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Conductive Polymer "Molecular Wires" For Neuro-Robotic Interfaces

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Abstract— **Direct electrical communication between living nervous systems and external devices would allow a wide variety of clinical and engineering applications. This neuro-robotic interface has not yet been fully achieved because existing interface systems cannot establish sufficiently close contact between neurons and communicating electrodes. We present here the synthesis and testing of a new class of conductive polymer that can be used to coat metal electrodes and achieve the necessary close contact. The polymer coatings can be made to incorporate biological adhesion proteins to maximize biocompatibility. Neurons extracted from mouse and rat brains were able to attach to the coatings, survive beyond five days, and grow out long, communicating processes. These new polymer films offer the potential for a new level of communication between robotic devices and the nervous system and may eventually make a high-bandwidth interface a reality.**

I. Introduction

A direct electrical interface to a living nervous system could be a valuable tool for roboticists. At the most basic level, chronic low-noise recordings of neural data would offer insight into human motor control, perception, and cognition that could then be used to develop new algorithms and actuators for robotics. Robotic mechanisms could be placed directly under the control of living neurons, either for clinical prostheses or for adaptive control of a robot [1], [2]. If the interface is bidirectional, the living organism could also be placed under control of an external electronic system. This has been demonstrated in "remote-controlled" rats and in functional electrical stimulation for paralyzed humans [3], [4].

Despite their promise, these applications have not been fully realized to date. A principal barrier is the need for better electrode systems to implement the interface. Present interface devices depend on arrays of fine metal wires or silicon shanks that are implanted into the target tissue. At present, the contacted neurons are not well-connected electrically to the metal or silicon electrodes being used to stimulate and record. Inflammatory tissue reaction to an implant can separate nerve cell and electrode [5]. Even when this does not happen, the stimulating currents used by existing interfaces are large enough to place cells into a partially unresponsive state that lasts as long as stimulation continues [6].

Conductive polymer coatings for interface electrodes have been proposed as a way to improve the intimacy of the connection. A nanostructured "fuzzy" polymer coat increases electrode surface area and thus lowers the electrical impedance [7]. Polymer coatings can also contain biomolecules to attract neurons and promote their adherence to the electrode [8]. Because they are softer and more compliant than metal or silicon, it has been suggested that these coatings will reduce inflammation caused by strain mismatch between tissue and electrode.

Although published conductive polymer coatings represent an improvement in electrode technology, the present method of electrodeposition allows only limited control over the nanoscale structure and thickness of the film. Moreover, the films are not covalently bound to the electrode and may detach from it under the harsh saline conditions found in living organisms [7]. Finally, the most conductive known polymer, regioregular poly(3-alkylthiophene) (PT) cannot be electropolymerized, as electrodeposition does not permit regioregular synthesis. In this work, we describe the deposition of a new conductive polymer electrode coating through single-molecule self-assembly. These monolayer coatings of "molecular wires" are chemically bound to the underlying metal electrode. By incorporating the neural cell adhesion molecule L1 into our monolayers, we demonstrate adhesion and long-term survival of primary rodent neurons with extensive neurite outgrowth. Our aim is to demonstrate the biocompatibility of this new coating system in preparation for *in vitro* electrophysiology experiments.

II. Methods and Materials

A. Synthesis of Polythiophenes

The polymer used in these experiments was end-thiolated poly(3-(2-ethylhexyl)-thiophene), hereafter referred to as EHPT. 2,5-dibromo-3-(2-ethylhexyl)thiophene (1.63g, 5.0 mmol) was dissolved in tetrahydrofuran (50 mL). tert-butylmagnesium chloride (2.5 mL, 5.0 mmol) was added via

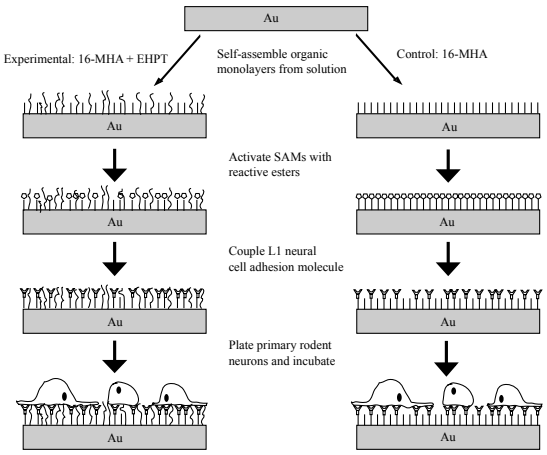


Fig. 1. Procedure for biocompatibility studies on organic monolayers. SAMs are assembled by overnight incubation of surface with end-thiolated molecules. Formed SAMs are activated with reactive esters and used to capture proteins. Primary rodent neurons are then plated and grown 1-5 days before cell fixation and staining. Control SAMs contain pure 16-MHA with coupled L1 and should show the maximum possible cell compatibility. Experimental SAMs contain varying proportions of EHPT (from pure EHPT to 2:1 MHA:EHPT) to test cell adhesion and outgrowth on this new conductive polymer electrode material.

syringe and the mixture was refluxed for 1.5 hours. The reaction mixture was then allowed to cool to room temperature and $\mathrm{Ni(dppp)Cl_2}$ (45 mg, 0.08 mmol) was added in one portion. The mixture was stirred for 10 minutes at room temperature, then poured into methanol to precipitate the polymer. The polymer was filtered into an extraction thimble and then washed by Soxhlet extraction with methanol, hexane and chloroform. The polymer was isolated from the chloroform extraction. Thiol end groups were added by lithiation of the bromine-terminated polymer followed by quenching with elemental sulfur and acetyl chloride. This results in the formation of a stable thiol acetate that prevents oxidation while the polymer is stored prior to use. End-group composition was evaluated with MALDI-TOF mass spectroscopy (Voyager-DE STR BioSpectrometry workstation by Biosystems, terthiophene matrix, sometimes performed before extractions), and confirmed with 1H nuclear magnetic resonance (Brucker 300 MHz instrument). The molecular weight of the polymer used in these experiments was found to be approximately 3000 g/mol.

B. Preparation of Gold Substrates

To produce cell culture substrates, 6 kiloAngstroms (kÅ) of gold with a 300 Å chromium adhesion layer were deposited by thermal evaporation from a tungsten boat (system provided by Denton Vacuum, Moorestown, NJ) onto a fresh <100> silicon wafer (Silicon Quest International, Santa Clara, CA). Individual samples (0.25 to 1 cm^2 area) were prepared from this wafer by cleaving along crystal planes. Samples were cleaned just before use by 10 minutes or more of immersion in a 1:1:5 solution of $\mathrm{HOOH : NH_4OH : H_2O}$ at 80° C followed by rinsing with absolute ethanol and blow-drying with a stream of pre-purified nitrogen.

For atomic force microscopy experiments, atomically flat Au(111) samples were obtained from Molecular Imaging Corporation (Tempe, Arizona) and used as received.

C. Formation of Pure and Mixed Self-Assembled Monolayers

Thiolated EHPT was dissolved in chloroform at a concentration of 20 mg/mL, a nearly saturated solution. To form a pure self-assembled monolayer (SAM) of EHPT, gold substrates prepared as above were immersed in this solution for 48 hours or longer. After removal from the SAM-forming solution, substrates were rinsed repeatedly with fresh chloroform followed by a half-hour soak in chloroform to further remove any unreacted or unthiolated polymer. Monolayer formation was initially verified by atomic force microscopy of an EHPT SAM formed on atomically flat gold, using a Digital Instruments NanoScope III AFM in tapping mode. For all cell culture experiments, SAM presence was assessed by visualizing the increased background fluorescence emitted by the conjugated polymer.

We prepared pure SAMs of EHPT and of 16-mercaptohexadecanoic acid (16-MHA) as well as mixed SAMs incorporating both EHPT and 16-MHA. Long-chain alkanethiols such as 16-MHA have previously been used to couple dense layers of biomolecules to gold surfaces [9]. 16-MHA (Sigma-Aldrich Chemical Co., used as received) was dissolved in chloroform at 20 mg/mL and mixed in various proportions with the EHPT SAM-forming solution described above. These mixed SAM samples were rinsed and soaked before use as per the pure EHPT SAMs. The solutions used in the experiments reported here were 0:1, 1:1, 2:1, 1:2, and 1:0 (v/v) MHA:EHPT.

D. Protein Incorporation in Mixed SAMs

We hypothesized that unmodified EHPT SAMs would be nonpermissive for neuron attachment and growth, and we therefore modified mixed EHPT/MHA SAMs by covalent coupling of the neural cell adhesion molecule L1 (purified from neonatal mouse brain extract as described in [10]) to the free carboxyl groups of the 16-MHA chains.

In order to verify coupling of protein to these mixed SAMs, we also coupled fluoroscein isothiocyanate (FITC) conjugated IgG (goat anti-mouse, Sigma-Aldrich Chemical Co., diluted to 15 μg/mL in PBS) to mixed SAMs formed from thiolated poly(3-hexylthiophene) (HPT, synthesized as per EHPT) and 11-mercaptoundecanoic acid (11-MUA, Sigma-Aldrich Chemical Co.). This alternative SAM system was chosen for evaluation because its components are chemically similar to EHPT and 16-MHA, but are less expensive, simpler to prepare, and of higher purity. Both the antibody and the polymer emit light under proper illumination. Fluorescence emission from these SAMs was quantified by image capture and pixel intensity analysis using a Nikon charge-coupled device digital camera. 162,144 intensity samples were acquired per SAM with five seconds of exposure per sample.

Coupling of both proteins to mixed SAMs was essentially as per the protocol in [9]. Activation with reactive esters was performed in absolute ethanol, and total activation time was two hours. Protein coupling time varied slightly but was never less than one hour.

E. Cell Culture and Evaluation

To assess compatibility of the pure and mixed EHPT SAMs with mammalian neurons, primary rodent CNS neuron cultures were prepared and maintained as described in [11]. Tissues evaluated included embryonic day 14 (E14) mouse spinal cord, post-natal day (PND) 1 rat cortex, and PND 6-7 mouse cerebellum. Cells were plated on pure and mixed SAMs (Figure 1) at an approximate density of 10,000 cells per sample chip. Control spots of L1 on nitrocellulose-coated coverslips were plated and maintained alongside all cultures in order to verify the activity of each L1 sample.

Cells were grown for 1 to 5 days *in vitro* (DIV) before fixation with paraformaldehyde and immunofluorescent staining. Stained chips were coverslipped and mounted in anti-fade medium before visualization by fluorescence microscopy. Anti-tubulin staining with NP40 permeabilization was used in most cases in order to maximize the detail of the neurite tree. 5 DIV was chosen as the endpoint due to our empirical experience that this is the age at which dissociated neurons begin to show electrical activity suitable for *in vitro* electrophysiology. By studying the cells up to 5 DIV and verifying that they are still viable at that age, we sought to understand how attachment and outgrowth differed from the normal progression on pure protein/MHA substrates and ensure that no detrimental effects occurred before we would be able to do electrical studies.

For quantification of cell attachment, roughly 30 high-power fields per chip were randomly selected and the cell bodies counted. For quantification of neurite length, roughly 25 cells were randomly selected on each chip. Selected cells were photographed using a CCD digital camera attached to a Zeiss Axiotech microscope. Overlapping fields were photographed to ensure capture of each cell's complete process tree. Images were manually merged and neurites measured using IPLab software, version 3.2.

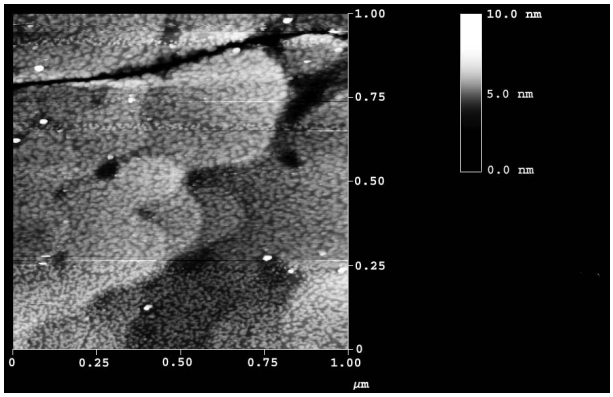
III. Results

A. Atomic Force Microscopy of EHPT SAMs

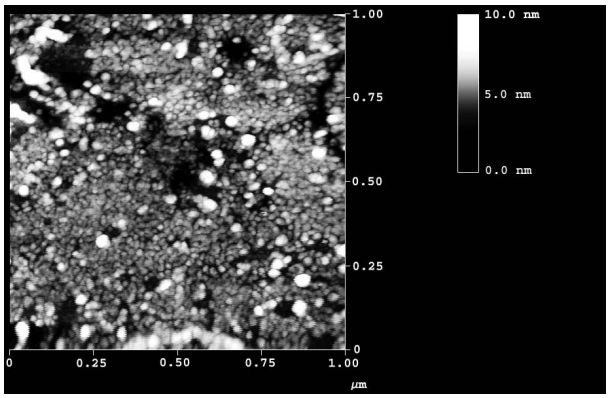
Results of the AFM study are shown in Figure 2. EHPT forms a loosely packed monolayer of varying height. It is noteworthy that one cannot see bundles of polymer laying flat on the surface as has been observed in AFMs of other PT preparations; the polymer wires appear to be standing straight up from the surface.

B. Fluorescence Measurement of Protein Incorporation

Figure 3 shows the results of fluorescence intensity measurements on SAMs formed from pure 11-MUA (with coupled FITC IgG), pure HPT, and a 1:1 (v/v) mixture of 20 mg/mL chloroform solutions of the two components (again with coupled IgG). The notable finding is that intensities of the 1:1



(a)



(b)

Fig. 2. AFM images of HPT SAM formation. (a) , bare terraces of atomically flat gold. (b) , gold sample after overnight incubation with saturated solution of thiolated HPT. Terraces are now covered by a loosely-packed monolayer with polymer chains oriented normal to the surface. The variation in length of the individual molecules is visible, although most of the polymer brushes extend above the scan range.

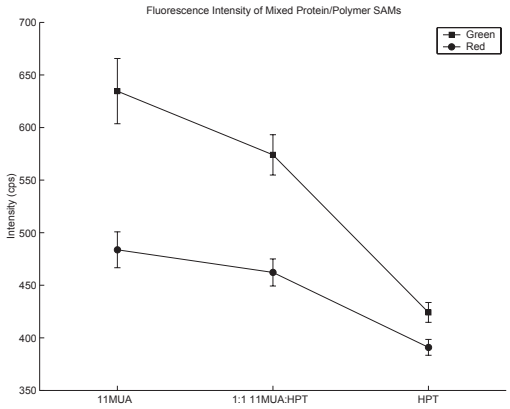


Fig. 3. Fluorescence of pure and mixed SAMs coupled with FITC IgG. Each point represents the mean fluorescence of 162,144 sampled pixels. In both red and green channels, emission of the mixed SAM is between those of the two pure SAMs. Mixed monolayer formation is therefore successful, and the mixed SAM remains capable of coupling to proteins.

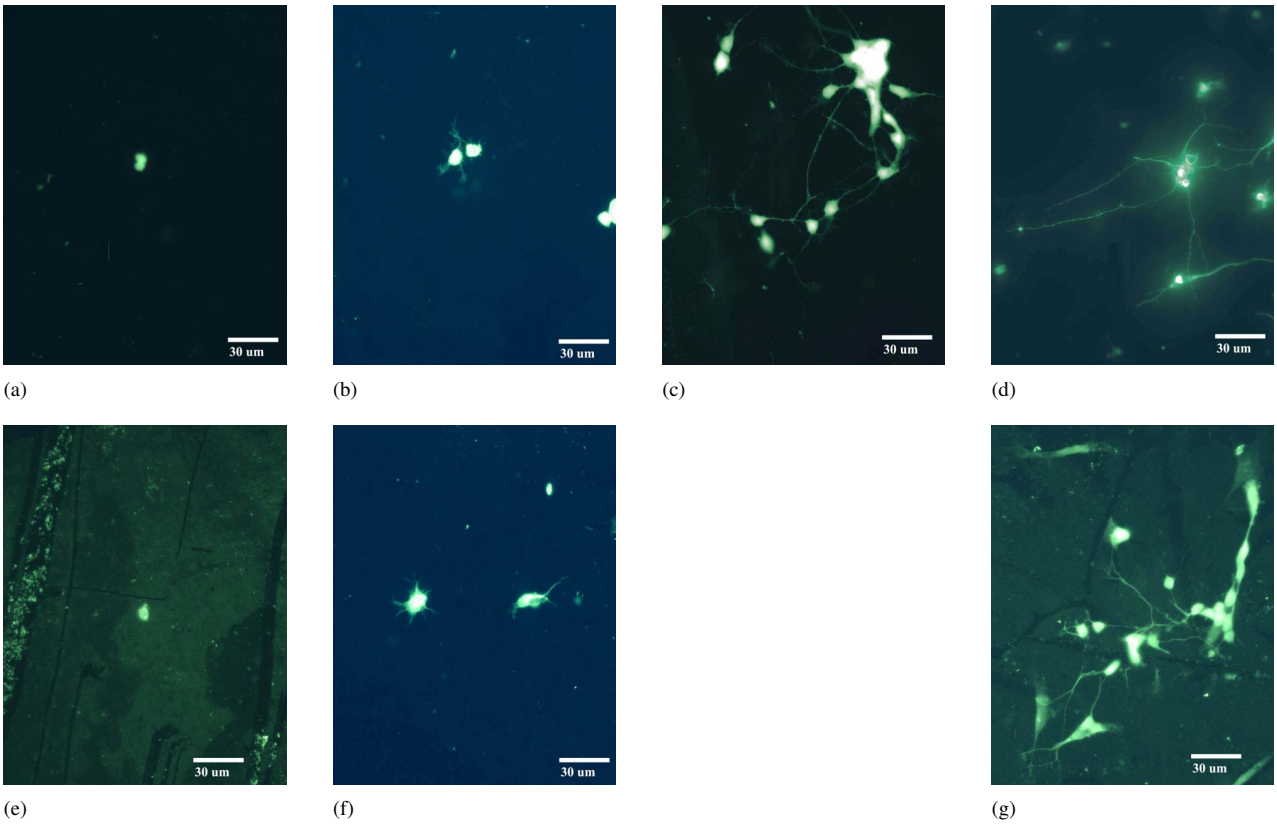


Fig. 4. Images of different species and neuron subtypes on mixed L1/EHPT SAMs, 1 day in vitro, 400x magnification. Top row, PND 1 rat cortical cells, anti-M6 stain. Bottom row, E14 mouse spinal cord cells, anti-tubulin stain. (a) and (e) , pure EHPT. Minimal cell attachment with no visible neurite outgrowth. (b) and (f) , L1 with 1:1 MHA:EHPT. Some small neurites are now visible. (c) , rat corticals on L1 with 2:1 MHA:EHPT. (d) and (g) , L1 on pure MHA. Dense cell attachment with outgrowth of multiple long neurites.

mixed SAM are between those of the two component SAMs in both red and green channels, implying that the SAM is in fact a mixture of polymer and MUA-coupled protein.

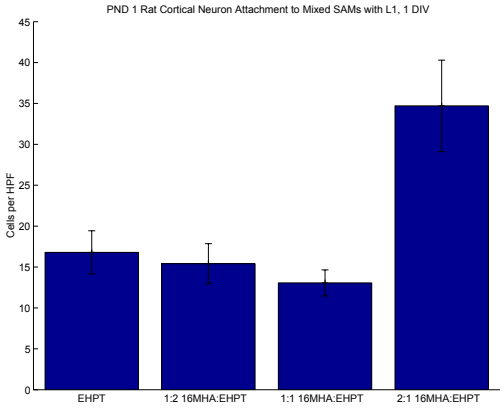


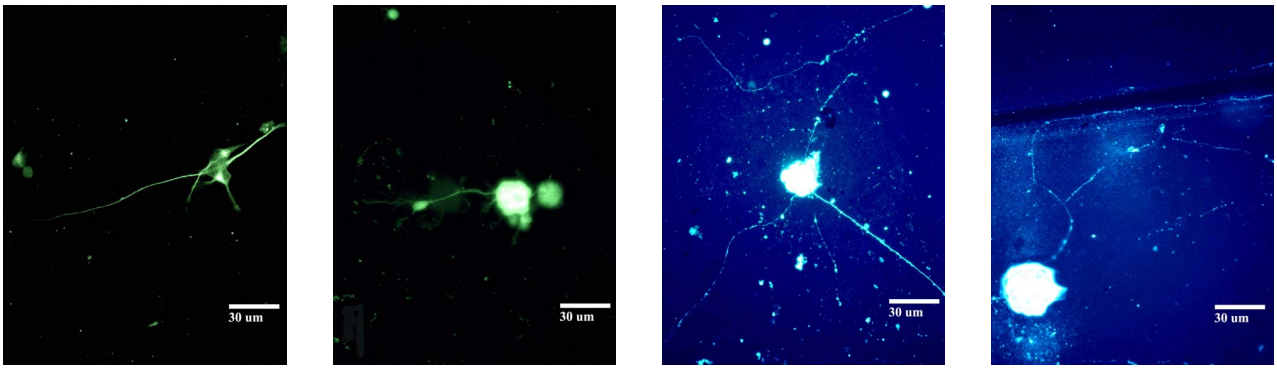
Fig. 5. Quantification of PND 1 rat cortical neuron adhesion to mixed SAMs of varying EHPT/MHA content (all MHA SAMs with coupled L1). Each bar represents the mean number of cells seen in one microscope field at 400x magnification. No significant difference is seen between pure EHPT and 2:1 or 1:1 EHPT:MHA ($p > 0.4$), but there is a sudden and statistically significant increase ($p < 0.002$) in adhesion when moving between 1:1 and 1:2 EHPT:MHA films.

C. Primary Neuron Culture on Mixed Protein/Polymer SAMs

We report the results of two sets of experiments: studies of adhesion of various CNS neuron subtypes to pure and mixed SAMs (evaluated at 1 DIV to minimize possible differences in cell survival under our culture conditions) and studies of longer-term neuron response to mixed SAMs as measured by neurite outgrowth over 1 to 5 DIV.

Images of various neuron phenotypes on a range of SAMs from pure EHPT to pure 16-MHA with L1 are shown in Figure 4. Results from cell attachment quantification of rat corticals are shown in Figure 5. Both photographs and cell counting show a marked increase in cell count once the solution that formed the mixed SAM is composed of more MHA (by volume) than EHPT. Presence of the EHPT is still visible in the background fluorescence of these SAMs, particularly under green illumination and red emission filters (data not shown).

Photographs of mouse cerebellar cells at 1 DIV and 5 DIV are shown in Figure 6, with the mean neurite lengths of cells from the same chips plotted in Figure 7. Pure EHPT SAMs were not evaluated in these long-term experiments due to their poor performance in the initial adhesion trials. 2:1 (v/v) MHA:EHPT SAMs were not significantly different ($p > 0.3$) from pure MHA and were significantly different



(a) (b) (c) (d)

Fig. 6. Images of PND 7 mouse cerebellar neurons at longer culture times. All images are 400x magnification with anti-tubulin stain. (a) , L1 with pure MHA at 1 DIV. Dispersed cell attachment with long neurite outgrowth. (b) , L1 with 2:1 MHA:EHPT at 2 DIV. Combination of clustered and disperse attachment, again with a few long neurites. (c) , L1 with pure MHA at 5 DIV. Condensation of cells into clusters, with increase in length and complexity of neurites. (d) , L1 with 2:1 MHA:EHPT at 5 DIV. Clusters and long branched neurites, very similar to pure L1 at 5 DIV. Data are not available for all days due to a limited availability of the necessary reagents (particularly purified L1) and the subsequent need to sacrifice different cultures on different days.

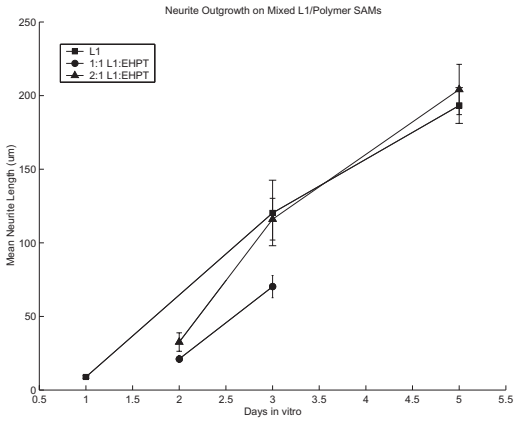


Fig. 7. Mean neurite lengths over five days of PND 7 mouse cerebellum culture on varying SAM compositions. 2:1 MHA:EHPT is significantly different ($p < 0.05$) from 1:1 MHA:EHPT over all days, but is not significantly different from L1 on pure MHA at any day ($p > 0.3$).

($p < 0.05$)from 1:1 SAMs after 2 DIV. Both 1:1 and 2:1 mixed SAMs continued to show polymer fluorescence under proper illumination.

IV. Discussion

This series of experiments indicates that conductive polythiophene coatings can be formed on metal surfaces similar to those found in brain implants, that biomolecules that promote neuron attachment can be incorporated into the coatings, and that neurons do in fact respond to the presence of those molecules by attaching and thriving. Together, these findings are promising for the development of more effective interfaces between the nervous system and external robotic devices.

A. Verification of SAM Formation

Self-assembly of an EHPT monolayer is clearly visible in Figure 2. This SAM is not as densely packed as SAMs of long-chain alkanethiols; this is to be expected, as EHPT chains are much longer and have side chains that presumably prevent dense packing. Loose packing is most likely acceptable for our purposes, since we will want to cover a significant portion of the surface with 16-MHA for protein coupling. If these mixed SAMs were to display phase separation, this would also most likely be tolerable, since a clustered distribution of adhesion molecules has been shown to be more effective than even dispersal [12]. Moreover, increased SAM regularity and a defect-reduced packing is probably possible by forming the SAM at elevated temperature. It is notable that the molecules in the SAM appear to be normal to the surface rather than flexing and lying flat. This verifies that we have formed the desired brush structure that will allow the polymer to bridge the cell-electrode gap and carry current directly to the cell.

B. Verification of Protein Incorporation

As noted above, the mixed SAM shows an emission behavior that is approximately intermediate between the two components. The 1:1 mixture more closely resembles the MUA than the HPT SAM. This is to be expected; at 20 mg/mL of each solution, the alkanethiol is at a much higher molarity and should comprise a majority of the mixed SAM. However, the decreased protein fluorescence of the 1:1 SAM compared to the MHA SAM also indicates that the polymer is able to compete successfully and incorporate into the SAM in significant quantities. These measurements verify that the adhesive biomolecule L1 can be coupled to the SAM in future experiments in order to improve neuron adhesion to the coating and produce a better electrode.

It is interesting that the HPT does not fluoresce brighter than the FITC-conjugated protein in the red channel, although it is still brighter than the bare gold (measured at roughly 345 counts per second). It is well known that fluorescence is quenched in general near metal surfaces due to dissipation of molecular excitation through the metal. This quenching effect may be particularly strong for a conductive polymer that is directly bound to the metal.

C. Neuron Attachment and Outgrowth on Mixed SAMs

There is a clear and statistically significant change in cell attachment and phenotype with increasing L1 concentration. The precise threshold for the observed sudden increase in attachment is yet to be determined. However, this dose-response behavior for both attachment and outgrowth is a strong indicator that L1 is in fact coupling to the MHA of the SAMs and promoting attachment and survival of the cultured neurons. Moreover, this type of thresholded behavior has been observed with other cell types on artificial peptide-coated surfaces [12]. 2:1 (v/v) mixtures of the SAM components appear particularly promising, since the cells on these SAMs appear to behave as though they were on a pure surface of coupled L1, but with polymer present in quantities that may still be sufficient to improve electrode performance. We can be fairly certain that this is a true covalent coupling and not a simple adsorption, as most adsorbed proteins will have been displaced from the surface after five days in cell culture conditions.

Although the results reported here only extend to 5 DIV, there is no indication that the cells cannot survive longer than that as long as the medium is appropriate. Our unpublished prior experience with cerebellar cultures includes preparations that have survived several weeks *in vitro*. The principal determinant of cell survival in our experiments was sterility; cultures that did not survive to or beyond 5 DIV failed due to microbe growth, presumably due to exogenous contamination. Far longer trials would obviously be needed before any future device could be used *in vivo*.

D. Future Directions: Towards the Neuro-Robotic Interface

We have demonstrated the formation of mixed SAMs of polymer nanowires and adhesion proteins and verified that primary neurons will adhere to these SAMs for sufficient periods of time to become electrically active. If electrodes with these same coatings are implanted into a living animal or used to communicate with cells in a culture, we expect that they will be able to produce higher-quality recordings and to stimulate the neurons without damage. Both of these capabilities are necessary in order to produce a high-bandwidth bidirectional interface that will allow effective control of external robotic devices and delivery of high-resolution sensory information to the neural circuitry.

The logical next step towards this ultimate goal is verification that these nanowire coatings improve cell-electrode contact and produce better stimulation and recording performance. We expect to carry out such studies in the near future using commercially available electrode arrays designed for *in vitro* electrophysiology. The work will begin with basic impedance spectroscopy, then proceed to stimulation and recording of cells *in vitro*. Potential performance metrics include noise level of recorded signals and current amplitudes necessary to trigger action potentials in stimulated cells.

Once this improved communication strategy is demonstrated, we can either move on to implantation of coated electrodes in a living animal or attempt to control a robot through the behavior of neurons cultured on those same *in vitro* arrays. Such work would essentially follow the same pathway as has been previously demonstrated with existing neural probes and *in vitro* networks [1], [2]. The goal would merely be to demonstrate that the use of these new polymer monolayer coatings produces superior performance over presently available technology.

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