were 101.7 μm for 10 neurites per cell, 91.63 μm for 11 neurites per cell, and 67.45 μm for six neurites per cell and the minimum neurite lengths were 2.7, 1.3, and 0.9 μm at one neurite per cell, respectively.

Summarizing the results, the average number of neurites per cell on diX H was the largest and the longest neurite length of a cell with increment of the number of neurites per cell was observed on diX H, whereas the outgrowth on diX AM was smaller than that on PS. Further, the ratio for less than three neurites per cell on diX H (about 50%) was much smaller than that on diX AM and PS, which were over 80%, meaning that larger numbers of neurites were well differentiated on diX H. This trend in increasing the number of neurites per cell leading to the extension of the neurite lengths per cell is consistent with other reports in indicating the degree of differentiation,[14,15] although the culture conditions of these references differed from those of our present study. These results suggest that diX H has the most suitable surface characteristics for the neurite outgrowths. Interestingly, there was no significant difference in cell adhesion between diX H and diX AM in our previous study,[8] but the degree of neurite outgrowth was significantly different between them. This implies that the aldehyde group (—CHO) in diX H, which is generally known to conjugate amines on proteins is relatively more effective to promote the differentiation of neurons than the amino group ($—NH_2$) in diX AM,[16,17] although the aldehyde group may react, not with the cell, but with protein in the medium. Combining the results, we conclude that the aldehyde group in diX H is the most suitable functional group among the diX family materials for supporting neuronal cells.

The conformal coating of diX H with high cell adhesiveness and capability to promote differentiation should be important advantages over conventional polymers for fabricating neural devices. This coating can be used for both two- or three-dimensional surfaces, and it is applicable to not only external surfaces but also inside surfaces of microfluidic structures, which would lead to the ability to seed cells on inside structure walls. Topographical patterning is also possible, which would provide anisotropic or directional growth for cells with regeneration of neural tissue and formation of neural networking.[18] The patterning capability of the coating can be expanded to application for *in vivo* neural prosthetics, such as providing stable control for autonomic neural signals of an artificial organ and peripheral neural systems of artificial limbs.[19,20]

In conclusion, we examined neuronal cell differentiation on the surface of newly commercialized diX H and diX AM materials. Although the observed differentiation of diX AM and diX H was almost equivalent to that of PS tissue culturing, diX H achieved a higher cellular differentiation of

neurite outgrowth, which was significantly larger than that of the standard PS tissue culture. Thus, diX H holds promise for use as a biocompatible coating material for a scaffold, which can be used in a substrate to get various configurations in neural devices.

Acknowledgments

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Literature Cited

1. Loeb GE, Bak MJ, Salcman M, Schmidt EM. Parylene as a chronically stable, reproducible microelectrode insulator. *IEEE Trans Biomed Eng*. 1977;24:121–128.
2. Takeuchi S, Ziegler D, Yoshida Y, Mabuchi K, Suzuki T. Parylene flexible neural probes integrated with microfluidic channels. *Lab Chip*. 2005;5:519–523.
3. Hassler C, von Metzen RP, Ruther P, Stieglitz T. Characterization of parylene C as an encapsulation material for implanted neural prostheses. *J Biomed Mater Res B Appl Biomater*. 2010;93:266–274.
4. Delivopoulos E, Murray AF, MacLeod NK, Curtis JC. Guided growth of neurons and glia using microfabricated patterns of parylene-C on a SiO_2 background. *Biomaterials*. 2009;30:2048–2058.
5. Delivopoulos E, Murray AF, Curtis JC. Effects of parylene-C photooxidation on serum-assisted glial and neuronal patterning. *J Biomed Mater Res A*. 2010;94:47–58.
6. Unsworth CP, Delivopoulos E, Gillespie T, Murray AF. Isolating single primary rat hippocampal neurons & astrocytes on ultra-thin patterned parylene-C/silicon dioxide substrates. *Biomaterials*. 2011;32:2566–2574.
7. Unsworth CP, Holloway H, Delivopoulos E, Murray AF, Simpson MC, Dickinson ME, Graham ES. Patterning and detailed study of human hNT astrocytes on parylene-C/silicon dioxide substrates to the single cell level. *Biomaterials*. 2011;32:6541–6550.
8. Kato YX, Saito I, Takano H, Mabuchi K, Hoshino T. Comparison of neuronal cell adhesiveness of materials in the diX (Parylene) family. *Neurosci Lett*. 2009;464:26–28.
9. Letourneau PC. Possible roles for cell-to-substratum adhesion in neuronal morphogenesis. *Dev Biol*. 1975a;44:77–91.
10. Letourneau PC. Cell-to-substratum adhesion and guidance of axonal elongation. *Dev Biol*. 1975b;44:92–101.
11. Waddell RL, Marra KG, Collins KL, Leung JT. Doctor JS. Using PC12 cells to evaluate poly(caprolactone) and collagenous microcarriers for applications in nerve guide fabrication. *Biotechnol Prog*. 2003;19:1767–1774.
12. Achyuta AK, Cieri R, Unger K, Murthy SK. Synergistic effect of immobilized laminin and nerve growth factor on PC12 neurite outgrowth. *Biotechnol Prog*. 2009;25:227–234.
13. Curtis AS, Forrester JV, McInnes C, Lawrie F. Adhesion of cells to polystyrene surfaces. *J Cell Biol*. 1983;97:1500–1506.
14. Attiah DG, Kopher RA, Desai TA. Characterization of PC12 cell proliferation and differentiation-stimulated by ECM adhesion proteins and neurotrophic factors. *J Mater Sci Mater Med*. 2003;14:1005–1009.
15. Lee SJ, Khang G, Lee YM, Lee HB. The effect of surface wettability on induction and growth of neurites from the PC-12 cell on a polymer surface. *J Colloid Interface Sci*. 2003;259:228–235.
16. MacBeath G, Schreiber SL. Printing proteins as microarrays for high-throughput function determination. *Science*. 2000;289:1760–1763.
17. Naka Y, Eda A, Takei H, Shimizu N. Neurite outgrowths of neurons on patterned self-assembled monolayers. *J Biosci Bioeng*. 2002;94:434–439.
18. Tai HC, Buettner HM. Neurite outgrowth and growth cone morphology on micropatterned surfaces. *Biotechnol Prog*. 1998;14:364–370.
19. Mitsui M, Suzuki T, Mabuchi K, Takiura K, Saito I, Chinzei T, Mochizuki S, Isoyama T, Abe Y, Imachi K. Development of a novel intrafascicular nerve electrode. *ASAIO J*. 2005;51:692–695.
20. Ramachandran A, Junk M, Koch K.P, Hoffmann KP. A Study of Parylene C Polymer deposition inside microscale gaps. *IEEE Trans Adv Packag*. 2007;30:712–724.

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