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Epoxy-silane linking of biomolecules is simple and effective for patterning neuronal cultures

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Abstract

Surface chemistry is one of the main factors that contributes to the longevity and compliance of cell patterning. Two to three weeks are required for dissociated, embryonic rat neuronal cultures to mature to the point that they regularly produce spontaneous and evoked responses. Though proper surface chemistry can be achieved through the use of covalent protein attachment, often it is not maintainable for the time periods necessary to study neuronal growth. Here we report a new and effective covalent linking approach using (3-glycidoxypropyl) trimethoxysilane (3-GPS) for creating long term neuronal patterns. Micrometer scale patterns of cell adhesive proteins were formed using microstamping; hippocampal neurons, cultured up to 1 month, followed those patterns. Cells did not grow on unmodified 3-GPS surfaces, producing non-permissive regions for the long-term cell patterning. Patterned neuronal networks were formed on two different types of MEA (polyimide or silicon nitride insulation) and maintained for 3 weeks. Even though the 3-GPS layer increased the impedance of metal electrodes by a factor of 2–3, final impedance levels were low enough that low noise extracellular recordings were achievable. Spontaneous neural activity was recorded as early as 10 days in vitro. Neural recording and stimulation were readily achieved from these networks. Our results showed that 3-GPS could be used on surfaces to immobilize biomolecules for a variety of neural engineering applications.

Keywords: Epoxy silane; Protein linking; Microcontact printing; Cell patterning; Multielectrode array; Hippocampal neurons

1. Introduction

Surface-patterning techniques have been applied to dissociated neuronal cultures to design ordered neuronal networks (Kleinfeld et al., 1988; Corey et al., 1991; Branch et al., 2000). The formation of functional synapses and synaptic plasticity in patterned networks has been reported by others (Ma et al., 1998; Ravenscroft et al., 1998; Liu et al., 2000; Matsuzawa et al., 2000; Wyart et al., 2002; Vogt et al., 2005). There has also been work suggesting the possibility of patterned networks with designed neuronal polarity (Stenger et al., 1998; Vogt et al., 2004). These neuronal patterning techniques were extended to planar multielectrode arrays (MEAs) with low density hippocampal neurons so that one can study the functional properties of neuronal networks related to the geometry of the network (Chang et al., 2000; Chang et al., 2001; James et al., 2004; Nam et al., 2004).

For dissociated neuronal cultures derived from rat embryos, 2–3 weeks are required for the maturation of neural networks that regularly produce spontaneous or evoked responses. In order to create the needed longer lasting surface patterns there have been several approaches using covalent linking, as opposed to physisorption, to modify the surface of MEAs (Chang, 2002; Nam et al., 2004). Covalent linking schemes also provide a way to create the cell repulsive background regions (off-pattern areas), which have been shown to be an important factor for the long-term compliance of neurons to the pattern (Branch et al., 2000). Previously, (3-mercaptopropyl) trimethoxysilane (3-MPS) and sulfo-GMBS (*N*-(γ-maleimidobutyrloxy) sulfosuccinimide ester) were used to create cell adhesive/repulsive regions on polyimide and silicon nitride insulated MEAs and a long lasting highly compliant pattern with hippocampal neurons was obtained (Chang, 2002). However, the protocol to form 3-MPS layers is complex and, more importantly, the 3-

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MPS passivated electrode surface has higher impedance and degraded performance for recording extracellular spikes. Alternately, alkanethiol based self-assembled monolayers were used by coating the entire surface of MEA with a thin gold film (Nam et al., 2004). This chemistry was very reproducible but it proved to be difficult to recycle the gold surface after the long term culture.

Here we report the use of (3-glycidoxypropyl) trimethoxysilane (3-GPS) to crosslink proteins for the purpose of cell patterning. This epoxy-silane has been shown to form a dense, homogeneous and complete monolayer (Luzinov et al., 2000). The epoxide group, which is the end terminal group of 3-GPS reacts with nucleophilic reagents including carboxylic acids and amines to form a neutral oxygen-hydrogen bond (Tarducci et al., 2000). The interaction between the nonbonded electron pair on the nitrogen of amine group and the carbon atom of the epoxy ring leads to the sequential processes of ring opening, electron rearrangement, and the formation of a neutral oxygen-hydrogen bond (Scheme 1 in Sales et al., 2002). Others have used this chemistry to immobilize various biomolecules including DNA (Pathak and Dentinger, 2003; Hang and Guiseppi-Elie, 2004), antibodies (Yang and Li, 2005), glucose oxidase (Van Gerwen et al., 1998), and poly(ethylene glycol) (Piehler et al., 2000) on various substrates including glass (Piehler et al., 2000; Hang and Guiseppi-Elie, 2004), silicon dioxide (Van Gerwen et al., 1998; Luzinov et al., 2000), and indium-tin oxide (Yang and Li, 2005). Robustness and the ease of the chemistry are the main advantages over the use of other organosilane linkers such as (3-mercaptopropyl) trimethoxysilane (Branch et al., 2001) or (3-aminopropyl) trimethoxysilane (Branch et al., 1998). Here we report the successful use of 3-GPS in cultures of hippocampal neurons on micropatterned poly-D-lysine (PDL) lines, including recording and stimulation of neural circuits after 3 weeks in culture. The culture results also show that 3-GPS is effective as a background cell repulsive material.

2. Methods

2.1. Substrate cleaning

MEAs were purchased either from Multi Channel Systems (30 μm TiN/200 μm spacing, 0.5 μm silicon nitride insulation, Reutlingen, Germany) or Elume, Inc. (20 μm × 20 μm or 30 μm × 30 μm indium tin-oxide (ITO)/200 μm spacing, 4 μm polyimide insulation, Simi Valley, CA). ITO electrodes were electroplated in platinic chloride (P-5775, Sigma–Aldrich) solution (3% (w/v) H_2PtCl_6 in deionized water) to create a platinum black electrode ($I = -0.7$ μA, 10–20 s).

Organic contaminants were removed by soaking MEAs in an acetone ultrasonication bath for 10 min followed by isopropanol (IPA) and deionized water (DI water) rinsing and nitrogen gas blow-drying. Then MEAs were further cleaned in a Planar Plasma Etch System (oxygen, process pressure 500 mT, power 300 W, Texas Instruments) for 10 min. Cleaned substrates were immediately transferred to an ethanol jar where they remained until use.

Glass coverslips (Assitent, No. 100, 22 mm × 22 mm) were acid-cleaned in piranha solution (H_2SO_4:H_2O_2 = 7:3) for 1 h and rinsed in running DI water for 15 min. Clean coverslips were stored in ethanol solution until use.

Before surface modification, substrates were thoroughly blown dry using pressurized nitrogen gas to remove any excessive moisture.

2.2. Surface modification by 3-GPS

One percent (3-glycidoxypropyl) trimethoxysilane (3-GPS, SIG5840.0, Gelest Inc., Morrisville, PA) was prepared by mixing 1 ml of 3-GPS solution with 99 mL of toluene in a beaker. The solution was stirred well and substrates (MEAs or coverslips) were soaked for 15–20 min. After the reaction, substrates were transferred to another beaker that contained fresh toluene and rinsed several times to remove any unbound 3-GPS molecules. Then the surface was blown dry using pressurized nitrogen gas and stored in an oven (110–120 °C) for 30 min.

To measure the water contact angle, a small drop (5 μl) of DI water was put on the substrate using a Hamilton syringe. A custom built goniometer was used to measure the contact angle.

2.3. Immobilization of protein by microcontact printing or flooding

To perform microcontact printing (μCP), PDMS (polydimethylsiloxane) stamps were created by a molding process. Silicon wafers (3 in or 2 in diameter) were cleaned with acetone, IPA and DI water, sequentially, then dried with pressurized nitrogen gas and left on a hotplate (125 °C) for 1 min. Photoresist (AZ4620, Clariant Corp., Sommerville, NJ) was spin-coated for 30 s at 3000 rpm (thickness 8–10 μm) and baked for 1 min at 110 °C. Coated wafers were brought into contact with a chromium mask using a contact mask aligner (Karl Suss MJB3, SUSS MicroTec Inc., Santa Clara, CA) and exposed under UV for 1 min. Wafers were developed with AZ developer (Clariant Corp., Sommerville, NJ) for 20–60 s depending on the feature size of the pattern. To harden the mold, wafers were baked for 1 min at 125 °C. To fabricate PDMS stamp, PDMS elastomer from Sylgard 184 kit (Dow Corning Corp., Midland, MI) was mixed with curing agent at the ratio of 10:1 (w/w) and stirred well. Bubbles generated during the stirring were removed by degassing the mixed solution under house vacuum. When the absence of bubbles was confirmed visually, the solution was poured onto the mold and cured at 60 °C for 12 h or at 90 °C for 1 h in a convection oven. Molded PDMS pieces were carefully removed from the mold and cut into the desired size and attached to coverslips which served as a backing substrate.

We followed the μCP procedure optimized for maximum protein transfer (Chang et al., 2003). The stamp surface was modified by soaking the stamp in aqueous SDS solution (10% w/v in DI water, sodium dodecyl sulfate, Bio-Rad Laboratories, CA) for 15 min. Excess SDS layers were removed by dipping the coated stamp into DI water once and drying under gentle nitrogen gas. Poly-D-lysine (PDL, MW 70,000–150,000, 0.1 mg/ml in 1 × PBS, Sigma–Aldrich) was loaded for 30 min and dried

gently using nitrogen gas. Each PDL-inked PDMS stamp was aligned to an MEA using a custom-built contact aligner and brought into contact for 3 min. Printed substrates were rinsed with DI water. Background regions were left with unconjugated 3-GPS. To image the printed patterns, either FITC conjugated poly-L-lysine (PLL-FITC, MW 70,000–150,000, 0.1 mg/ml in 1 × PBS, Sigma–Aldrich) or a mixture of PDL and PLL-FITC was stamped in a same way. Fluorescent images were acquired using Olympus IX-51 microscope.

For uniform PDL coatings, we used a flooding process. First, a drop of PDL solution (0.5 ml) was put on top of the substrate (MEA, glass coverslip, SiO_2 wafer) for 1 h. Then, the PDL drop was removed by dipping the substrate in DI water. We rinsed the substrate in fresh DI water twice to remove any unbound PDL and dried the sample using nitrogen gas.

2.4. X-ray photoelectron spectroscopy (XPS) analysis

Control samples of polyimide (PI2771, HD Microsystems, fabricated by Elume Inc.) and SiO_2 on Si wafer (SiO_2/Si) were oxygen plasma treated and stored in ethanol until analyzed. 3-GPS treated samples were prepared following the same process described above and PDL treated samples were made by loading PDL (0.1 mg/ml in 0.5 × PBS) for 60 min followed by rinsing in DI water. All samples were stored in an air tight container until analyzed. XPS analysis was performed with a Kratos axis ULTRA imaging X-ray photoelectron spectrometer (Kratos Analytical Inc., Chestnut Ridge, NY). An Al Kα source was used for all substrates. Survey spectra were obtained at a constant pass energy of 160 eV and swept four times in the range of 0–1100 eV. For high resolution spectra, carbon (C) 1s (278–296 eV), nitrogen (N) 1s (392–410 eV), and silicon (Si) 2p (95–113 eV) were scanned at a pass energy of 40 eV and 10 sweeps were performed for each scan.

2.5. Cell cultures and microscopy

Dissected hippocampal tissues (18-day gestation Sprague/Dawley rat hippocampus) were purchased from Brain Bits™ (http://www.brainbitsllc.com/). The tissues arrived in 2 ml tube containing embryonic day 18 hippocampus in B27/Hibernate. This was immediately stored at 4–8 °C until cell plating (typically within 7 days). Tissues were mechanically dissociated and plated in serum-free B27/Neurobasal medium (Invitrogen, Gaithersburg, MD) with 0.5 mM glutamine and 25 μM glutamate at the density of 75–200 cells/mm^2. Cultures were stored in an incubator at 37 °C, 5% CO_2, 9% O_2. After 4 days in vitro (DIV), the medium was changed to serum free B27/Neurobasal medium with 0.5 mM glutamine. Thereafter, half of the medium was changed weekly or after recording if necessary. Phase contrast images of cultures were taken weekly to record the status of the culture using an Olympus IX-51 inverted microscope. Live cell counts were carried out from phase contrast images taken with 10× objectives in order to compare relative cell survival between PDL linked (PDL/3-GPS) and PDL unlinked 3-GPS surfaces.

For cultures on MEAs, a FEP (fluorinated ethylene-propylene) membrane (ALA Scientific Instruments, Inc., Westbury, NY) was installed to cover the culture and minimize contamination and evaporation (Potter and DeMarse, 2001).

2.6. AC impedance measurement

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

2.7. Neural recording/stimulation and data analysis

Each MEA was connected with the MEA1060 amplifier (Gain 1200, 10 Hz–3 kHz, Multi Channel Systems, Reutlingen, Germany) and amplified signals were fed to MC card (sampling rate 40 kHz, Multi Channel Systems, Reutlingen, Germany). Software (MC Rack) provided by the manufacturer (Multi Channel Systems) was used to display and store the digitized data in hard disk.

Positive first biphasic current pulses were delivered by STG-1008 (Multi Channel Systems). The magnitude of the current pulses varied from 10 to 30 μA with a pulse width of 100 μs for each phase. The frequency of the stimulation was fixed at 0.2 Hz.

Recorded raw data were filtered with a digital second order Butterworth filter (high pass filter, cut-off frequency 200 Hz) and spikes were detected if their amplitude exceeded ± 5 times standard deviation. Electrodes with a mean firing rate of 0.1 spikes/s was considered as active electrodes. Noise RMS (root mean square) value was estimated by calculating standard deviation of 10 s recording trace and SNR (signal-to-noise ratio) was calculated by taking the ratio of signal (spike) amplitude from zero to peak and estimated noise RMS.

3. Results

3.1. 3-GPS deposition on substrates

The deposition of 3-GPS layer was confirmed by two different measurement techniques – water contact angle and XPS analysis. Both measurements were performed on three different model substrates – glass coverslip, SiO_2/Si, and polyimide.

Here we used the water contact angle to quickly evaluate the success of 3-GPS reaction by preparing negative control samples that were reacted in solvent without 3-GPS. Table 1 shows the representative water contact angles before and after 3-GPS reaction for a single experiment. The water contact angles of 3-GPS reacted samples had larger changes than those of control groups. Initially, clean glass coverslips were completely wettable (water contact angle <5°). Water contact angles of 3-GPS reacted samples were 29.5° ± 0.2° (N = 3, S.E.) and angles of control groups were 7.9° ± 0.5° (N = 2, S.E.). Both SiO_2/Si and polyimide samples also showed increase in

Table 1
Water contact angles for different substrates

	Clean surface[a]	3-GPS	PDL
Glass	<5.0°	29.5° ± 0.2° (N = 3)	21.8° ± 2.5° (N = 2)
SiO_2/Si	25.6° ± 0.8° (N = 6)	43.9° ± 0.9° (N = 3)	26.0° (N = 1)
Polyimide	6.5° (N = 1)	11.0° (N = 1)	Not tested

Mean ± S.E.
[a] Piranha cleaning or oxygen plasma treatment (10 min).

contact angle after the 3-GPS reaction (Table 1), consistent with a previous report (Luzinov et al., 2000). Water contact angles were reproducible over day-to-day experiments. Water contact angles of glass coverslips from five different experiments were 32.3° ± 0.5° (N = 5, S.E.). Small variance in day-to-day experiments implied that the 3-GPS reaction was reproducible.

The XPS spectra of carbon (C 1s) further verified the presence of 3-GPS terminal, epoxide group. Fig. 1(a) shows the XPS spectra of 3-GPS reacted SiO_2/Si. Compared with the control sample, the split of two peaks corresponding to C–O and C–H (C–C) after the 3-GPS reaction was a direct indication of the presence of the epoxide group (Luzinov et al., 2000). This was also the case for an oxidized polyimide sample. Two glass samples prepared from different batches produced identical peaks, which further supports the reproducibility of the current 3-GPS chemistry (data not shown).

Based on these two measurements, we concluded that our process successfully deposited a 3-GPS layer on model substrates and would produce similar results for MEAs with similar surface conditions.

3.2. Functionalization of 3-GPS surface with polylysine

We determined the successful functionalization of the 3-GPS to PDL using XPS and water contact angle measurements. In Fig. 1(b), we observed the N 1s spectra of 3-GPS treated SiO_2/Si with and without PDL reaction. The lack of peaks for the control and samples prepared with only 3-GPS are a strong indication that epoxide has not reacted with amines. For PDL reacted samples, we observed a large distinct peak at 399.5 eV on PDL reacted 3-GPS samples. Collectively, our findings indicate that the PDL was believed to be covalently linked to the surface. The formation of a covalent linking was not explicitly tested further in this work.

Contact angle measurements also indicated the change of the surface properties after PDL reaction. 3-GPS surfaces became more hydrophilic due to the charged amine groups ($–NH_3^+$) in poly-D-lysine (Table 1).

3.3. Microcontact printing of polylysine on 3-GPS surface

Fig. 2 shows the transferred PLL-FITC pattern on 3-GPS modified surfaces, including a glass coverslip, a Si_3N_4 MEA surface, and a polyimide MEA surface. Microstamping on a 3-GPS modified surface was moderately superior to microstamping on an unmodified glass surface as judged by the fluorescent intensity of micropatterned PLL-FITC. After subtracting the background mean value, the adjusted fluorescent values were 85.3 ± 0.9 (a.u., N = 4, mean ± S.E.) and 72.3 ± 3.6 (a.u., N = 2, mean ± S.E.) for 3-GPS modified glass and bare glass, respectively ($p < 0.05$, Student's t-test). This indicated that more protein linked to the 3-GPS modified glass than to the unmodified glass surface.

3.4. Long-term cell growth on PDL/3-GPS modified substrates

Neurons grew well on uniform or patterned PDL functionalized 3-GPS surfaces. On uniformly coated PDL surfaces, cells readily spread and attached to the surface and formed dense networks for a month. On patterned PDL surfaces, neural growth was confined to the restricted cell adhesive area. Fig. 3(a) is an example of confined growth of hippocampal neurons on a patterned PDL/3-GPS glass coverslip at 30 DIV. The micropatterned PDL was a 10 μm wide grid with 200 μm spacing and background regions were a bare 3-GPS surface. Long term healthy cell growth implied that 3-GPS is biocompatible with embryonic tissue cultures. In addition, the long term growth on covalently linked substrates supported our hypothesis that 3-GPS provides long-term linking of protein to surfaces.

Similar quality long term cell growth was achieved on 3-GPS modified MEAs. Fig. 3(b) and (c) show patterned hippocampal neurons grown on an MEA either with Si_3N_4 (Fig. 3(b)) or with polyimide (Fig. 3(c)) at 16 DIV. Overlaying the printed surface

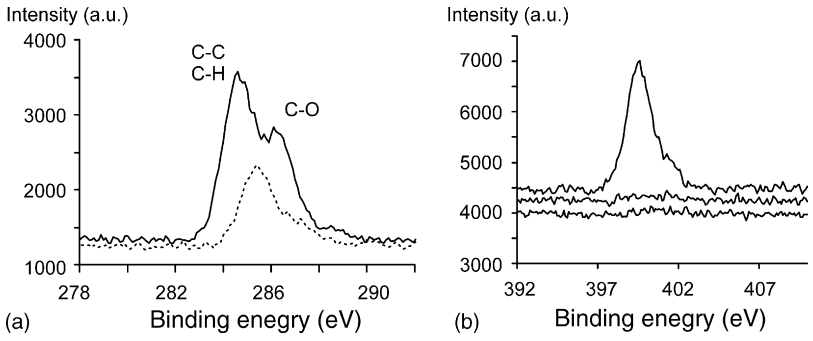

Fig. 1. XPS study: (a) C 1s peak before (dotted line) and after (solid line) 3-GPS formation, (b) N 1s peak without 3-GPS (bottom trace), with 3-GPS (middle trace) and after PDL linking (top trace).

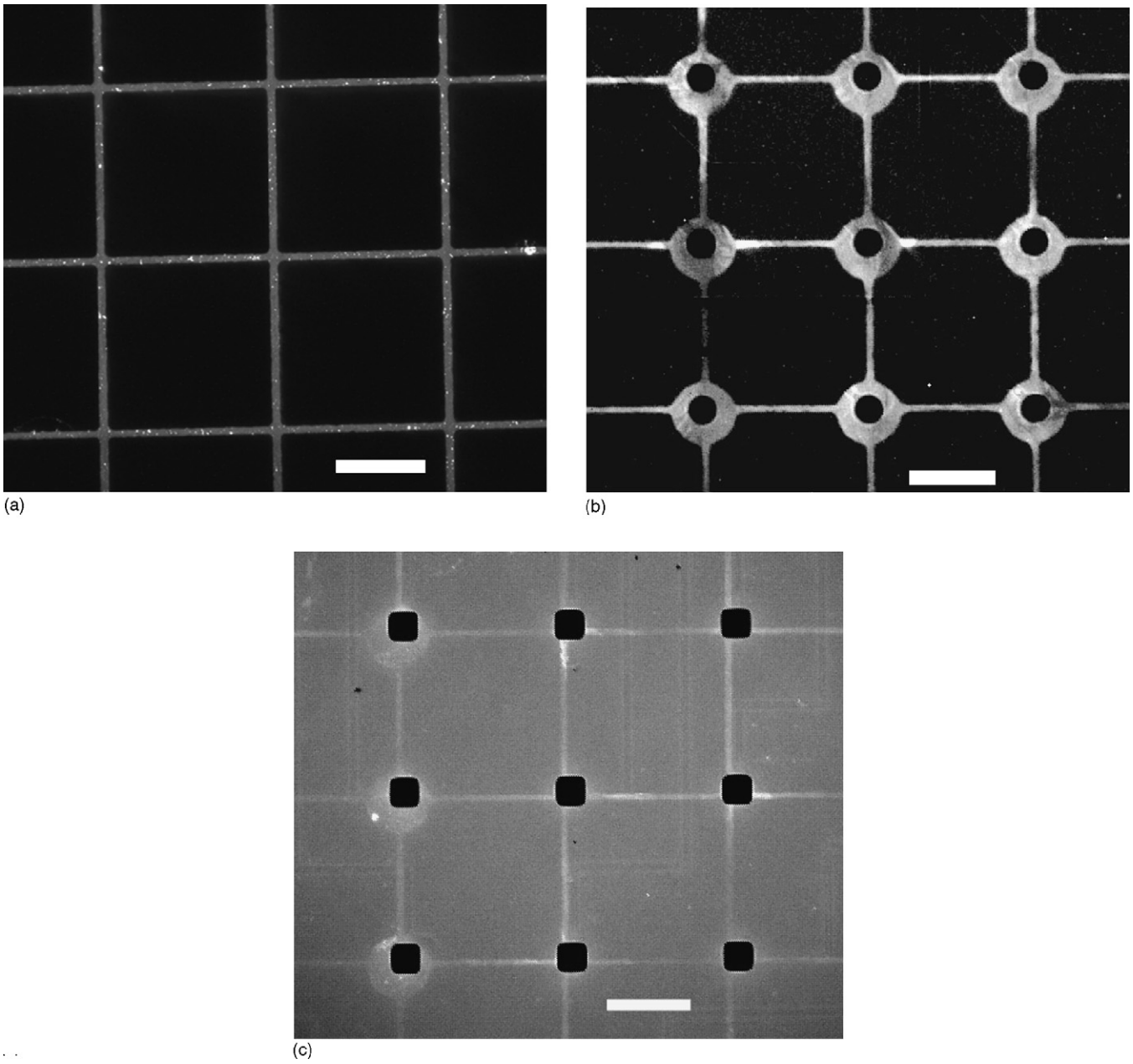

Fig. 2. PLL-FITC pattern (bright lines) on 3-GPS linked (a) glass, (b), Si_3N_4 MEA, and (c) polyimide MEA. The stamped pattern in (c) was designed to have circular nodes only in the left column. Scale bar: 100 μm.

pattern and the cell growth confirmed that neurons indeed follow the pattern on the surface.

As demonstrated in Fig. 3, only a few cells spread and extended neurites on a background surface coated with unconjugated 3-GPS. To test the hypothesis that 3-GPS does not support the cell growth, neurons growing on PDL linked or unlinked 3-GPS surface in the same dish were counted at 4 DIV. Unlike neurons growing on PDL surfaces elsewhere on the same substrate, most of the live cells on the 3-GPS surface were clumped or clustered and individual somata could not be resolved from phase contrast images. There were 42.2 ± 5.9 cells ($N = 3$ cultures, mean ± S.E.) growing on PDL linked 3-GPS surfaces while only 2.7 ± 0.4 cells ($N = 3$ cultures, mean ± S.E.) were growing 3-GPS surfaces (field of view: 0.25 mm^2). These results support the hypothesis that an unmodified 3-GPS surface is an effective non-permissive background material.

To test the utilization of 3-GPS surface as a cell repulsive area, we measured the soma compliance to the PDL pattern (with the same dimension as in Fig. 3(b)) on a 3-GPS modified Si_3N_4 MEA. Soma compliance was measured by counting soma attached in the on-pattern or off-pattern areas. At 1 DIV, 47.4% of total neurons in fields of view (1 mm^2) were on pattern and 57.6% were growing off the pattern. At 4 DIV, 82.1% of neurons were on pattern, which suggests the migration of somata from nonadhesive areas to adhesive (on-pattern) areas. Soma compliance was 88.8% and 88.5% after 1 week and 2 weeks,

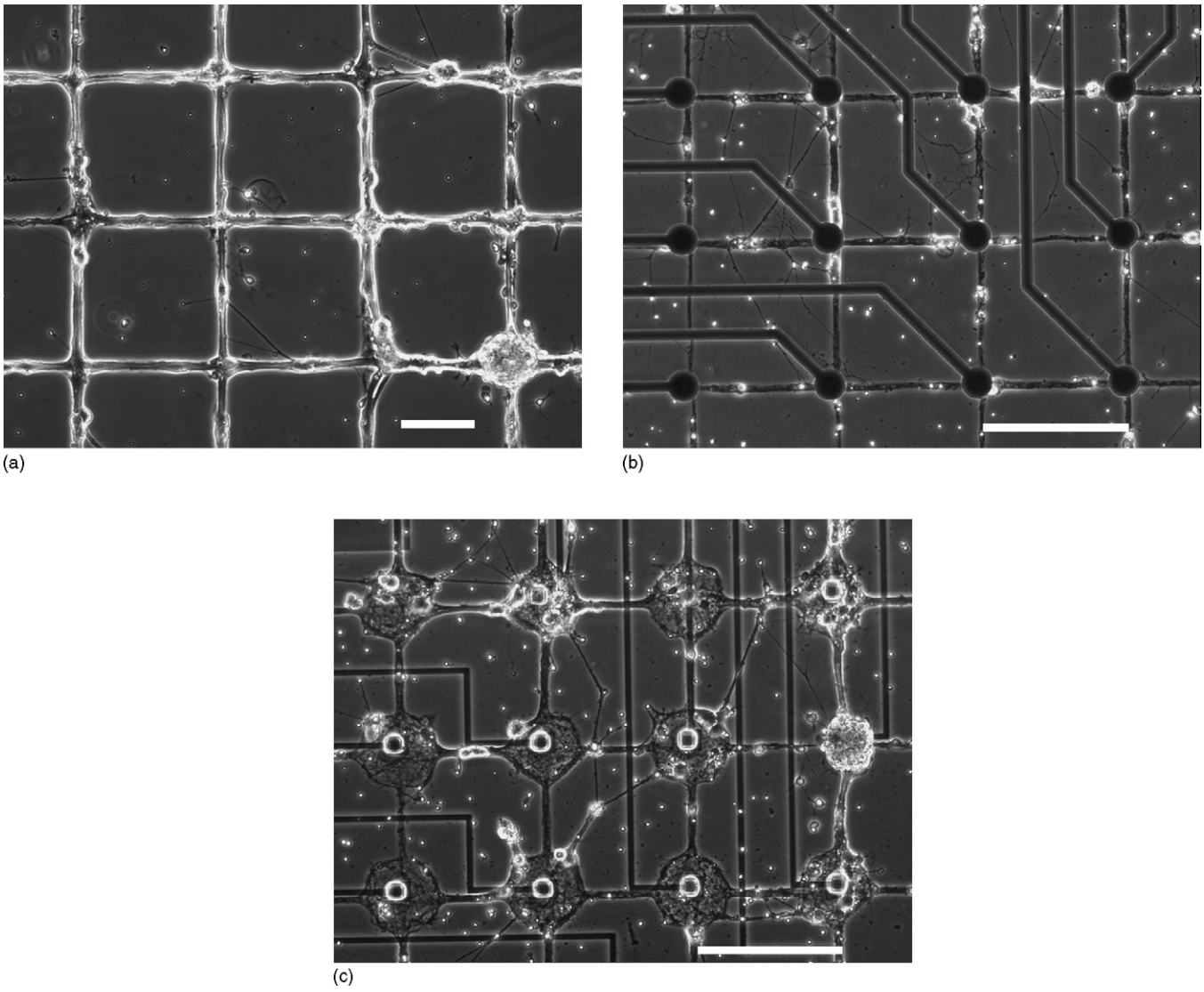

Fig. 3. Patterned neuronal cultures on 3-GPS linked (a) glass (30 DIV), (b) Si_3N_4 MEA (16 DIV), and (c) polyimide MEA (16 DIV). The printed protein pattern in (b) does not have any nodes, while (c) has square nodes. White dots in background regions are cell debris of detached cells. Scale bar: (a) 100 μm, (b) and (c) 200 μm.

respectively. Even though cells initially adhered to the background area, the 3-GPS surface was insufficiently attractive so that cells moved to the adhesive area to form the desired network pattern. The soma compliance and the trend of cell migration were consistent with results from other reports (Corey et al., 1991; Branch et al., 2000).

3.5. Effect of 3-GPS layer on electrode impedance

The degree of electrode passivation was tested by measuring the electrode impedance before and after the 3-GPS deposition on TiN electrodes. The electrode passivation could be problematic if the final electrode impedance value is too high to obtain low noise neural recordings. Table 2 summarizes the measurements of impedance changes before and after 3-GPS deposition obtained from five different 3-GPS preparations by recycling two MEAs with TiN electrodes. The mean of nine electrodes and corresponding standard deviations are reported here. 3-GPS increased the electrode impedance ($f = 1$ kHz, 10 mV_{rms}) by the factor of 2.8 ± 0.2 ($N = 5$, S.E.). Since the impedance of negative control groups, that were treated identically but without 3-GPS, remained unchanged, the increase of the impedance was due to the 3-GPS layer deposited on the TiN surface. For the electrodes used here the resulting thermal noise (2.1–2.7 μV_{rms}; $E_{rms} = (4\,kTRB)^{1/2}$, where k = Boltzmann constant 1.38×10^{-23} J/K, T = absolute temperature 310 K, R: electrode resistance, B: bandwidth 3000 Hz) is low enough not to affect neural recordings. However, noise could be an issue for much smaller electrodes, e.g. with 10 μm diameter.

3.6. Neural recordings and electrical stimulation

We tested five cultures (four patterned and one random) for neural recording and stimulation. Fig. 4(a) illustrates the neural activity recorded from patterned cultures. Spontaneous activity was readily recordable at 10–12 DIV. In the experiments

Table 2
Impedance change of TiN due to 3-GPS treatment

MEA	Trial[a]	|Z| (kohm)			Phase (degree)	
		Before	After	Ratio	Before	After
Array 1	1	31.8 ± 2.1	94.6 ± 21.3	3.0 ± 0.8	−51 ± 1	−66 ± 2
	2	44.4 ± 4.3	123.0 ± 23.8	2.8 ± 0.3	−60 ± 1	−69 ± 2
	3	62.0 ± 8.5	132.0 ± 23.1	2.1 ± 0.1	−62 ± 2	−68 ± 2
Array 2	1	30.1 ± 0.5	130.8 ± 16.0	4.4 ± 0.5	−50 ± 1	−51 ± 2
	2	56.0 ± 1.5	93.4 ± 3.1	1.7 ± 0.0	−63 ± 1	−67 ± 1

$N = 9$ (electrodes), mean ± S.D. Impedance measured at $V = 10\,mV_{rms}$, $f = 1$ kHz.
[a] Array 1 and Array 2 were recycled 3 and 2 times, respectively.

reported here both patterned and unpatterned cultures had four to eight active electrodes. The mean amplitude of extracellular spikes was 25 μV_{pp} at 7 DIV and 50 μV_{pp} at 20–24 DIV. Background noise levels were low (2.7 and 2.9 μV_{rms} at 7 and 20 DIV, respectively) permitting the recording of small spikes with peak amplitudes about 15 μV (roughly 5 times the noise RMS value). Large spikes with mean amplitudes of 115 μV_{pp} were also recorded. The SNR of active electrodes ranged from 4.5 to 15.8, with a mean SNR of 6.5 after 2 weeks in culture. With 3-GPS modified TiN electrodes, it was possible to deliver biphasic current pulses (10–20 μA, 100 μs) to stimulate neurons near the electrodes. Time locked evoked responses were recorded from other electrodes (Fig. 4(b)).

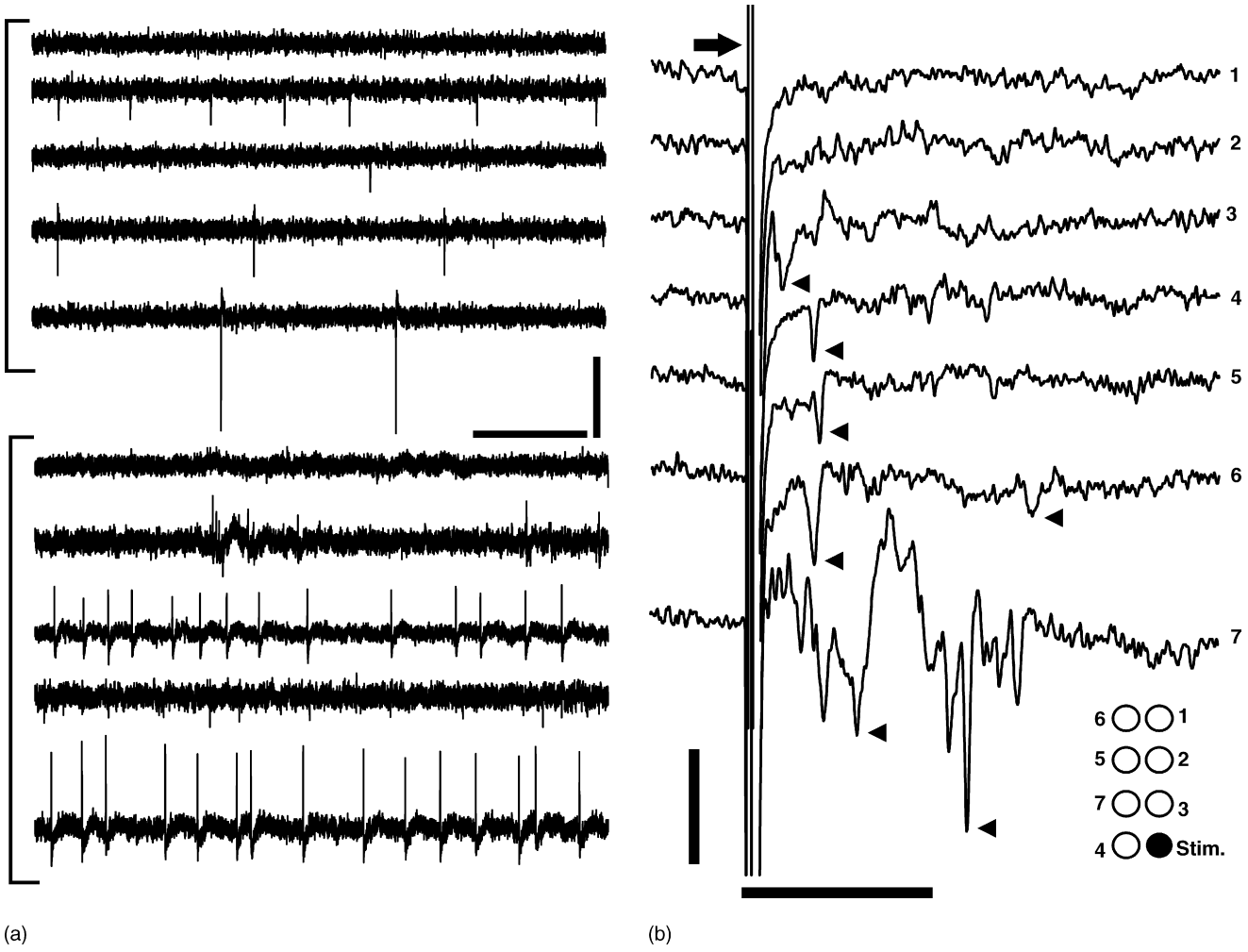

Fig. 4. Multichannel recording and stimulation from 3-GPS modified electrodes. (a) Neural recording from two patterned cultures. Top five traces from TiN electrodes at 16 DIV, bottom five traces from platinized electrodes at 24 DIV. Scale bar: 400 ms, 100 μV. (b) Evoked responses after stimulating by biphasic current pulses. Arrow heads indicate time-locked responses after averaging ten repeated stimulations. Stimulation artifacts are indicated by an arrow (top left). Scale bar: 10 ms, 20 μV. From unpatterned cultures.

4. Discussion

The main focus of this work was to develop a reliable process to easily link cell adhesive biomolecules using an organosilane (3-GPS) based SAM on various surfaces for cell culture applications. We used 3-GPS for the following reasons: (a) the epoxide group readily forms covalent links with amines, (b) the silanization process is simple and robust, (c) no cross-linker is necessary (one-step linking). For cell culture applications, the substrate preparation steps should be simple so as to make quality control easy as well as to produce good results in cell culture. Also, simple preparation steps can attract more researchers whose chemistry background is minimal. Direct linking of protein without any cross-linker is an important part of simplifying the preparation.

Various tests showed that we could activate insulating materials (silicon oxide, silicon nitride, and polyimide) with 3-GPS and link PDL on them. Successful reaction with various substrates implied that quality protein linking can be achieved over a range of original surface conditions. The cell culture assays show that the processes and parameters (e.g., cleaning, reaction, and baking times) that were used for 3-GPS reaction are sufficient to form a stable PDL layer on the surface sufficient to maintain the long term growth of the neuronal culture. Preliminary investigations with laminin showed success similar to that reported above with poly-D-lysine.

In contrast to excellent neural growth on PDL functionalized 3-GPS surfaces, the neural growth on unmodified 3-GPS surfaces showed clumping, infrequent extension of neurites, and frequent cell death. We had originally intended to link polyethylene glycol to the background 3-GPS to suppress cell growth, but found this additional step was unnecessary due to the highly effective non-permissive character of 3-GPS. Although the 3-GPS region was sufficient for repelling neural growth for a few weeks, the current results do not imply that this is a universal property of 3-GPS. It would have to be tested with other cell types such as fibroblasts or endothelial cells which are of interest in tissue engineering. Comparison between protein resistant coatings such as polyethylene glycol and untreated 3-GPS should be investigated further.

Electrode impedance is closely related to the background noise level in recordings and to the safely deliverable charge in stimulation. If the electrode impedance is too high, the thermal noise generated by the electrode becomes too high to record spikes. Moreover, high electrode impedance limits the amount of charge that can be delivered, reducing the likelihood of stimulating nearby neurons. The modest increase in impedance change with the 3-GPS layer is promising for neural applications, and is considerably less than that previously reported for the silane 3-MPS, used in an analogous linking scheme (Chang, 2002).

5. Conclusions

A simple and efficient protein linking scheme for cell adhesive proteins using organosilane based SAM has been introduced here. 3-GPS deposition of various substrate including glass, silicon dioxide, silicon nitride and polyimide made it easier to create protein patterns on any of these substrates using micro-contact printing technique. Hippocampal neurons grew in patterns for 2–3 weeks with excellent compliance to the pattern. An unexpected finding is that unlinked 3-GPS proved cell repulsive, further facilitating the preparation of surfaces for patterned neuronal growth.

As a demonstration of the utility of 3-GPS based preparation, the microcontact printing technique was applied to create micropatterns of protein and grow neurons to form ordered networks on planar multielectrode arrays. Neural recording and stimulation after long-term culture demonstrated the utility of using a 3-GPS toward the study of patterned neuronal networks in vitro. The techniques could be applied to various neural engineering applications including the surface modification of neural probes used for chronic implantation in an in vivo study or cell-based biosensors using dissociated neuronal cultures.

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