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Self-assembled monolayers of alkanethiols on gold modulate electrophysiological parameters and cellular morphology of cultured neurons

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Abstract

Self-assembled monolayers (SAMs) of ω-substituted alkanethiols on gold have been explored as well defined in vitro model surfaces for the investigation of neuronal growth and function. When used as cell culture substrates, surfaces with monolayers functionalized with terminal $-$COOH groups support neuron attachment and growth even without an intermediate protein layer. Addition of a poly-L-lysine layer (PLL) to the $-$COOH terminated monolayers significantly increases total neurite outgrowth. Mixed monolayers containing $-$COOH and $-CH_3$ terminal groups in 1:10 and 1:100 ratios poorly support neuron adhesion and preclude neurite extension. A layer of PLL improves the ability of mixed monolayer surfaces to support neuronal growth in culture. The morphology of cultured neurons depends on the chemical composition of SAMs on the support surface. Using glass microelectrode intracellular recording, the properties of cell culture substrates modulate the dynamic properties of action potentials of cultured neurons. These findings provide insight into the cellular responses of excitable cells to the chemical details of a surface and, thus, may help direct the rational design of biologically active materials.

Keywords: Aplysia; Cell culture; Cell morphology; Electrophysiology; Neural cell

1. Introduction

What is the role of substrate chemical structure in the regulation of nerve cell function? This question is relevant to understanding cell adhesion and growth, nerve regeneration, signal transduction, biocompatibility, and modeling of biomaterial performance. While many aspects of cell–substrate coupling would better be understood if studied in vivo, the structural and chemical complexity of the mammalian nervous system precludes detailed investigation of how individual factors and mechanisms combine to form an integrated cellular response. Such constraints are largely circumvented in cell culture, which make in vitro culture models attractive for studying fundamental neurobiological problems.

Investigations of cell–surface interactions in culture, however, require a reliable way of preparing model substrates with defined properties of a biocompatible material support. Self-assembled monolayers (SAMs) of alkanethiols on gold offer an attractive route to the preparation of model surfaces with spatially and temporally tunable chemical properties, including biocompatible surfaces [1]. Close packing of the SAM molecules assures that the properties of the SAM surfaces are dominated by the terminal functional groups present at the liquid–solid interface. For example, variation of the reactive end-group can change the hydrophobicity and electrical charge of the monolayer, which is believed to strongly influence cell adhesion [2]. SAMs may also be terminated with reactive functionalities and thereby form the starting point for

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chemical immobilization strategies for chemical coupling of proteins via covalent binding from solution [3]. Although SAMs may be rendered biocompatible, the adsorbates used for SAM formation are themselves typically foreign to the in vivo cell environment and may, therefore, induce unexpected physiological responses. Cellular morphology and strength of adhesion have been shown to be strongly influenced by the composition of the underlying monolayer [4]. For a given surface, cellular adhesiveness also appears to be highly dependent on a number of parameters, including cell type and the details of medium composition [5]. Therefore, cell function data obtained using functionalized SAM substrates must be carefully validated for its relevance to different model systems.

We take advantage of the more simply organized central nervous system (CNS) of the marine mollusk, *Aplysia californica*, whose neurons are relatively large, easily identifiable and robust in culture [6–8]. To gain insights into mechanisms underlying the cellular response from excitable cells to varied surface properties, we analyze how SAM composition alone, and with an added protein layer, affects the morphology and electrophysiological parameters of cultured *Aplysia* bag cell neurons (BCNs). For surface modification, we use a common synthetic protein, PLL, known to promote the adhesion and growth of many cell types in culture [9,10]. The PLL molecules have been shown to bind to the −COOH terminated surface of a mercaptoundecanoic acid (MUA) monolayer in the extended conformation, in which some free lysine residues are available for interaction with molecules other than MUA [11]. Thus, multilayer constructs consisting of MUA and PLL should present multiple positive charges on free amino groups in the PLL side chain, which would be available to interact with the negatively charged cell membrane, thereby providing non-specific cell adhesion to PLL-coated SAM surfaces. Our findings suggest that the detailed composition and manner of construction of the support surface are directly linked to modulation of the electrophysiological parameters and cellular morphology of cultured neurons. Potential mechanisms mediating the observed physiological changes are discussed.

2. Materials and methods

2.1. SAM preparation

A thin layer of Au (30 nm) with an intermediate thin (~1 nm) adhesive layer of titanium, was produced by vacuum evaporation on 18 mm square glass coverslips pre-cleaned in piranha (4:1 H_2SO_4:H_2O_2) solution (CAUTION: *Piranha is a vigorous oxidant and should be used with extreme caution*). Gold-covered substrates were stored under N_2 until use. Prior to thiol assembly, the gold surfaces were cleaned with O_3 for 30 min and rinsed with ethanol. O_3 was generated upstream of the samples by illuminating the air with an Hg pen lamp. Cleaned film samples were then immediately soaked in 1 mM ethanolic thiol solution for 1 h, after which they were rinsed with, and stored in, EtOH until use. SAMs of varying densities of the terminal functional groups were formed from the following thiol solutions: 11-MUA, a 1:10 mixture of MUA and octanethiol (OT), and a 1:100 mixture of MUA and OT. It is important to note that the ratio of MUA to OT in solution may not necessarily translate to the same mixture of functional groups in the monolayer [12]. For PLL (MW ~150,000–300,000) attachment, SAM surfaces were exposed to an aqueous solution of 75 mM 1-ethyl-3,3-(dimethylaminopropyl)carbodiimide (EDC) and 15 mM *N*-hydroxysuccinimide (NHS) for 1 h [13], followed by 2 h incubation in 0.01% PLL solution in artificial sea water (ASW: 460 mM NaCl, 10 mM KCl, 10 mM $CaCl_2$, 22 mM $MgCl_2$, 26 mM $MgSO_4$, 10 mM HEPES, pH 7.8) at pH 7.8. Unreacted surface succinimidyl esters were removed by 15-min incubation of films in 0.025 M NaOH [11]. Surfaces were subsequently rinsed with distilled H_2O and ASW and used as cell culture substrates.

2.2. Cell culture bioassay

Specimens of *Aplysia californica*, weighing 30–120g, were obtained from the Aplysia Research Facility (Miami, FL, USA) and maintained in a tank with circulating artificial seawater (Instant Ocean; Aquarium Systems, Mentor, OH) at 14 °C until use. BCNs were mechanically dissociated from the bag cell clusters of the *Aplysia* CNS after 3-h incubation in 1% protease (Type IX: Bacterial; Sigma, St. Louis, MO) at 34 °C. Individual BCNs, 10–15/0.5–1 cm^2 SAM film, were plated and maintained at room temperature for several hours and then at +14 °C for 3–7 d in ASW-antibiotic solution. For comparison, BCNs were cultured on regular glass coverslips incubated in PLL solution identical to that used for PLL SAMs. The cultured neurons were photographed on day 3 of each experiment using either a Zeiss Axiovert 100 inverted microscope (Carl Zeiss, Inc., Thornwood, NY, USA) equipped with a *Coolsnap fx* color camera (Roper Industries, Inc., Duluth, GA, USA) or a Zeiss Axiovert 25 equipped with a Zeiss Axiocam MRC color camera (Carl Zeiss Inc., Göttingen, Germany). Images were adjusted for brightness/contrast in Adobe Photoshop 6.0 (Adobe Systems, Inc., San Jose, CA, USA). Neurolucida and StereoInvestigator software (MicroBrightField, Colchester, VT, USA) were used to assess the cell morphology. Statistical comparisons were made between all SAM surfaces versus the PLL on glass surface with one-way ANOVA followed by Dunnet test for multiple comparisons using GraphPad InStat 3 software (GraphPad Software, San Diego, CA, USA).

2.3. Electrophysiology

Intracellular recording and stimulation were made with glass microelectrodes (8–15 MΩ) pulled from 1 mm borosilicate glass capillaries (WPI, Sarasota, FL, USA) and filled with 506.2 mM KCl, 5 mM HEPES solution (pH 7.6). Signals were amplified with an AxoClamp 2B amplifier (Axon Instruments, Union City, CA, USA), monitored and stored on a PC using a Digidata® 1322A D/A–A/D converter (Axon Instruments). The software package pClamp 8 (Axon Instruments) was used for data acquisition and analysis. Records were digitized at 5.1 kHz. After microelectrode impalement, membrane potential (MP) was measured and manually clamped at −40 mV using the current clamp mode. Action potentials (APs) were stimulated in normally silent cultured BCN by current injection (thirty 58.5 ms 0.5–1.0 nA square pulses, 132.6 ms interpulse interval). All experiments were performed at room temperature (23–24 °C). Results were analyzed using Student's *t*-test and single factor ANOVA incorporated into Excel (Microsoft Corp., Redmond, WA, USA).

3. Results

3.1. Nerve cell growth on SAM surfaces

We explored the application of SAM surfaces with various densities of active ligand −COOH [14] as substrates for culturing neurons. We found that only hydrophilic MUA substrates support BCN attachment and