Comprehensive Evaluation of Stable Neuronal Cell Adhesion and Culture on One-Step Modified Polydimethylsiloxane Using Functionalized Pluronic

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ABSTRACT: Polydimethylsiloxane (PDMS) is a popular and property-advantageous material for developing biomedical microsystems and advancing cell microengineering. The requirement of constructing a robust cell-adhesive PDMS interface drives the exploration of simple, straightforward, and applicable surface modification methods. Here, a comprehensive evaluation of highly stable neuronal cell adhesion and culture on the PDMS surface modified in one step using functionalized Pluronic is presented. According to multiple comparative tests, this modification is sufficiently verified to enable more significant cell adhesion and spreading in both quantity and stability, higher neuronal differentiation and viability/growth, more complete formation of the neuronal network, and stabler neuronal cell culture than the common coating tools on the PDMS substrate. The comparable and even superior cellular effects of this modification on PDMS to the standard coating of polystyrene for in vitro neurological research are demonstrated. Long-term microfluidic neuron culture with stable adhesion and high differentiation on the modified PDMS interface is accomplished, too. The achievement provides a detailed experimental demonstration of this simple and effective modification for strengthening neuronal cell culture on the PDMS substrate, which is useful for potential applications in the fields of neurobiology, neuron microengineering, and brain-on-a-chip.

INTRODUCTION

Polydimethylsiloxane (PDMS) is a widely used silicon-based polymer for diverse applications ranging from elastomers, medical devices, surfactants, to lubricants, as well as in daily life (e.g., shampoos to make hair slippery and foods for antifoaming).1−3 Recently, PDMS has become pretty popular as a stamp resin in the soft lithography procedure, which makes it one of the most common materials used in microfluidics (i.e., lab-on-a-chip), a promising science and technology of miniaturized systems in the 21st century for multiple disciplines of natural sciences.4,5 Especially for life sciences such as cell/tissue microengineering, the PDMS material, being inexpensive, flexible, chemically inert, optically transparent, nontoxic to cells, impermeable to water, permeable to gases, used in microprototyping, and fabrication-convenient, enables itself advantageous to conduct various biological studies on the microscale.6,7 Unfortunately, it is known that the PDMS surface is hydrophobic and cell-repellent, which result in poor adhesion and culture of mammalian cells (particularly neuronal cells, being very sensitive to the extracellular microenvironment).5,9 The defective problems extremely minify the cell compatibility of PDMS microsystems like cell-based microplatforms and chips. Therefore, the methodological development of an effective surface modification to promote cell adhesion on PDMS-related interfaces for expanding cell-culture-based microengineering applications is highly desirable.10

To this end, different strategies have been developed by research groups worldwide to modify PDMS for establishing cell-adhesive surfaces.11 Generally, two categories including physical coating and chemical coupling are involved.12 Physical coating of substances (e.g., collagen, laminin, and polylysine) to the PDMS surface can improve cell adhesion,13−15 but is unable to maintain it in a long-term cultivation, i.e., low adhesion stability. On the other hand, chemical coupling to form an adhesive PDMS interface is quite stable.16−20 However, it is commonly cumbersome and difficult to perform due to the chemical inertness of PDMS. Multistep reactions required in this process, as well as the use of various organic solvents, oxidants, or toxic reactants, invisibly hinder the popularization and application of chemical coupling for cell-adhesive modification in microengineering and biomedical microplatforms with PDMS.21,22 Accordingly, constructing a
facile, effective, and feasible method for stable and long-lasting cell adhesion/culture on the PDMS surface is necessary. Recently, we have reported a simple, straightforward, and practicable tool to enhance neuron adhesion on PDMS by one-step surface modification using functionalized Pluronic (poly-D-lysine-conjugated Pluronic F127, F127-PDL). Facile manipulation of F127-PDL coating was presented, and its ability to promote neuronal cell adhesion was found and preliminarily verified. However, its comprehensive demonstration involving multiple comparative and quantitative neuron behaviors for supporting stable and reliable neuronal cell culture on PDMS and in PDMS microfluidics is necessary.

In this study, to confirm a robust performance in the manipulation of microenvironment-sensitive neurons on the PDMS material, we systematically investigated neuronal cell adhesion and culture on the PDMS surface modified by the established simple-to-operate F127-PDL treating process. The adhesion (cell density and area), differentiation (axon-dendrite formation, axon branching, dendrite and multidendrite generation), and growth (axon and dendrite elongation) of primary neuronal cells along with the culture on the unmodified PDMS and the modified PDMS interfaces with F127-PDL, Pluronic F127 (F127), poly-D-lysine (PDL), or poly-D-lysine (PLL), as well as on the polystyrene (PS) surfaces coated with PDL or PLL, were comprehensively evaluated. Further, the neuronal cell activities associated with the adhesion, viability, and neuronal network formation during long-term on-chip and off-chip cultivation on the F127-PDL-modified PDMS surfaces were analyzed based on optical, fluorescence, and immunocytochemical detections. Meanwhile, a comparative assessment of long-term neuronal cell culture between different modified PDMS and PS surfaces was conducted, too.

RESULTS AND DISCUSSION

Neuronal Cell Adhesion on F127-PDL-Modified PDMS. For the remarkable effect on cell adhesion, the F127-PDL molecule (Figure 1) was designed to combine the excellent stability of the Pluronic F127 coating on the hydrophobic PDMS surface24,25 and the well-known cell-adhesive feature of PDL by electrostatic cell attraction. Consequently, the functionalization of Pluronic F127 with a cell-attracting PDL assists PDMS in producing a highly stable neuron-adhesive interface. Furthermore, the process of F127-PDL modification on the PDMS substrate is quite simple (i.e., one step) and similar to the conventional PDL coating.27 In general, the F127-PDL solution is added onto the PDMS surface, and an assembled cell-adhesive layer is spontaneously formed and stabilized in the solution. The one-step modification is quite simpler than the previous multistep methods.18,19 Theoretically, the F127-PDL layer on PDMS is able to cause more significant neuronal cell adhesion in quantity and stability than PDL. This enhanced cell compatibility is critical for performing robust culture-based neuron manipulation and analysis on the PDMS interface or PDMS-involved microsystems and microfluidic platforms.

To systematically demonstrate the positive effect of F127-PDL on cell adhesion and its potential application merit, we first investigate the adhesion of neuronal cells on the modified PDMS substrate. The F127-PDL solution was pipetted onto the PDMS surface followed by a 2 h incubation and a rinse. The chemical and hydrophilic characterization results presented that F127-PDL can be well coated onto the PDMS substrate (Figure S1) and largely increased its wettability (Figure S2) and hydrophilic stability (at least 12 days, Figure S3). Primary neuronal cells were then seeded on the modified substrate, and the cultivation was carried out. For comparison, the neuronal cell cultures on the unmodified PDMS substrate as blank control, the modified PDMS substrates (with F127, PLL, or PDL), and the modified PS substrates (with PLL or PDL, which is the representative adhesive molecule for standard in vitro neuron cultivation17,26,27) were applied here. To evaluate the adhesion, the culture medium was replaced with a fresh medium to remove the suspended cells after culture for 0.5 day. The neuronal cells on different PDMS interfaces were optically imaged and quantitatively analyzed. The results are shown in Figure 2. We found 47, 259, and 6918% increases in the number (i.e., density) of neuronal cells adhered on F127-PDL-modified PDMS (F127-PDL/PDMS) compared to the respective PDL-modified PDMS (PDL/PDMS), PLL-modified PDMS (PLL/PDMS), and unmodified PDMS after 0.5 day in culture. The adhesion of neuronal cells on F127-PDL/PDMS was quantitatively similar and slightly higher than PDL-modified PS (PDL/PS), which was known to help more cells to adhere (over 60% increase in this study) than PLL-modified PS (PLL/PS). In addition, we also observed that there were not any cells that remained on the PDMS surface treated with F127, which was demonstrated to exhibit intense and constant protein/cell repellency. Opposite to our purpose for the adhesion, F127 is popularly used for stable inhibition of protein adsorption and cell adhesion on hydrophobic materials like PDMS.25,29

Next, the quantitative results (Figure 2C) showed over 25, 89, and 98% increases in cell spreading (i.e., neuronal cell area) on F127-PDL/PDMS compared to the respective PDL/PDMS, PLL/PDMS, and unmodified PDMS after 0.5 day in culture. The F127-PDL modification on PDMS achieved a similar neuron spreading effect to the classic PDL coating on PS, a very popular material to fabricate in vitro neuron culture-related Petri dish.30 Furthermore, we observed whole adhesion of all neuronal cells without any self-aggregation on both the F127-PDL/PDMS and PDL/PDMS substrates within 1 day of culture. They were quite different from the PLL/PDMS and control PDMS surfaces. Therefore, we confirmed a high positive impact of one-step F127-PDL modification on the
neuronal cell adhesion in terms of quantity and extensibility on the PDMS surface, which was comparable to the typical neuron-adhesive achievement using the classic PDL-modified PS interface.31

**Neuronal Cell Differentiation and Growth on F127-PDL-Modified PDMS.** The most distinctive characteristic of neurons is their morphological polarization, typically involving the differentiation of a single axon and multiple dendrites.32 The differentiation of axons and dendrites is a crucial process in neuronal development and is an important section of evaluation for in vitro neurobiology.32,33 We further completely estimated the differentiation of neurons on the aforementioned seven substrates. Associated with neuronal differentiation and development, the axon-dendrite differentiation (axon-dendrite formation, Figure 3A), axon branching (Figure 3B), dendrite and multidendrite generation (Figure 3C,D), and axon and dendrite elongation (Figure 3E,F) were included for quantification and assessment.34,35 It was shown that most neurons (over 97%) presented axon-dendritic differentiation (i.e., the formation of either axon or dendrite) on F127-PDL/PDMS, which was quantitatively more than the other five types of substrates (PDL/PDMS, PLL/PDMS, F127/PDMS, control PDMS, and PLL/PS) except PDL/PS. The results of axon growth presented a similar tendency to the axon-dendritic differentiation on the substrates within 1 day of culture. The F127-PDL/PDMS surface caused better axon extension (over 2.5 μm per hour) than the other tested substrates (all less than 2.3 μm per hour). Based on the in-depth analysis of neuronal differentiation and growth in different culture tests, we found that there were only three types of substrates (F127-PDL/PDMS, PDL/PDMS, PDL/PS) allowing axon branching, dendrite generation, and elongation. As exhibited in Figure 3, the axon-dendrite activities of neurons on F127-PDL/PDMS, like PDL/PS, were more active (540, 189, 283, and 36% increases in axon branching, dendrite generation, multiple dendrite generation, and dendrite growth, respectively) than that on PDL/PDMS. These activities during the neurodevelopment process expedite the generation of the neural communication network, which constitutively guides the directional signal/information transfer in the central nervous system.33 The results suggested that F127-PDL modification enables brilliant neuronal cell differentiation and growth, being critical for performing culture-based neuron microengineering and microfluidics. We experimentally demonstrated from different aspects that the F127-PDL molecules presented more satisfactory effects on neuronal cell adhesion, differentiation, and growth on different PDMS and PS substrates. The respective modified PDMS with F127-PDL, PDL, PLL, and F127, the unmodified PDMS as blank control, and the modified PS with either PDL or PLL were involved. (A,B) Optical images of primary neuronal cells on various PDMS (A) and PS (B) substrates within 1 day of culture. (C) The density (top) and area (bottom) of the neuronal cells adhering on different PDMS and PS substrates after 0.5 day in culture. ***P < 0.001. **Figure 2.** Neuronal cell adhesion, differentiation, and growth on different PDMS and PS substrates. The respective modified PDMS with F127-PDL, PDL, PLL, and F127, the unmodified PDMS as blank control, and the modified PS with either PDL or PLL were involved. (A,B) Optical images of primary neuronal cells on various PDMS (A) and PS (B) substrates within 1 day of culture. (C) The density (top) and area (bottom) of the neuronal cells adhering on different PDMS and PS substrates after 0.5 day in culture. ***P < 0.001.
neuron development than the other routine molecules for one-step PDMS modification.

Neuronal Cell Culture on F127-PDL-Modified PDMS. Based on the cellular achievements related to surface modification mentioned above, we next evaluated the neuronal cell culture on the F127-PDL/PDMS, PDL/PDMS, and PDL/PS substrates. Cell cultivation for a total of 7 days was performed here. The microscopic results showed (Figure 4A) that the neuronal cells gradually aggregated along with the cultivation and finally formed the neuronal spheroids on PDL/PDMS. The axon connections between the spheroids became quantity-dropped and space-floating, suggesting the process of neurodegeneration, which is unsuitable for long-term culture-based in vitro neuronal investigation.36,37 Cheerfully, we noticed that F127-PDL modification rendered the neuronal cells robustly adhesive to the PDMS surface within the whole 7 days of culture, being like the classic PDL modification of PS in the Petri dish. The neuronal network based on the axon and dendrite connections progressively formed and appeared denser along the culture process. Moreover, the quantitative analysis presented that the density of neuronal cells on F127-PDL/PDMS was higher than that of PDL/PS during the culture, especially after culture for 7 days (Figure 4B). The neuronal cell area associated with F127-PDL/PDMS (Figure 4C) increased with cultivation time and was significantly higher than that involving PDL/PDMS, which induced the formation of self-aggregated neuronal spheroids. The F127-PDL/PDMS interface also presented better potential, to a certain extent, for neuron spreading than PDL/PS. The results suggested that stable neuronal cell adhesion and spreading, as well as the formation of the neuronal network, can be constructed on the F127-PDL/PDMS surface in long-term culture. In addition, it was hard to prolong the testing time after 1 day in culture for quantitative comparison of axon/dendrite growth on different substrates due to the formation of an intricate neuronal network on F127-PDL/PDMS, as well as the aggregation of neuronal cells on PDL/PDMS.
Viability and immunocytochemical analyses were performed to further assess the neuronal cell culture on the F127-PDL/PDMS substrate. We used the common FDA/PI double staining to fluorescently present the live/dead (green/red) neuronal cells on different substrates at the time points within 7 days of culture.38 The images and the quantified results are shown in Figure 5. The number of cell deaths on the PDL/PDMS increased with culture time. Plenty of red fluorescent dots in the neuronal spheroids on the PDL/PDMS surface appeared after 7 days in culture. Comparatively, the viability of neuronal cells on either F127-PDL/PDMS or PDL/PS remained invariant (nearly 99%) along with the cultivation.

We next immunocytochemically stained the neurons on both F127-PDL/PDMS and PDL/PS after 7 days in culture (Figure 6 and Figure S4). The representative axon-specific (SMI-312) and somatodendritic (MAP2) markers were involved here for neuronal identification. The fluorescence imaging results showed that neurons on F127-PDL/PDMS polarized as they were on the standard PDL/PS substrate. Long/slender (i.e., the axons) and short (i.e., the dendrites) protoplasmic extensions of the neurons built a complex neuronal network, which is indispensable for neurocommunication.39,40 These results suggested that the F127-PDL molecules played a critical role to maintain the viability and polarization of the neuronal cells on the PDMS substrate, presenting a remarkable cell compatibility. Totally, we demonstrated that a stable adhesion with high quantity and extensibility, high differentiation, and long-term culture of neurons can be smoothly completed on the PDMS surface after the one-step F127-PDL modification. The simplicity of this modification follows the representative operation in standard dish cultures.

Microfluidic Neuronal Cell Culture Based on F127-PDL Modification. Microfluidic chips bring new opportunities for controlling cell activity in time and space and rebuilding in vivo-like complex biochemical and geometric microenvironments in microchambers and channels.41 Over the past 20 years, microfluidic systems have become popular, too, as potential microplatforms for neurobiology.42-44 Depending on the robust neuron culture related to F127-PDL, we compared the neuronal cell culture in a microfluidic chip containing the F127-PDL/PDMS surface to the off-chip F127-PDL/PDMS and PDL/PS surface substrates. The rule of one-step treatment was also followed for the internal surface modification of PDMS microfluidics. An F127-PDL solution was introduced into a device, and the incubation was performed for 2 h. The device was ready for use to perform neuronal cell culture after a rinsing process. The optical and fluorescence imaging results (Figure 7A and Figure S5) showed that high cell viability and neuronal polarization happened in the microfluidic culture for 7 days after F127-PDL treatment on the PDMS substrate. Further, the comparative analyses (Figure 7B) showed that F127-PDL-assisted microfluidic culture was quite favorable for neuronal cell spreading and axon-dendrite differentiation. In detail, the area of the...
neurons in the F127-PDL/PDMS chip was almost equal to that on the off-chip F127-PDL/PDMS and was higher than that on the common PDL/PS. The ratio of differentiation between on-chip F127-PDL/PDMS and off-chip F127-PDL/PDMS or PDL/PS was close to 1, implying a similar neuronal differentiation of F127-PDL/PDMS-associated on-chip culture to the other off-chip cultures with high adhesion and viability mentioned above. These results demonstrated the ability and applicability of the F127-PDL molecules for PDMS substrate modification in microfluidics for facile, highly effective, stable, and feasible on-chip neuronal cell culture, which was not the case for the previous microfluidic neuron devices generally with an adhesive substrate like PS and glass.45–48

CONCLUSIONS

In summary, we comprehensively explored the effects of F127-PDL on neuronal cell adhesion and culture on the PDMS substrate based on the one-step modification. Diverse neuronal cell activities associated with the adhesion, differentiation, and growth on either F127-PDL/PDMS or other modified substrates were comparatively and entirely investigated. We demonstrated from multiple aspects very positive impacts of F127-PDL on stable cell adhesion in quantity and extensibility, high differentiation and viability, and significant neuronal growth and network formation during the culture on PDMS, which was stronger than the commonly used PDL and PLL for one-step treatment on either the PDMS or standard PS interfaces. The F127-PDL-aided microfluidic long-term neuron culture on PDMS was verified to be pretty feasible and highly effective. The experimental achievements and systematic demonstrations support the advantageous features of the F127-PDL modification for robust neuronal cell culture on the PDMS substrate with largely enhanced cell compatibility. Considering the stability, simplicity, efficiency, feasibility, and applicability, this progressed PDMS surface modification may facilitate the development of microsystems and microfluidic platforms involving living neuronal cells for various applications in nervous tissue microenvironment establishment, neuronal sensing, and pharmaceutical analysis.

EXPERIMENTAL SECTION

Materials and Reagents. Poly(ethylene oxide)-poly-(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) block copolymer (Pluronic F127, average MW: 12,600), poly-d-lysine (PDL, MW: 150,000–300,000), fluorescein diacetate (FDA), propidium iodide (PI), and Hoechst 33258 were obtained from Sigma-Aldrich (MO, USA). F127-PDL was prepared according to our reported synthesis method.23 PDMS prepolymer (RTV 615A and curing agent (RTV 615B) were purchased from Momentive Performance Materials (Waterford, NY, USA). The SU-82025 photoresist and developer were from Microchem (Newton, MA, USA). Primary antibodies SMI-312 and MAP2 were purchased from Abcam (Cambridge, UK). Cell culture medium, fetal bovine serum (FBS), and Alexa Fluor conjugated secondary antibodies were purchased from Thermo Fisher Scientific (Waltham, MA, USA). All solvents and other chemicals of analytical reagent grade were purchased from local commercial suppliers, unless otherwise stated. All solutions were prepared using ultrapurified water supplied by a Milli-Q system (Millipore).

PDMS Substrate and Microfluidic Device Fabrication. To prepare a PDMS substrate, a well-mixed PDMS prepolymer [RTV 615 A and B (10:1)] was added (five drops) onto a 35 mm Petri dish (Corning), which was then spin-coated to form a thin PDMS film (3000 rpm for 30 s) and ready for use after baking at 80 °C for 30 min.

The microfluidic device was fabricated using soft lithography.49,50 The fluidic mold was produced via ultraviolet patterning of the SU-8 photoresist on a silicon wafer. To fabricate the PDMS device, the fluidic mold was exposed to trimethylchlorosilane vapor for 3 min. PDMS RTV 615 A and B were mixed in a 10:1 ratio, poured onto the molds, degassed in a desiccator, and cured in an 80 °C oven for 35 min. After incubation, the PDMS layer was peeled off the mold. Holes were punched using a hole puncher at the terminals of the channels. Next, the fluidic layer was placed on top of a glass slide, which was spin-coated with a thin PDMS film [RTV 615 A and B (5:1)], 3000 rpm for 60 s, and cured at 80 °C for 10 min. The microfluidic device was then ready for use after baking at 80 °C for 48 h.

Neuronal Cell Preparation. Primary cortical neurons were obtained from embryonic C57BL/6 mouse pups following the standard protocol.51,52 Briefly, the mouse pups were sacrificed by cervical dislocation, and the cortex was separated from the brain and dissociated with trypsin for 20 min at 37 °C. Next, neurons were collected by centrifugation and resuspended in the neurobasal medium for use. The animal experiment was approved by Institutional Animal Care and Use Committee of Central South University. This study was performed in accordance with the institutional guidelines (Central South University).

Neuronal Cell Culture on the PDMS and PS Substrates. For neuronal cell culture, the PDMS substrate on the 35 mm Petri dish was UV-sterilized and then modified using F127-PDL solution (100 μg/mL) or other molecules (F127, PLL, or PDL, 100 μg/mL) for 2 h at room temperature (RT) followed by phosphate-buffered saline (PBS, 0.01 M, pH 7.4) rinsing. The primary neuronal cells (1.0 × 10^6 cells/mL, 2 mL) were seeded in the PDMS-contained dish and cultured using a fresh supplemented neurobasal medium (a total of 2 mL in a 35 mm Petri dish) with 2% B27 (Gibco) at 37 °C in a humidified atmosphere with 5% CO₂. The cells were fed every third day by aspirating half of the medium (1 mL) from the dish and replacing it with the fresh supplemented neurobasal medium (1 mL). Additionally, the Petri dish in PS modified using PLL or PDL solution (100 μg/mL) was used, too, for neuronal cell culture.

Microfluidic Neuronal Cell Culture. For microfluidic neuronal cell culture, the PDMS device was UV-sterilized, gel-preloaded, and prepared for culture by coating the channels and chambers with F127-PDL solution (100 μg/mL) for 2 h at RT followed by PBS rinsing. The coated channels and chambers were then equilibrated with the fresh neurobasal medium. Neuronal cells were loaded into the device by flowing the suspension for 5 min at a cell density of 2.0 × 10^6 cells/mL. The device was incubated at 37 °C for 2 h to allow cell adhesion. The loaded cells in the device were cultured using the fresh supplemented neurobasal medium at 37 °C in a humidified atmosphere with 5% CO₂.

Cell Staining. Cell viability was evaluated using an FDA/PI staining method. After removing the culture medium from cells and rinsing with PBS, the FDA/PI (10 μg/mL of each in PBS) staining solution was added, and the staining process was
performed for 15 min at 37 °C. Afterward, PBS was introduced for 10 min as a final rinse.

For immunocytochemical analysis, neurons were fixed with 4% paraformaldehyde in PBS for 10 min at RT, permeabilized and blocked in PBS containing 0.3% Triton X-100 and 5% bovine serum albumin for 30 min at RT, and incubated with the primary antibody (1:200 for SMI-312 as the axon-specific marker and MAP2 as the somatodendritic marker) in the same buffer for 12 h at 4 °C followed by secondary antibodies conjugated with Alexa Fluor 555 (1:500) and Alexa Fluor 488 (1:500) for 2 h at RT for visualization. Cell nuclei were counterstained using a Hoechst staining solution (H33258, 0.5 μg/mL in PBS).

**Microscopy and Image Analysis.** A confocal laser scanning microscope (Olympus, FV1000) and an inverted microscope (Olympus, CKX41) with a mercury lamp (Olympus, U-RFLT50) were used to acquire phase-contrast and fluorescence images. Image-Pro Plus 6.0 (Media Cybernetics, Silver Spring, MD) and ImageJ plugin NeuronJ (NIH) were employed to conduct image analysis. SPSS 12.0 (SPSS Inc.) was used to perform data statistical analysis. Values are represented as means ± SD, and statistical comparisons of means were made using one-way analysis of variance (ANOVA). For all statistical tests, ***P < 0.001 was used as the criterion for statistical significance.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c05190.

Additional data associated with chemical and hydrophilic characterization, immunocytochemistry, and microfluidic neuronal cell culture (PDF)

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