Gold-Coated Microelectrode Array With Thiol Linked Self-Assembled Monolayers for Engineering Neuronal Cultures

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***Abstract*—We report the use of a gold coating on microelectrode arrays (MEAs) to enable the use of the relatively reliable surface modification chemistry afforded by alkanethiol self-assembled monolayers (SAMs). The concept is simple and begins with planar MEAs, which are commercially available for neuronal cell culture and for brain slice studies. A gold film, with an intermediate adhesive layer of titanium, is deposited over the insulation of an existing MEA in a manner so as to be thin enough for transmission light microscopy as well as to avoid electrical contact to the electrodes. The alkanethiol-based linking chemistry is then applied for the desired experimental purpose. Here we show that polylysine linked to alkanethiol SAM can control the geometry of an *in vitro* hippocampal neuronal network grown on the MEA. Furthermore, recordings of neuronal action potentials from random and patterned networks suggest that the gold coating does not significantly alter the electrode properties. This design scheme may be useful for increasing the number of neurons located in close proximity to the electrodes. Realization of *in vitro* neuronal circuits on MEAs may significantly benefit basic neuroscience studies, as well as provide the insight relevant to applications such as neural prostheses or cell-based biosensors. The gold coating technique makes it possible to use the rich set of thiol-based surface modification techniques in combination with MEA recording.**

***Index Terms*—Cell patterning, hippocampal pyramidal cells, MEA, neural recording, neuronal network, self-assembled monolayer (SAM).**

I. Introduction

PLANAR microelectrode arrays (MEAs) have been used to characterize the electrophysiological properties of excitable tissues derived from both the brain and the heart. Two popular uses of MEAs are with brain slices [1], [2] and with dissociated cell culture, including spinal cord neurons [3], cardiac myocytes [4], [5], cortical neurons [6], [7], and hippocampal neurons [8], [9]. Electrical activity can be modulated by electrical stimulation through individual electrodes [3], [7] or by a direct drug injection into the culture chamber [10]. These publications have shown that MEAs provide stable long-term interface between the biological tissues and the electrodes.

A growing number of researchers use microlithographic techniques to control surface chemistry which in turn controls the spatial development of cultured neurons [11]–[14]. One aspect of this paper is the guidance of cultured neurons to overlie MEA electrodes to facilitate recording and stimulation. Cell patterning techniques vary according to the identity of the cell, the geometry of the pattern, the bioactive molecule, the substrate surface, and the means for linking that molecule to the surface. For the work done here, the surface and the linking scheme are of interest.

Most cell patterning work has been done with tissue-culture plastics, cover glass, and gold-coated surfaces, although materials associated with the electronics industry, including polyimide, silicon, silicon dioxide, and silicon nitride, have also been used. Physisorption of protein to any of these substrates is the most common method of deposition because of its ease of use. Alternatively one can create free functional groups on a surface using a self-assembled monolayer (SAM) technique and use it either directly or as a means of linking a self-assembled protein layer [15]. With glassy and plastic substrates, proteins and other biomolecules can be linked using organosilanes and cross-linkers [16], [17], with the advantage of higher stability and the disadvantage of greatly increased experimental difficulty as compared with physisorption. More common, however, is the use of organosulfur (disulfide or alkanethiol) species linked to gold. Alkanethiol-based SAMs are well studied due to their simplicity and reproducibility and are widely used to create model surfaces to study the interaction between the protein and cells [18]–[20].

Electrode array insulator surfaces to date have either been glassy (silicon dioxide [5] or silicon nitride [21], which tends to oxidize rapidly when exposed to air) or plastic (including polyimide [1] and polysiloxane [3]). Array surface modification schemes include physisorption of polylysine or laminin. Our group has used both physisorption [22] and silane linking of polylysine to MEA surfaces [17] in order to promote cell patterning on top of the MEA electrodes. Offenhäusser's group also used the silane linking to immobilize and pattern cell adhesive proteins on silicon dioxide surfaces of field-effect transistor-

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based MEAs [14]. To our knowledge, the use of gold/organothiol linking techniques in combination with MEAs is unexplored.

This paper reports that by using a simple modification to MEAs-gold coating-one can use alkanethiol-based SAM techniques to create controlled surfaces on the MEAs. This paper shows that the gold overcoat can be created easily and without affecting the electrical properties of the array. The purpose of this technique is to make accessible the entire set of organothiol linking techniques and results to researchers who wish to modify surfaces for biosensor applications or for studying the growth, attachment and development of biological tissues. Our demonstrations of the function of the technique relate to our laboratory's work in recording electrical activity from cultured dissociated hippocampal neurons and, in particular, our use of microcontact printing (μCP) to create spatially precise patterned neuronal networks from these cells. The demonstration indicates that gold-coated MEAs could significantly facilitate research into the creation and understanding of patterned neuronal networks.

The report of our work includes MEA fabrication and reconditioning, deposition of a thin film of gold over the array without shorting the electrodes while preserving transmitted light microscopy, chemical linking of growth promoting polylysine and cell resistant polyethylene glycol to the gold in patterns, verification of polylysine linking, sustained growth of neurons in patterns, and electrical recordings of spontaneous action potentials from both unpatterned and patterned neuronal networks.

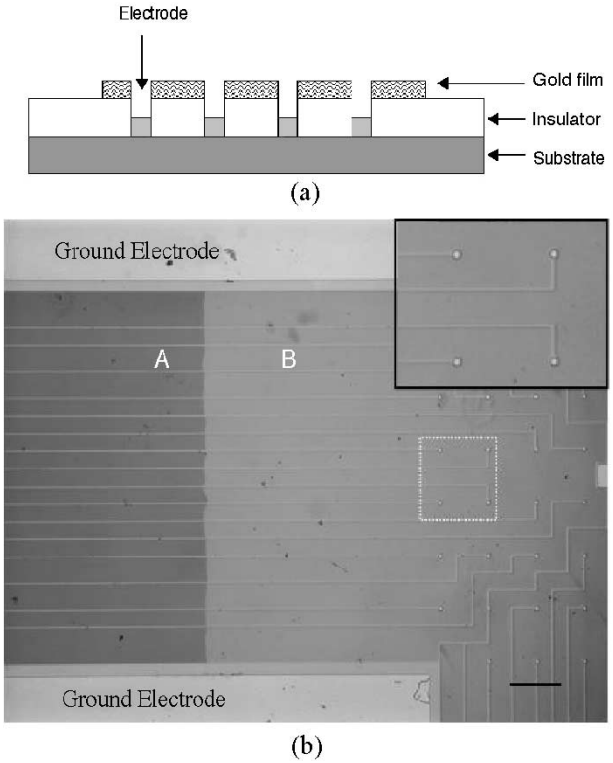


Fig. 1. Gold-coated MEA. (a) Schematic of partial gold coating on MEA, not to scale. (b) Bright field image (5×) highlighting the original insulator (A) and the gold overlay (B), (inset) microelectrodes, large ground electrodes are seen at top left and bottom left of the picture. Scale bar = 200 μm.

II. Methodology

A. MEA Fabrication

Two types of MEAs were used. MEAs insulated with Si_3N_4(0.5 μm) from Multi Channel Systems (Reutlingen, Germany) were ordered through ALA Scientific Instruments (Westbury, NY). These MEAs had TiN electrodes (10 μm in diameter) and electrodes were spaced by 200 μm. MEAs with polymer insulation were fabricated at the Micro and Nanotechnology Laboratory at the University of Illinois at Urbana-Champaign (Illinois MEA). To fabricate the Illinois MEAs, we began with chrome mask masters created in the NanoBio Engineering Research Center at Seoul National University in Korea. Glass microscope slides (2 in × 3 in, Fisher Scientific) for MEA substrates were cut into 4.9 cm × 4.9 cm and coated with a three-metal conductor layer (Ti/Au/Ti, 500/1000/200 Å) by e-beam and thermal evaporation using an NRC evaporator. Titanium was used to increase the adhesion of the gold layer to the glass substrate and to the overlying insulator. These layers were patterned by standard photolithography and wet etching processes. Polyimide (HD Microsystems, PI2611) was spin coated at 4000 rpm after application of an adhesion promoter (VM651, HD Microsystems). Samples were cured on a hot plate at 175 °C for 15 min followed by 350 °C for 30 min and then cooled to room temperature. To open electrode holes and contact pads, a titanium mask layer was deposited by e-beam evaporation and patterned using photolithography and wet-etching. Exposed PI2611 was etched by oxygen reactive ion etching (RIE) to form electrode sites (12.5 μm in diameter) and external contact pads. After RIE, the mask layer was removed by 5% HF (Hydrofluoric Acid). The final thickness of the insulator was measured with Tencor Alpha-Step 200 Profilometer (KLA-Tencor Corp., San Jose, CA). Three used MEAs (MultiChannel Systems, Reutlingen, Germany) were reconditioned by coating with PI2611 following the same process described above.

B. Gold Film Coating

A rectangular region (3.4 mm × 3.8 mm) to be coated with gold film was defined by standard photolithography using a dark field mask (Fig. 1). Titanium (30 Å, serving as an adhesion layer) and gold (50 ∼ 80 Å) were deposited using an NRC evaporator and the gold layer on the surrounding area was removed by the lift-off process. After lift-off, oxygen plasma treatment (300 W, 500 mT, 2 min) was used to clean the gold surface. Samples were kept in ethanol (Ethyl Alcohol USP, PROOF 200, AAPER Alcohol and Chemical Co.) to minimize surface contamination. For some of the culture studies, glass coverslips (22 mm × 22 mm, VWR Scientific Inc., West Chester, PA) were coated with gold using the same evaporation process.

C. Electrical Characterization

Short-circuit tests between the gold film and each electrode were done using a DC multimeter. AC impedances at 19 different frequencies (0.1–100 kHz, 10 mV_{rms}) were measured with an HP 4284A Precision LCR meter, with an Ag/AgCl electrode as the reference electrode in 1x PBS solution. In order to measure the shunt capacitance between the metal lines and

the solution ground plane, electrodes were blocked by a drop of photoresist (AZ5214; baked at 125 °C for 2 min), eliminating the series electrode conductance to the PBS solution.

D. Surface Modification and Protein Delivery

Gold-coated substrates (MEAs or glass coverslips) were treated with an ozone generator (custom built) for 30 min followed by 1-min ethanol ultra-sonication to remove any organic contaminants that would affect the quality of the SAM [23]. Then the array was immersed in 1 mM ethanolic 11-mercaptoundecanoic acid (MUA, Aldrich) for 2 h before being rinsed with ethanol and then dipped in deionzed water (DI) for 10 s. Subsequently, the MUA SAM was exposed to a mixture of 75-mM EDC (1-ethyl-3-[3(dimethylamino) propyl]carodiimide hydrochloride, Sigma Chemical Co.) and 15-mM NHS (N-hydroxysuccinimide, Pierce, Rockford, IL) in DI for 10 min to form an NHS ester [Fig. 2(a)] [24]. A uniform background coating of poly-D-lysine [PDL, 130 000 MW, Sigma Chemical Co.; 0.1 mg/ml in PBS (pH 8.0)] was created by flooding; patterns of PDL were created using microcontact printing.

E. Microcontact Printing (μCP)

PDMS (polydimethoxylsiloxane, Sylgard 184) stamps were prepared by a casting process in which molds were created by reactive ion etching of polyimide (PI2611, 20 μm thick) [11]. The liquid prepolymer of PDMS was poured into a mold and a glass slide was added to create a stamp with a rigid back. After curing for 12 h at 90 °C, the PDMS stamp was released from its mold. The stamp preparation was adopted from [25]. Stamps were cleaned by acetone for 5 min in an ultra-sonication bath and rinsed with IPA (isoprophyl alcohol) and DI water successively. They were immersed in 10% sodium dodecyl sulfate (SDS, Bio-Rad Laboratories, CA) for 15 min to enhance PDL adsorption. PDL solution (50–100 μL, 0.1 mg/ml in 1x PBS) was dropped on each stamp and allowed to adsorb for 60 min. A custom built contact aligner was used to align the stamp to the electrodes on the arrays. MEAs and glass coverslips were left in contact with a PDL loaded stamp for 20 min at room temperature after visible contact was formed between the stamp and the array [Fig. 2(b)]. After stamping, the array was rinsed with DI water to remove unbound PDL residues.

Unstamped areas (background areas) were covered with 1 mg/ml ω-hydroxy-α-amine polyethylene glycol ($\mathrm{PEG-NH_2}$, MW 3400, Shearwater Polymers Inc., Huntsville, AL) in PBS (pH 7.0) for 30 min. This molecule has been shown to be effective in prohibiting cell growth [11]. The amine groups ($-\mathrm{NH_2}$) formed covalent bonds with the unbound NHS ester [Fig. 2(c)]. The array was rinsed in DI to remove the unlinked molecules and dried using nitrogen gas.

F. Fluorescence Imaging

To visualize the printed micropattern, fluorescent doped carboxylate-modified microspheres (F-8811, yellow/green fluorescent, Molecular Probes, Inc., Eugene, OR) were used to tag the patterned PDL. The bead (microsphere) solution was prepared by mixing 2.65×10^{10} beads/ml with 1.4×10^{-5} M EDC and 1.4×10^{-5} M NHS for 15 min. This process allowed NHS esters to be formed on the microsphere surface so they can react selectively with the PDL printed on the substrate. The PDL patterned substrate was submerged into this bead solution for 1.5 h. Images of PDL patterns on MUA-SAM were acquired with a fluorescence microscope (Zeiss Axiovert 100 inverted research-grade microscope).

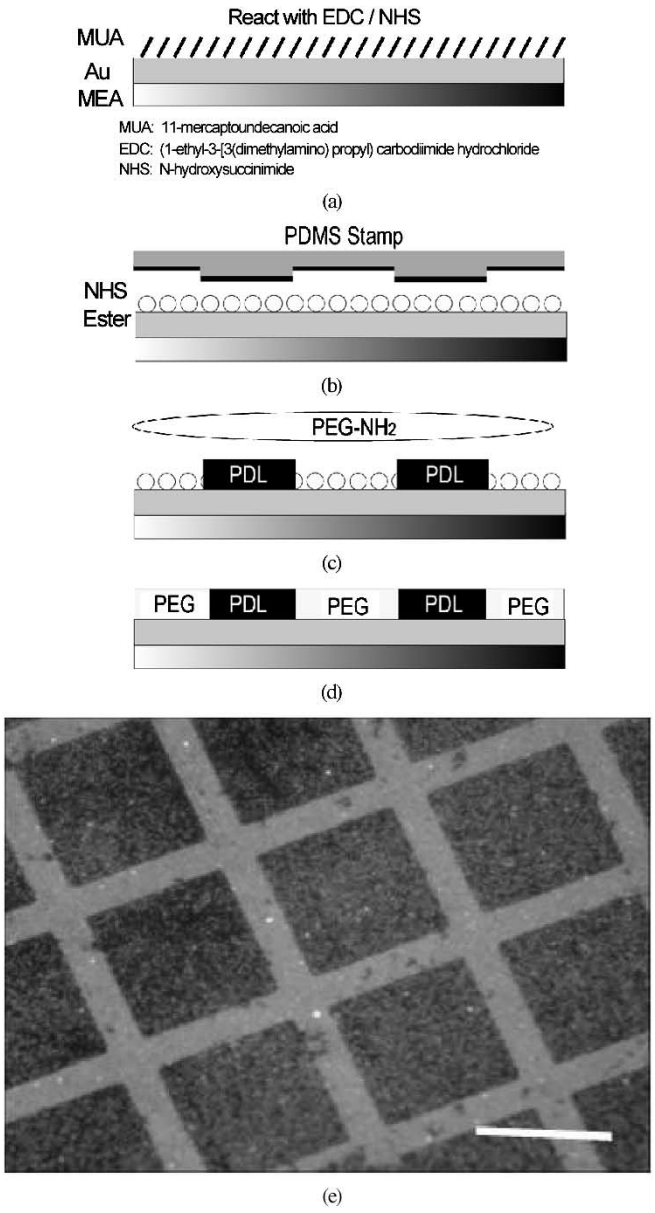


Fig. 2. Schematic of surface reaction, (a) NHS ester formation on MUA-SAM by reacting with EDC/NHS, (b) PDL pattern transfer by μCP on NHS ester surface (PDL: poly-D-lysine), (c) background treatment by polyethylene glycol (PEG) flooding, (d) final surface pattern of PDL versus PEG, and (e) fluorescence image of PDL pattern on gold-coated coverslip, light color region represents the grid pattern, 10 μm wide. Scale bar = 60 μm. From *Proc. 2nd Annu. Int. IEEE-EMBS Special Topic Conf. on Microtechnologies in Medicine & Biology*.

G. Recording

MEAs were placed in the MEA1060 Amplifier (Gain 1200x, 10–3000 Hz, Multi Channel Systems) and either the MEA60 system (MultiChannel Systems) or the Multichannel Acquisi-

tion Processor (Plexon Inc., Dallas, TX) was used to digitize 60 channel analog data at a sampling frequency of 25 kHz. If needed, software digital filters (200-Hz high-pass filter or 60-Hz notch filter) were used to remove the electrical interference while preserving the fast extracellular action potentials of interest in this study. pCLAMP 8 (Axon Instruments Inc., Union City, CA) hardware and software were also used to monitor and record real time signals from selected electrodes coupled through the Multichannel Acquisition Processor. Data collected through MEA60 system were replayed after recording to extract the spikes and transferred to the dedicated software ("Off-line sorter," Plexon Inc., Dallas, TX) to analyze the neural signals from each channel.

H. Cell Culture

Hippocampal neurons (Brain Bits, www.siumed.edu/brainbits) from 18-day gestation Sprague-Dawley rat embryos were dissected mechanically and cultured at 37 °C, 5% CO_2, 9% O_2, and 86% N_2, in serum free B27/Neurobasal medium (Invitrogen, Gaithersburg, MD) with 0.5 mM glutamine and 25 μM glutamate [26]. Cells were plated at a density of 100–400 $\mathrm{cells/mm^2}$ onto the center of MEA in a region secured by a PDMS ring which can contain up to 3 ml of media. Additional serum-free medium was added to bring the total volume to 2 ml. The array was stored in a 15-cm-diameter Petri dish. Half of the medium was changed semi-weekly, without glutamate.

III. Results

A. MEA Fabrication

Each electrode site was slightly over-etched to form a via-hole of 15 μm in diameter. MEAs, which were originally purchased from Multi Channels Systems and reconditioned with PI2611, showed stable adhesion of the polyimide to the manufacturer's existing insulator (0.5 μm, Si_3N_4); the AC impedance of the electrodes was $229\,\mathrm{k\Omega}\pm10\,\mathrm{k\Omega}\angle-75.9°\pm0.7°$ [$\mathrm{n} = 14$, standard deviation (SD)] which indicated that the original TiN electrodes were preserved during the insulation process. The final thickness of PI2611 was 3–4 μm which we believe was sufficiently thick to provide a stable insulation under very long term exposure to the culture media. The AC impedance of the Illinois MEA fabricated gold electrodes was $2.5\ \mathrm{M\Omega} \pm 0.6\ \mathrm{M\Omega}\angle - 80.5° \pm 2.3°$ ($\mathrm{n} = 50$, SD). For the Illinois MEAs, the normalized shunt impedance between the conductor line and the ground plane (the culture media) was $6.8 \pm 1.3\ \Omega\mathrm{m}^2$ ($\mathrm{n} = 5$, SD), equivalent to an impedance greater than 100 MΩ at 1 kHz for the $5 \times 10^{-8}\mathrm{m}^2$ (5 mm × 10μm) conductor line which is defined by our culture chamber. Since the electrode impedance was at most in the few MΩ range, signal attenuation by shunt capacitance could be neglected.

B. Gold-Coated MEAs

The bright field image of the Illinois MEA with gold film is shown in Fig. 1(b). As indicated in Fig. 1(a), the thin gold coating was confined to the center area that contains 60 electrodes. The thickness of the gold layer varied between 50 Å and 80 Å, which was sufficiently thick for the subsequent surface chemistry. The 30 Å thickness of the titanium layer was sufficient for the adhesion of gold on insulators. Although the combined film was thin, there was no sign of degradation of metal layers during rinsing, ultra-sonication, and long-term culture. Furthermore, the steep sidewalls of the electrodes prevented the background gold film from short-circuiting the electrodes, as verified by the DC short circuit test. Although the electrode impedance after gold coating increased slightly to $2.9\ \mathrm{M\Omega} \pm 0.6\ \mathrm{M\Omega}\angle - 81.2° \pm 1.4°$ ($\mathrm{n} = 50$, SD) at 1 kHz, impedance spectroscopy before and after gold coating suggests that the electrode properties were not altered by the coating procedure (Fig. 3), which agrees with the literature [27], [28].

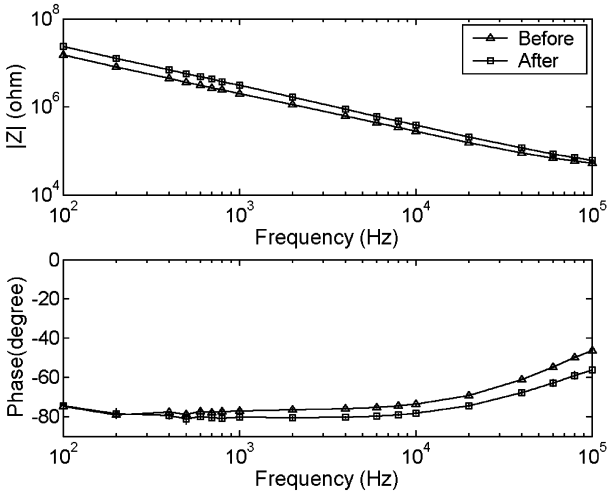


Fig. 3. Impedance spectroscopy of four electrodes before and after gold coating. Averaged values at 1 kHz are $\mathrm{Z\ (before)} = 2.0\ \mathrm{M\Omega} \pm 0.5\ \mathrm{M\Omega}\angle - 77.2° \pm 0.7°$ and $\mathrm{Z\ (after)} = 3.1\ \mathrm{M\Omega} \pm 0.4\ \mathrm{M\Omega}\angle - 80.4° \pm 0.9°$ ($\mathrm{n} = 4$, SD).

C. Neural Growth on a Uniform PDL Layer on Gold Surfaces

SPR (surface plasmon resonance) angle shifts indicated completeness of the covalent reaction between PDL and MUA (Data not shown). The viability of neurons on a PDL linked gold surface was compared with neurons grown on a controlled glass area on the same sample. Good cell growth occurred on both surfaces over several weeks [Fig. 4(a)]. Growth on MEAs followed a similar course.

D. Controlled Growth of Neurons on Micropatterned Protein Layer on Gold Surface

Fig. 2(e) illustrates successful protein imprinting on a gold-coated coverslip of a well-resolved stamped grid pattern of 10 μm wide lines (light colored region). No separate study was done in this work to quantify the amount of PDL transferred to substrate. Cells on patterned substrates showed good compliance to the predefined pattern for more than two weeks. As reported in [11], the covalently linked PEG layer was repulsive to cell attachment and growth for an extended period of time (up to 18 days in the present study). As shown in the SEM image [Fig. 4(b)], cell growth could be confined successfully to 5 μm thin lines, suggesting the utility of the technique for creating geometrically controlled neuronal networks *in vitro*. On gold-coated MEAs, the pattern was aligned so as to overlie

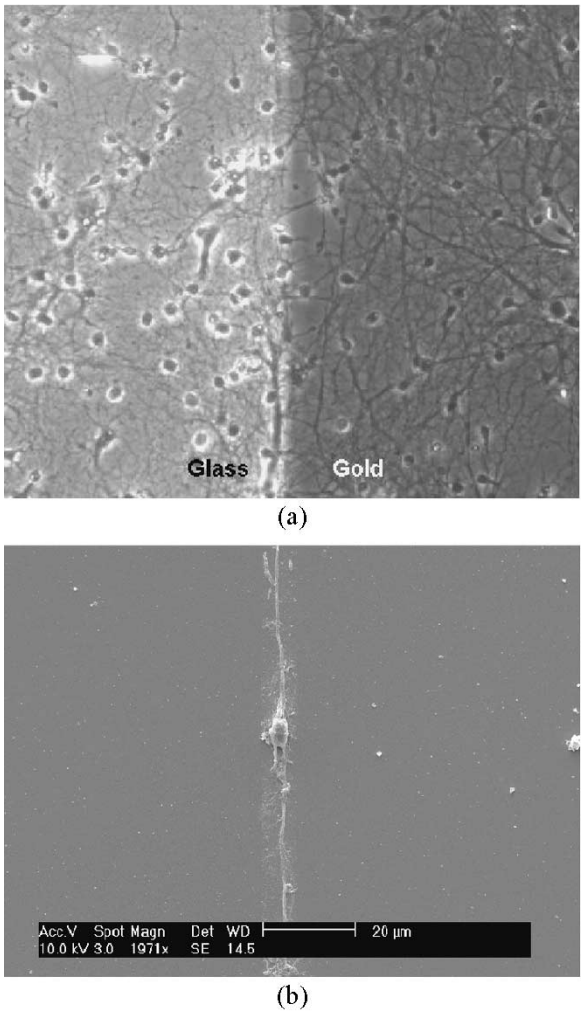


Fig. 4. Cell growth on gold-coated glass coverslips. (a) Transmitted light microscope (phase contrast) image of cell growth on uniform PDL layer on glass cover slip partially coated with gold. The image contrast is reduced in the area of the thin gold film. 18DIV. (b) Scanning electron microscope (SEM) image of guided cell growth on 5-μm thin line PDL line against a background treated with PEG, 14 DIV.

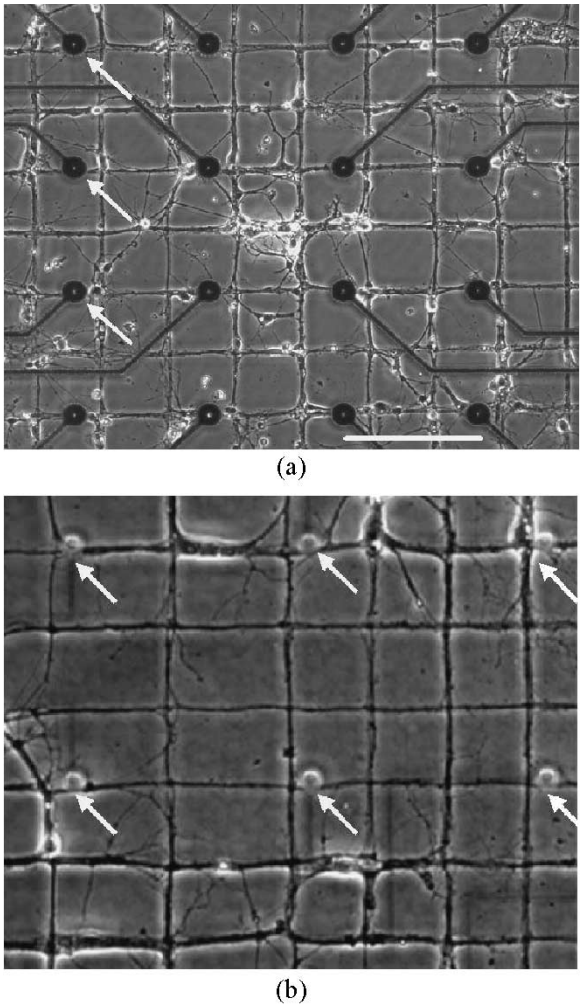


Fig. 5. Neuronal circuit created on gold-coated MEA whose original insulator is Si_3N_4 (a) or polyimide (b). Cell growth near electrodes (arrows) was induced by precise alignment of predefined pattern to electrode layout though μCP. The grid patterns in (a) and (b) have a different spacing. Cell density: 100 $\mathrm{cells/mm^2}$. Pictures taken at 12 DIV (a) and 15DIV (b). Scale bar = 200 μm. From *Proc. 25th IEEE EMBS Annu. Int. Conf.*

the electrodes, resulting in neurons and neurites that are either on or near the electrodes (Fig. 5). The high-quality of patterns was maintained across the different insulations tested (Fig. 5, Si_3N_4 and polyimide).

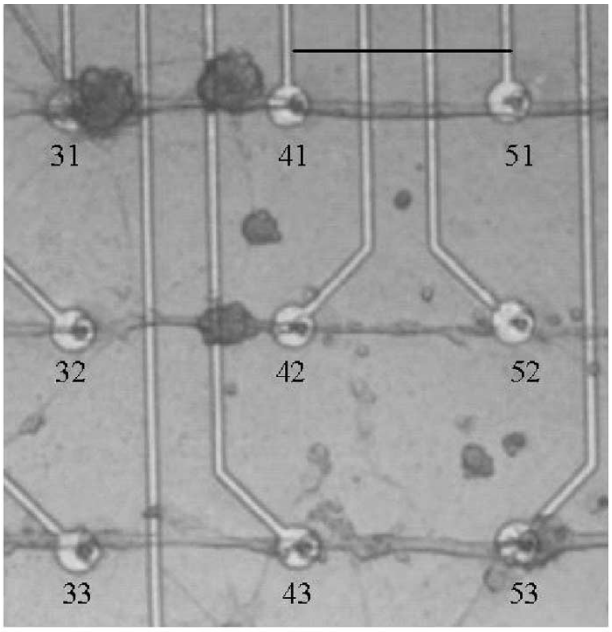
E. Neural Recording

To demonstrate the recording properties of a gold-coated MEA, we recorded spontaneous action potentials from hippocampal neurons cultured on both unpatterned and patterned PDL layers. In the unpatterned culture, spontaneous activity was recordable from cells near a few of the electrodes after 14 days *in vitro* (DIV). The action potentials were usually triphasic in shape with amplitudes of 100 μV_{p-p} [29].

Fig. 6(a) shows the confined linear growth of neurites and somata after 17 DIV. The overall pattern consisted of thin lines (5 μm in width) and large rectangular moat regions [800 μm $\times$ 200 μm; out of the field of view in Fig. 6(a)]. The shapes of spontaneous single unit action potentials recorded with the array were consistent with the literature [22], [30], including signals from neurons in the large rectangular moat regions and from neurons lying on the thin lines. Representative signals are shown in Fig. 6(b). Action potentials recorded from densely populated moat regions showed synchronized bursting activity with amplitudes of 500–700 μV_{p-p} and durations of 2–3 ms [Channels 14, 16, and 17 in Fig. 6(b)], and action potentials from the thin patterns of neurites were smaller, 100–200 μV_{p-p}, with durations ranging from 1.5–2.5 ms [CH 31–77 in Fig. 6(b)]. However, there were some channels that showed no neural signals even though visual inspection confirmed the neurites were located at the electrode [e.g., electrodes 41, 32, 33, 34, and 43 in Fig. 6(a)].

IV. Discussion

We found that a 50–80 Å gold layer, on top of a 30 Å titanium layer, was thin enough to ensure that one could monitor the cells with a transmitted light microscope, as well as thick enough to provide a reliable layer for the intended surface chemistry. Judging from the long-term neural growth on gold-coated substrates, a stable PDL layer was achieved by linking to an



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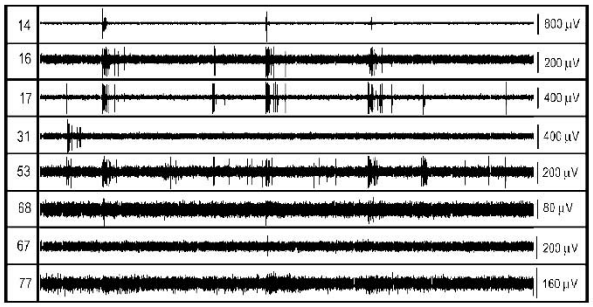

(b)

Fig. 6. Electrical recording from neurons cultured on gold-coated MEA. (a) Confined linear growth of neurites (bright field). Several clusters of cells are seen near electrodes of 31, 41, 42, and 53. 400 $\mathrm{cells/mm^2}$, 17 DIV. Scale bar = 200 μm (b) Multichannel recording from (a). Channels 14, 16, and 17 are within the moat area (not shown). Channels 67, 68, and 77 are from the regions patterned similarly to (a). Each trace is 25 s long.

alkanethiol SAM. This linking scheme is appropriate for PDL, proteins, and other biomolecules containing amine groups, such as the modified PEG used here. Microcontact printing allows us to define two (or more) different surface conditions-one adhesive and the other repulsive to cell attachment-with results similar to previous work with glassy substrates [11], [17]. In this paper, we have shown that this design has provided the chance to utilize state-of-the-art organothiol chemistry-based cell patterning techniques to MEAs. Our only concern with the process is the need to develop a reliable means to clean the gold surface for reuse of a coated array.

The thin gold film does not affect the electrical recording properties of the MEA, including shunt resistance, electrode impedance, and the apparent amplitude and time course of the recorded extracellular action potentials [8], [22], [30]. It is expected that extracellular current flows primarily through the highly conductive extracellular medium and does not couple to the gold due to the high gold/solution interfacial impedance. Thus, the gold-coated surface should not interfere with the neuronal network or extracellular signals.

Guiding the growth of neurons over the electrodes, arguably made easier by the organothiol chemistry, increases the strength of recorded electrical signals. Patterning of neurons over electrodes appears to enhance the detectability of action potentials [8]. We have recorded some signals with amplitudes of nearly 500 $\mu\mathrm{V_{p-p}}$ from neurons guided over electrodes by thin line patterns, although the 100–200 $\mu\mathrm{V_{p-p}}$ signals of Fig. 6(b) are more common. Larger amplitudes were more common in densely populated regions, suggesting that cell bodies lie directly on top of, and seal off, the underlying electrodes. Other researchers have modeled this effect [31]–[34]. It is unclear why some channels [e.g., electrodes 41, 32, 33, 34, and 43 in Fig. 6(a)] did not detect any signals at all. The cause may be the lack of neuronal activity or insufficient coupling of the signal to the electrode for one or more reasons, including small diameter axons and hence extracellular currents, distance between neurons and electrode, and isolation of the electrode by glial overgrowth.

Gold coating might inspire the use of other linking schemes to obtain novel biosurfaces. The EDC/NHS linking scheme that we used in this paper was chosen because it is well-known [24]. Alternatively, one might use microcontact printing of either amine- or PEG-terminated alkanethiol to achieve surfaces similar to those reported here, but with simpler processing [18], [19]. Other micropatterning techniques based on alkanethiol SAMs could also be applied to this MEA platform [35], [36].

V. Conclusion

MEAs can be coated with a gold background surface over the insulating material without compromising the electrical properties of the electrodes in the array. The advantage of this approach is the ability to make use of the relatively easy and versatile alkanethiol-based chemistry for modifying the surface, enabling a variety of experiments. Here the technique was used to realize covalently linked patterns of polylysine versus polyethylene glycol that in turn permitted the control of neuronal cells to lie near and in some cases over individual electrodes, perhaps enhancing the effectiveness of this neuronal network investigation technology. Increasing the versatility of the MEA surface without compromising its function should be beneficial to those researchers in related neuronal and cell culture areas.

Acknowledgment

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