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A biofunctionalization scheme for neural interfaces using polydopamine polymer

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ABSTRACT

Chemical surface modification of neuron-surface interfaces is essential for the development of biologically active and functional neural interfaces. Different types of surface modification schemes are required to derivatize either electrode or insulator surfaces, which limits the surface chemistry based neural interface design. Herein, we report a novel and powerful approach for modifying neuron-surface interfaces using mussel-inspired polymer ('polydopamine film(polyDA)') for generating effective chemical platforms on both electrode and insulator surfaces simultaneously. We applied polyDAs to common neural interface surfaces (gold, glass, platinum, indium tin oxide, liquid crystal polymer) and subsequently functionalized them by covalently linking biomolecules. The surfaces coated with polyDAs exhibited uniform and reproducible surface properties and they all became neuron-adhesive after linking with poly-D-lysine. In addition, polydopamine-coated microelectrode arrays were readily functional such that spontaneous and evoked neural activities could be recorded from cultured neuronal networks. We have successfully showed that a novel polyDA can be effectively used for the neural interface design.

1. Introduction

Neuron-surface interfaces have become indispensable in engineering problems including neural prostheses [1,2], neuron-based cell chips [3,4], and tissue engineering [5]. For neural prostheses, there were concentrated efforts for reducing the effects of inflammatory responses from the biosystems by modifying the surfaces of the devices, which construct neuron-material interfaces at the ends [6]. For example, bioactive molecules such as fibronectin [7], cell adhesion peptide [8], and neurotrophins [9] have been immobilized to the surfaces inspired by the idea that proximity of neurons to the electrodes could minimize the effects of the immune responses. There were also approaches using polyethylene glycol (PEG)-based coatings utilizing distinguishing non-biofouling effect of PEG brushes [10,11]. In neuron-based cell chip designs, surfaces are functionalized with various biomolecules to convert surfaces into cell-adhesive or neurite guiding surfaces. For example, ECM proteins were immobilized to gold surfaces to induce the attachment and differentiation of DRG neurons [12]; Polyaminoacids or polycations were used to position the neurons and guide the outgrowth of the neurites on gold [13] or polymer microparticles [14]; In addition, PEG or serine were used to obtain long-term stable patterning of hippocampal neurons on silicon-based surfaces (e.g. glass or silicon oxide) [15,16].

Surface modification schemes are required to functionalize neuron-surface interfaces. In neural probe or array designs, surfaces are composed of metal electrode and inorganic or organic insulators [17]. For insulators, silicon dioxide, silicon nitride or polyimide were derivatized by using organosilane with various functional groups. Mercapto groups were used to link biomolecules through heterobifunctional linker (sulfo-GMBS) [18], while epoxide groups were used for direct linking with an additional linker layer [16]. An organic insulator such polyimide could also be derivatized by organosilane chemistry. For metal electrodes, bioactive peptides were immobilized on electrodes by electroplating with conductive polymer coatings [19]. Despite these developments, there is still a need for some chemical modification schemes that can derivatize a variety of neural materials used for insulators and electrodes.

In this report, we demonstrate an approach for modifying neuron-surface interfaces using polydopamine films, mussel-inspired polymers, for the effective modification of the neuron-material interfaces. Polydopamine films (polyDAs) have recently been introduced by Messersmith et al., who reported their versatility for various substrates and feasibility for facile functionalization [20]. PolyDAs have exhibited uniform chemical

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characteristics independent of substrate materials, and facile covalent conjugation with nucleophiles (amines and thiols) taking advantage of catechol/quinone equilibrium. Here we applied polyDAs to investigate the application of polyDAs to neural interface designs. PolyDAs were applied to common neural interface materials (gold, glass, platinum, indium tin oxide, liquid crystal polymer) and converted them into biologically functional surfaces by covalently linking polylysines (Fig. 1). Primary neuronal cultures were used to test biocompatibility of polyDAs and cell viability was quantified. To demonstrate the applicability on actual neural devices, a planar microelectrode array surface was biofunctionalized using polyDAs and neural recording and stimulation were successfully obtained from cultured neuronal networks at 3 weeks *in vitro*.

2. Experimental section

2.1. Materials

2-Propanol (Merck, Germany), acetone (extra pure, Dae Jung Chemical & Metal Co., Ltd., Siheung, Korea), and Hank's Buffered Salt Solution (HBSS) (WelGENE Inc., Korea), were used as received. Sprague-Dawley rat embryonic stage 18 was purchased from Koatech, Co., Korea. Trypsin, 2.5% (10X), liquid, neurobasal, B-27 serum-free supplement, L-glutamine, penicillin-streptomycin, live/dead viability/cytotoxicity kit (L-3224), and phosphate buffered saline (PBS) 7.4 (1X), liquid were bought from Invitrogen and used as received. Trizma® hydrochloride (reagent grade, minimum 99%), trizma® base (minimum 99.9%), dopamine hydrochloride, and L-glutamic acid (meets EP testing specifications, cell culture tested, 98.5 ~ 100.5%, from non-animal source) were purchased from Sigma. For mounting of samples, Faramount Mounting Medium (Dako, Denmark) was used. Ultra pure water (18.3 MΩ cm) from the Human Ultra Pure System (Human Corp., Korea) was used.

2.2. Formation of polyDAs

Substrates were sonicated in acetone and 2-propanol for 5 min each, and dried under a stream of argon before use. Cleaned substrates were then immersed into a solution of dopamine (2 mg/mL in 10 mM Tris–HCl (pH 8.5)) for a desired reaction time (1 h-overnight). Because of the microparticle formation in the dopamine solution, the solution with substrates was needed to be vigorously stirred during the reaction to protect substrates from the particle deposition. The coated substrates were thoroughly rinsed with deionized water and dried under a stream of argon. The polyDAs remained stable at room temperature.

2.3. Polylysine linking on the polyDAs

Polydopamine-coated substrates were immersed in a buffered solution of poly-L-lysine (0.1 mg/mL in 10 mM Tris buffer, pH 8.5) for 1 h to overnight to yield polylysine-linked polyDAs. After the reaction, the substrates were thoroughly rinsed with deionized water and dried under a stream of argon. Above 1 h, changing reaction time did not make any significant difference to the linking.

2.4. Cell cultures

Primary hippocampal neurons were cultured in serum-free condition. Hippocampus from E-18 Sprague-Dawley rat was incubated with 0.25% trypsin for 15 min at 37 °C and residual trypsin was rinsed with Hank's Balanced Salt Solution (HBSS). The digested hippocampus was triturated in 1 ml of HBSS using a fire-polished Pasteur pipette. The cell suspension was centrifuged for 3 min at 1000 rpm and cell pallet was extracted. The cell pallet was suspended again in Neurobasal media supplemented with B-27, 2 mM L-glutamine, 12.5 μM L-glutamic acid, and Penicillin-Streptomycin using Pasteur pipette. Dissociated cells were seeded at the density of 200 cells/mm^2 on the prepared substrates. For microelectrode recordings, cells were seeded at the density of 1000 cells/mm^2. Cultures were maintained in an incubator (5% CO_2 and 37 °C) and the half of media was replaced with fresh culture media without L-glutamic acid supplement every 3 ~ 4 days. To image neurons on opaque substrates, samples were stained at 7 days *in vitro* (DIV) using a LIVE/DEAD Viability/Cytotoxicity Kit (Invitrogen, Carlsbad,

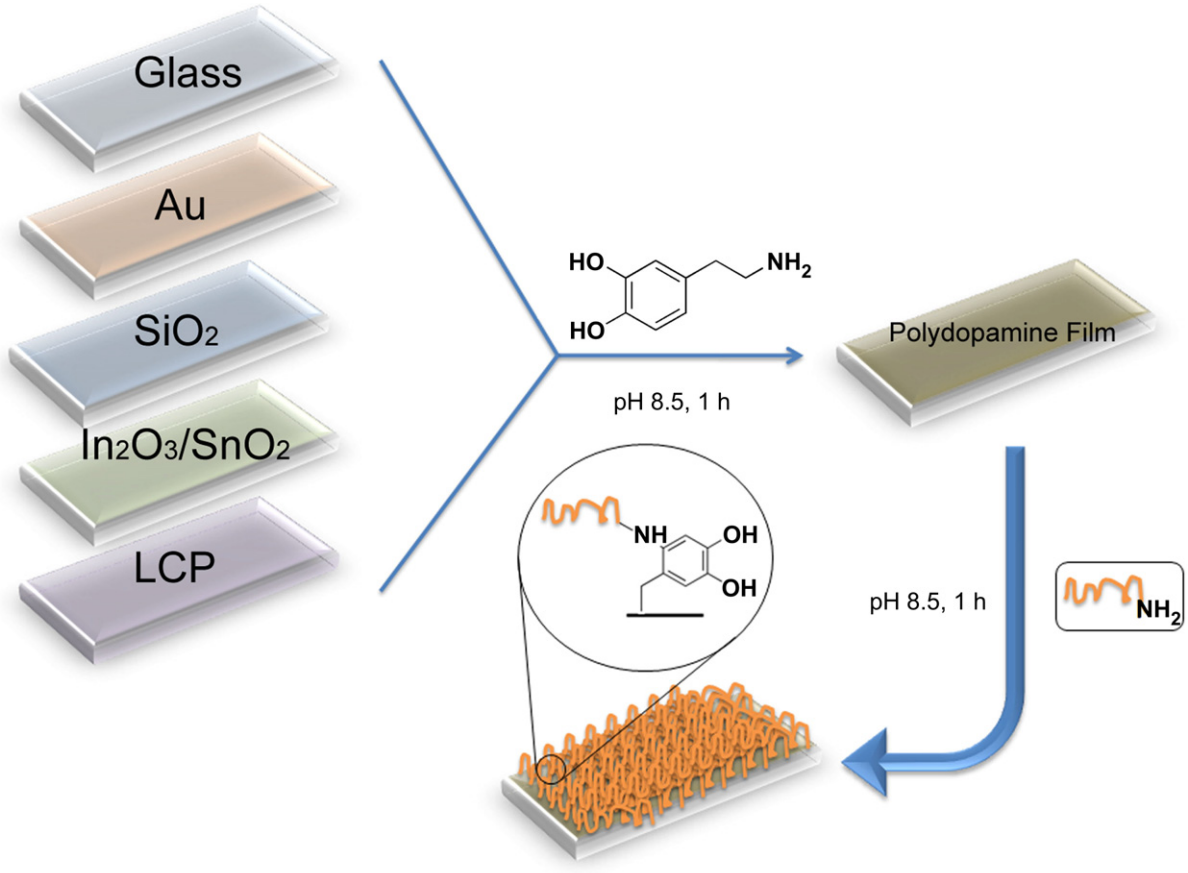

Fig. 1. Schematic illustration of the deposition of polyDA on various substrates and subsequent linking of amine molecules.

California), and the number of live/dead cell bodies were counted by the ImageJ software. This study was approved by IACUC of KAIST.

2.5. Instruments and characterizations

The formation of the polydopamine-coating on the substrates was confirmed by X-ray photoelectron spectroscopy (XPS) and contact angle goniometry. XPS study was performed with a VG-Scientific ESCALAB 250 spectrometer (U.K.) with a monochromatized Al Kα X-ray source (1486.6 eV). Emitted photoelectrons were detected by a multichannel detector at a take-off angle of 90 ° relative to the surface. During the measurements, the base pressure was 10^{-9}–10^{-10} Torr. Survey spectrum was obtained at a resolution of 1 eV from 1 scan. Contact angle measurements were performed using a Phoenix 300 goniometer (Surface Electro Optics Co., Ltd., Korea). Fluorescence microscopic images of neuron cultures were obtained using Olympus BX51M (Olympus Corp., Japan) equipped with a CCD camera (DP71, Olympus).

2.6. AC impedance measurement

The AC (alternating current) impedance at 1 kHz for each electrode was measured with an HP 4284A Precision LCR meter (Agilent Technologies, Inc., Palo Alto, CA) using 10 mV_{rms} as input voltage. Measurements were performed in PBS (phosphate buffered saline, pH 7.4) with a silver chloride wire as a reference electrode.

2.7. Neural recording/stimulation and data analysis

A planar microelectrode array (MEA) was composed of 60 gold microelectrodes with 4 types of electrodes (diameters of 5, 10, 30, and 50 μm) and silicon nitride (plasma-enhanced chemical vapor deposition, 500 nm) insulators. It was fabricated by standard photolithography, metal evaporation, and thin film deposition process provided by National Nanofabrication Center (Daejeon, KAIST).

An MEA 1060 amplifier (gain: 1200, bandwidth: 10 Hz–5 kHz, MultiChannel Systems, Germany) was used to record the electrical signals from the neuronal networks cultured on MEAs. The amplified signals were displayed and stored as digitized data (sampling rate: 40 kHz) by software (MC Rack, MultiChannel Systems, Germany).

Negative-first biphasic current pulses which were 9–20 μA in amplitude and 200 μs in width were delivered by STG 1004 stimulator (MultiChannel Systems, Germany). Responsive neural signals (extracellular action potentials) for each stimulus were recorded from other electrodes which were adjacent from the stimulating electrode. During the recording and stimulation experiments, the MEA was heated to maintain biologically relevant atmosphere (37 °C, 5% CO_2) and media evaporation was prevented by installing a fluorinated ethylene-propylene membrane on the MEA [21].

3. Results and discussion

3.1. Characterization of polyDAs and polylysine-linked PolyDAs

The formation of polyDAs were confirmed by measuring film thickness and water contact angle. Samples were simply dipped into a buffered aqueous solution of dopamine (2 mg of dopamine per milliliter of 10 mM tris, pH 8.5) for a desired reaction time (1 h-overnight). We could verify the formation of polyDAs by characteristic dark color of treated substrates. The film thickness measured by ellipsometry confirmed that more polyDAs were deposited as immersion time increased which was consistent with the previous report (Fig. 2(a)) [20]. The thickness of polyDA reached up to 100 nm on gold substrate by 23 h-reaction. Next, we applied the film deposition on glass, gold, indium tin oxide, platinum, and liquid crystal polymer. These materials were chosen as test materials since they were often used to design *in vitro* or *in vivo*

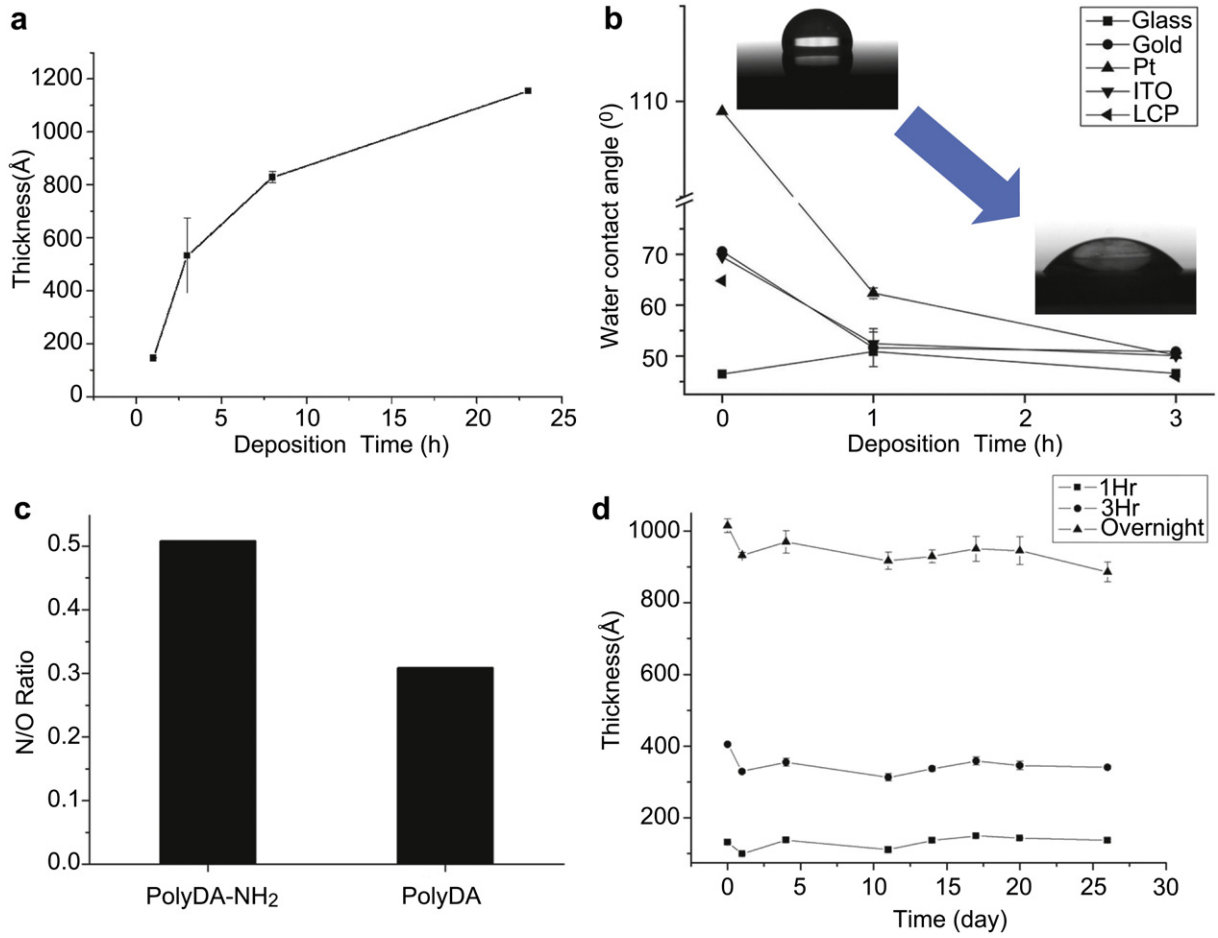

Fig. 2. (a) Growth of polyDAs on gold surfaces. (b) The change of water contact angles after the deposition of polyDA films on various substrates. (c) The N/O ratio of polyDA films with and without polylysine linking reaction. (d) Long-term stability of polyDA film thickness in biological enviroment (5% CO_2 at 37 °C).

microelectrode arrays [22,23]. We measured the change of wettability to confirm the film formation. The water contact angles converged into narrow region (46–51 °) on diverse surfaces after 3 h-deposition, which indicated that 3 h-deposition of polydopamine was enough to generate films with uniform chemical structures independent of the substrate composition (Fig. 2(b)).

For neuronal applications, polyDAs were tailored to be cell-adhesive by covalently immobilizing polylysines. This was also performed through the dip-coating of polydopamine-coated substrates into a buffered aqueous solution of polylysine (0.1 mg of polylysine per milliliter of 10 mM tris, pH 8.5). PolyDAs have been reported to be covalently linked with thiol- and amine-containing molecules through thiol- and amine-catechol adduct formation [20]. We confirmed the immobilization of polylysines on the polyDAs deposited on glass substrates by XPS analysis. The untreated polyDA showed 0.110 of nitrogen-to-carbon (N/C) ratio, which was similar with published value [20]. However, nitrogen-to-oxygen (N/O) ratio was changed from 0.3084 to 0.5077 by the overnight reaction with polylysine (Fig. 2(c)). This increase indicated successful immobilization of polylysines, because polylysine has higher N/O ratio than dopamine.

As the final characterization of polyDAs, we investigated the stability of the films at a biological environment. Gold substrates coated with polyDAs under three different conditions (1 h-, 3 h-, or overnight-deposition) were soaked in pH 7.4 phosphate buffered saline solution with physiological condition (5% CO_2 at 37 °C). Fig. 2(d) shows the temporal change of the film thickness over four weeks. The thicknesses of polyDAs decreased by 4.19%, 15.8%, and 12.7% during 26 days for 1 h-, 3 h-, and overnight-deposited films, respectively (Fig. 2(d)). For all of the substrates, the decrease mainly occurred within 1 day, and there were no change in the thickness after 2 days. This initial collapse might come from unpolymerized dopamine monomers or oligomers which were

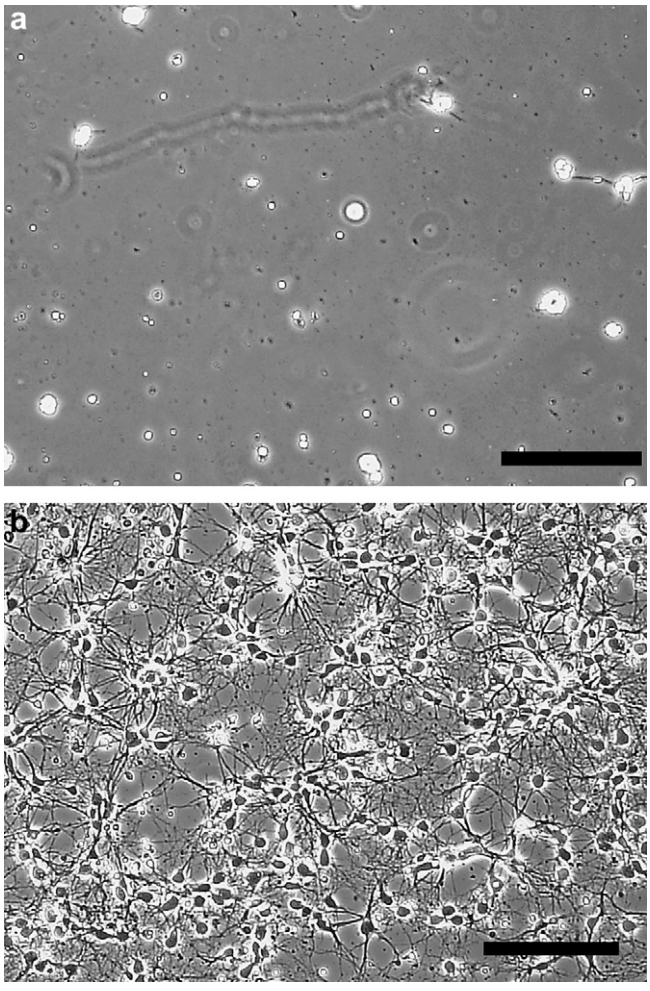

Fig. 3. Hippocampal neurons cultured on (a) bare polyDAs or (b) PDL-linked polyDAs at 6 DIV. The base culture substrate is a glass coverslip. Scale bars: 100 μm.

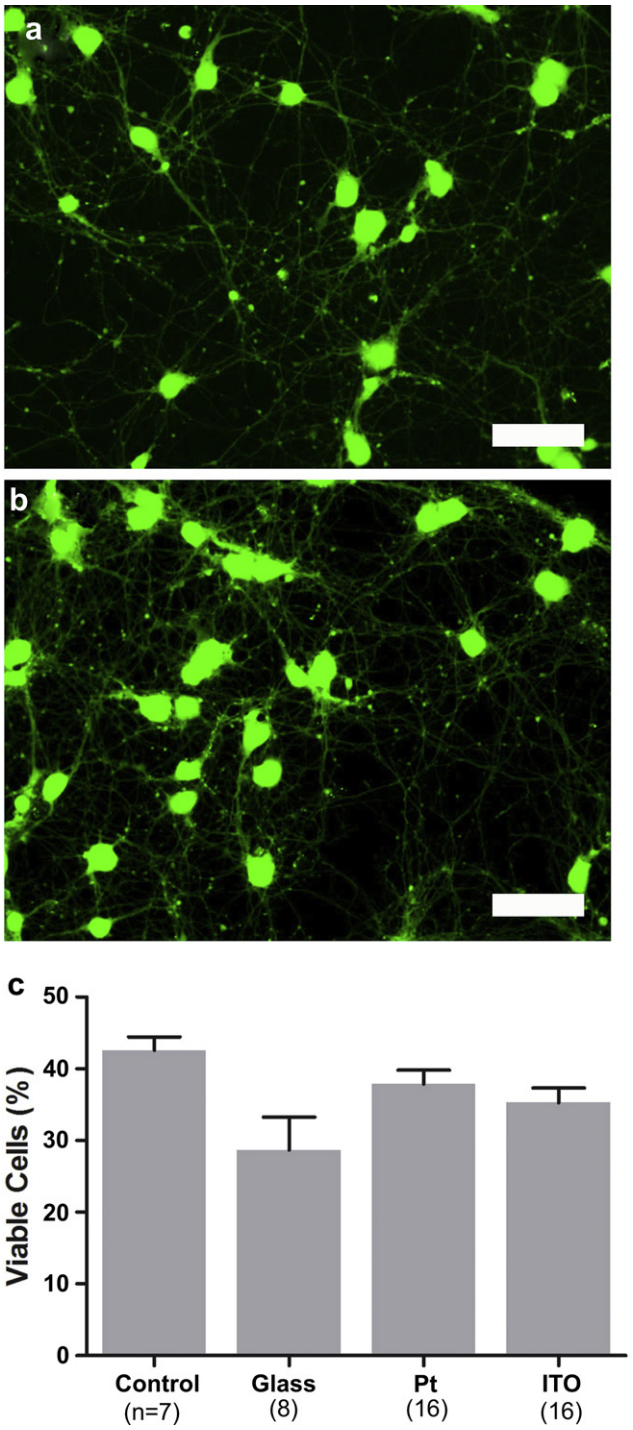

Fig. 4. Fluorescence micrographs of hippocampal neurons cultured on PDL-linked polyDAs grown on (a) ITO and (b) Pt, stained with calcein AM. Images taken at 7 DIV. Scale bars: 50 μm (c) Cell viability at 7 DIV on control, glass, Pt, and ITO substrates (mean ± S.E.)

physically trapped inside the polymer. This result indicates that polyDAs can be used for neuron-material coating that requires long-term stability.

3.2. PolyDA based neural surface functionalization

In order to confirm the biocompatibility of polyDAs, hippocampal neurons were cultured on untreated and polylysine-linked polyDAs. Based on the observation that short-time deposition of polydopamine was enough to impose desired chemical characteristics on the surfaces, we constructed facile process for generating cell-adhesive polyDAs. The process was composed of 1 h-polyDA deposition and 1 h polylysine linking, both of which were based on a dip-coating process. A substrate was dipped in polyDA solution for a certain period of time to form a polyDA on the surface. In contrast to the surface coating methods in other reports, the dip-coating process was simple and easy to be reproduced. We confirmed the feasibility of this process on glass substrates (Fig. 3). Neurons cultured on the untreated polyDA did not survive (Fig. 3(a)), whereas neurons on polylysine-linked polyDA showed good growth and the formation of neuronal networks at 6 DIV (Fig. 3(b)). On the polylysine-linked polyDA, most of neurons showed good soma spreading and active formation of neurites and few clumping, which indicated that polylysine molecules were linked uniformly over the surface.

A good uniform neuronal growth showed that the short-time dip-coating of polydopamine followed by 1 h polylysine linking was efficient for the preparation of neuronal culture substrates. The process was simple, time-saving and reproducible to produce neuronal culture substrates on various substrates. According to the conventional neuronal culture protocol, cell culture substrates had to go through relatively long substrate cleaning, polylysine coating, and subsequent rinsing steps [24,25]. Furthermore, covalently linking polylysine with polyDA coated surfaces could reduce the sample-to-sample variances in terms of the chemical environments for cells, especially when the samples are needed to be reused quite many times (e.g. bio-chips or microelectrode arrays).

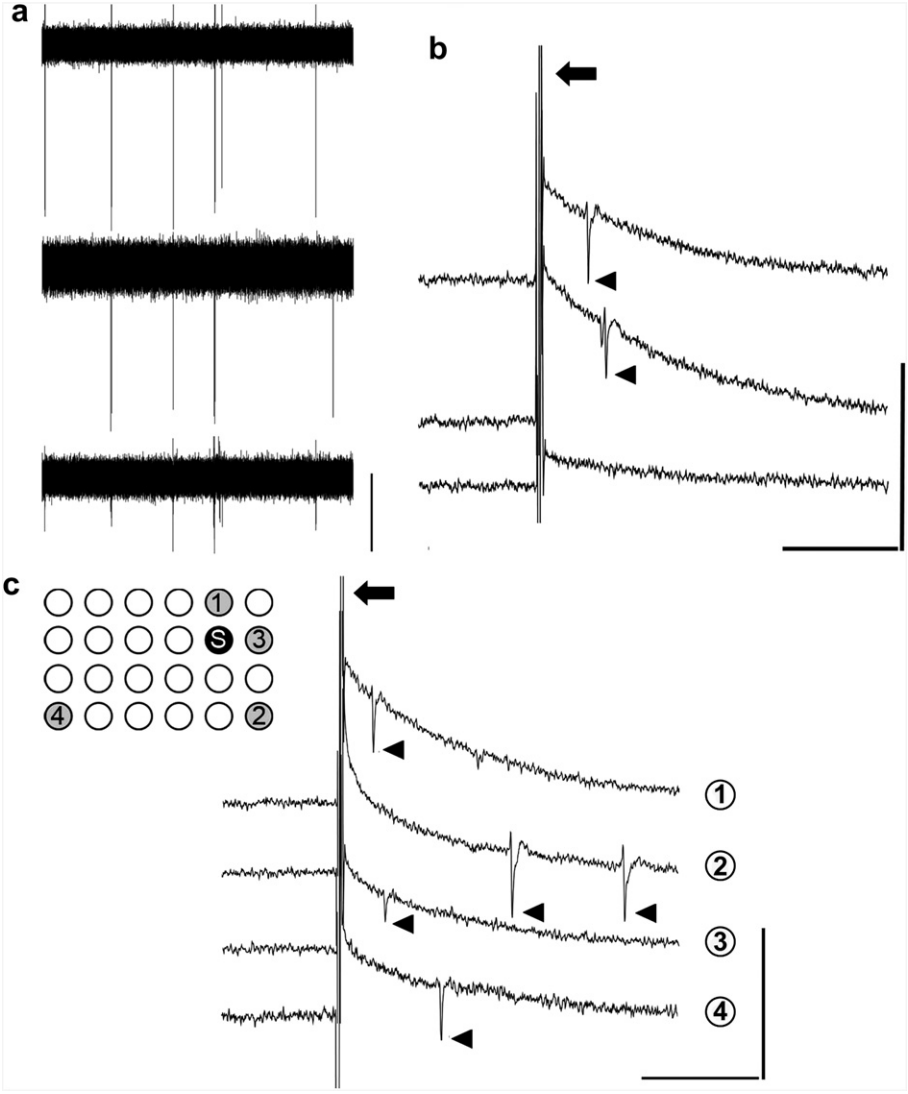

Fig. 5. Multichannel recording and stimulation using polydopamine-coated electrodes at 20 DIV. (a) Multichannel recordings of spontaneous neural activities from three different microelectrodes. Each trace is 20 s. Scale bar: 40 μV. (b) Recordings of extracellular neural spikes from a same microelectrode followed by three different current levels (9, 11, and 13 μV). (c) Multichannel recording after delivering a biphasic current pulse (20 μV) at a single site. The location of stimulation site ('S') and recording electrodes are superimposed on an MEA layout. Bold arrows indicate the stimulation artifacts, and arrowheads point out the evoked extracellular spikes. Scale bars: 10 ms, 200 μV.

3.3. Biofunctionalization of various substrates and neuronal growth

The versatility of the polyDAs for various materials was confirmed on glass, platinum, and indium tin oxide (ITO) surfaces. We used the same 'short' protocol used in the previous section for all substrates, and it resulted in a uniform deposition of polyDAs on the surfaces. For the quantitative analysis of biocompatibility of the films on the multiple substrates, we stained the neurons with a LIVE/DEAD Viability/Cytotoxicity kit. Fig. 4(a) and (b) are representative images of neurons cultured on polylysine-linked platinum and ITO surfaces. It shows that both surfaces were functionalized properly to promote good neuronal adhesion and growth. On platinum and ITO substrates, neurons developed well without clumping of somata, which indicated that the surface of these substrates were converted to be cell-adhesive and non-cytotoxic. Fig. 4(c) show that the viability of neurons on these substrates. All of the substrates exhibited viability within 30–40%, and the values were quite comparable with that of a PDL-coated glass control. This indicated that the performance of the polyDAs was not affected by the base substrate materials and the linked PDL layers were as good as conventional culture surfaces.

3.4. Neural recordings and electrical stimulation from polyDA-MEA

To demonstrate the application of polyDAs to the neuron-electrode interfaces, hippocampal neurons were cultured on polyDA coated on a gold electrode MEA. The polyDAs for gold MEA were prepared by 1 h-dip-coating of chips in buffered dopamine solutions. Subsequently, the films were linked with polylysines by overnight dip-coating in buffered polylysine solution. At this stage, the chemically treated MEAs were used for impedance measurement ($f = 1$ kHz, 10 mV_{rms}). The measured Z value was 209.7 ± 16.5 kΩ with phase angle of −68.8 ± 0.4° ($n = 58$, mean ± S.E.). Neurons were plated at relatively high density (1000 cells/mm^2) compared to the cultures from previous sections, and formed dense networks on the coated MEA, maintaining fine viability up to a month. Fig. 5(a) shows spontaneous activities from the neurons on the polyDA were recorded at 20 DIV. Most of spikes were synchronized even though some pairs of them were not adjacent, indicating neuronal networks formed on the film span the entire chip. Recorded extracellular spikes were usually triphasic, and the mean amplitudes of extracellular spikes were 95, 49, and 121 μV_{pp}, with background noise levels of 6.28, 8.80, and 6.76 μV_{rms} for each trace, which resulted in the SNR of 5–18.

Neurons could be stimulated by delivering biphasic current pulses (duration of 200 ms) through the polydopamine-coated microelectrodes. Current pulses were applied to one of the electrodes which had spontaneous activity, and evoked responses were recorded from the other electrodes. In order to obtain the threshold current to evoke the activities, responses for increasing current pulses (9, 11, and 13 μA) were recorded from the same electrode (Fig. 5(b)). As represented by traces of Fig. 5(b), we could clearly see the stimulus amplitude-dependent responses. Here, we found the threshold value around 10 μA, as 9 μA pulse could not evoke any responses while 11 μA pulse could. Spatial distribution of evoked responses through the chip was also addressed (Fig. 5(c)). The 4 different electrodes recorded 4 distinct evoked responses with different delays from the same stimuli (20 μA, 200 μs). The first trace was recorded from the most nearby electrode from the stimulating electrode, and exhibited the shortest delay (3 ms) in comparison to others (15.43, 4.26, and 9.35 ms).

4. Conclusion

In summary, we report here an approach using mussel-inspired surface chemistry for generating effective platforms for dissociated neuronal cultures, and demonstrate their performances at the recording of neural signals on MEAs. By exploring chemical properties of the polyDAs with varying the deposition time, we found that 1–3 h of deposition was enough for generating reproducible chemical properties on the surfaces. Applying this facile protocol, we could successfully culture neuronal networks on glass, platinum, ITO, and LCP surfaces, and all of them showed comparable and plausible viability. We also demonstrated the possible application of the films by showing their usage as an interface platform at the surface of MEAs by which we could successfully record neural signals. We believe that the chemistry reported here could help many researchers to overcome many limitations from the conventional surface modification method for neuron-surface interfaces.

Acknowledgements

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