Improving Neuron-to-Electrode Surface Attachment via Alkanethiol Self-Assembly: An Alternating Current Impedance Study

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Received March 29, 2004. In Final Form: May 30, 2004

In this work, the ω-amine alkanethiols, cysteamine (CA) and 11-amino-1-undecanethiol (11-AUT), were chemisorbed as self-assembled monolayers (SAMs) onto 250 μm gold microelectrodes that were micro- lithographically fabricated within eight-well cell culture plates and investigated as a means to improve neuron-to-electrode surface attachment (NESA). Dynamic contact angle (DCA) measurements showed similar advancing, θa (69° and 65°), but contrasting receding contact angles, θr (9 and 30°) for CA- and 11-AUT-SAMs, respectively. The corresponding hysteresis (Δθ = 60 and 35°, respectively) indicates the CA-SAM displays greater amphiphilic character than the 11-AUT-SAM. A portion of the greater Δθr for CA-SAMs may arise from surface heterogeneity, as compared to sputter-deposited gold and 11-AUT-SAMs. Tapping mode atomic force microscopy (AFM) confirmed a 6% increase (CA-SAM) and a 22% decrease (11-AUT-SAM) in surface roughness when compared to clean but unmodified, sputter-deposited gold. The extracellular matrix cell adhesion proteins, collagen, fibronectin, and laminin, were covalently coupled to the aminoalkanethiol-decorated gold electrodes via acid-amine heterobifunctional cross-linking. Using fluorescein isothiocyanate-tagged laminin, confocal fluorescence microscopy of both CA- and 11-AUT-SAM-modified and unmodified gold microelectrodes confirmed coupling of the protein to the electrode and was readily distinguishable from nonspecifically adsorbed protein. DCA measurements of laminin–modified and CA- or 11-AUT-SAM had similar advancing (ca. 63° and 65°) and receding (ca. 7°–9°) contact angles. Tapping mode AFM of these protein-bearing surfaces likewise showed dimerized protein aggregates of similar surface roughness. PC-12 cells cultured to confluence on both unmodified and SAM-modified, protein-derivatized gold microelectrodes were examined by alternating current impedance (50 mV p–p at 4 kHz). CA- and 11-AUT-SAM-modified surfaces when serving as a foundation or covalently immobilized adhesion proteins produced highly stable and reproducible temporal impedance responses. On the basis of the magnitude and the reproducibility of the impedance responses, the CA-SAM-modified surfaces were identified as being best suited for optimal neuron-to-electrode contact with laminin. Laminin performed best when compared to collagen and fibronectin. Covalent immobilization of the adhesion-promoting proteins results in enhanced NESA by tightly anchoring cells to the electrode.

Introduction

Traditionally, toxicity assessments involve animal studies, which is both time-intensive and costly.1 More importantly, the use of animals in research when alternative technologies exist may be socially objectionable. This creates opportunities for the development of cell-based, high-throughput screening techniques that may be applied to toxicity assessments and drug development. Cell-based biosensors are a key component in the development of practical methods for the screening of drugs for possible toxic side effects and for the monitoring of the effects of biochemical warfare agents, thus, minimizing the use of experimental animals. Our laboratory has long-standing interest in the development of a cytotoxicity biosensor based on differentiated neuronal stem cells (ES J 1) and pheochromocytoma cells (PC-12) that may emulate components of the central nervous system.2 Cell-based biosensors hold out the promise of presenting the intact sub-cellular machinery of the living cell within a diagnostic or monitoring device format—a “cellular sentinel”. However, interrogating environmentally induced changes to the physiologic responses of whole cells in a device format is by no means a trivial matter. Cells are active, dynamic entities, not given to permanent localization on a device to allow easy interrogation. The current manuscript represents a significant contribution to the generally emerging area of cell-to-electrode interactions that are important in the development of biosensors and prosthetic devices.

Introduction

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Advanced instrumentation has allowed electrical impedance techniques to be used to study the electrical properties of anchorage-dependent cells in culture. In this approach, cells were cultured on microelectrode arrays (MEAs) and the metastability/viability/electrophysiology of the cells was electrically detected and monitored. The interaction and spreading of cells on the electrode surface results in a significant change in the measured impedance of the electrodes. Impedance changes, when measured as a function of time (over minutes to hours), are mainly the result of the attachment and motion of cells on the electrodes, although electrophysiologic responses reflecting ion permeability changes within the cell walls may also contribute to the observed impedance. These changes continue as the cell layers become confluent.

Previous studies of the electrical impedance characteristics of anchorage-dependent cultured cells are represented by the work of Giaever and Keese and Connolly et al. Giaever and Keese cultured human lung fibroblasts in a modified tissue culture dish that allowed measurement of cell-to-electrode impedance with a lock-in amplifier. Connolly et al. used the methods of Giaever and Keese with a few modifications. In 1989, Connolly et al. fabricated electrodes on a standard Petri dish, which allowed a culture of cells on half of the dish while “control” electrodes without cells were maintained in the other half of the dish. They observed that cell attachment could be significantly improved by increasing surface roughness, resulting in improved electrical signal capture.

Many studies since Connolly et al. have investigated different designs pertaining to the combination of neuronal cells and electrodes. Maher et al. and Gross et al. coated electrodes, via physical adsorption, with extracellular matrix (ECM) proteins, which induced secretion of neuron adherence molecules that act as receptors in the neuron cell membrane and offer a wider range of potential adhesive interactions. Recently, three other groups using different electrode structures have performed impedance studies of anchorage-dependent cultured cells. Wegener et al. described an electrode system similar to that of Giaever and Keese that utilized a voltage divider technique for determination of the unknown impedance across a range of frequencies (1 Hz to 10 kHz). Ehret et al. described multiple interdigitated electrodes (IDEs) that allowed measurement of eight IDEs in parallel, as well as demonstrated that time-dependent cellular membrane destruction by toxic concentrations of cadmium could be detected. Highly stable self-assembling molecular layers (SAMs) have been applied to the development of electrochemical detection systems and chemical sensors and for controlling the wetting behavior of surfaces. Self-assembled monolayers based on alkanethiols are the result of chemisorption of single ion layers, which forms thiolate molecules to adsorb commensurate with the gold lattice. The combination of organo-sulfur compounds and gold has been well-established, especially those leading to the formation of alkanethiol SAMs, because they are easy to prepare and give rise to highly stable and reproducible close-packed structures. In addition, the surface properties of SAMs may be specifically tuned by varying the terminal functionalities of the monolayers as well as the molar composition of the starting alkanethiol solution. Alkanethiols terminated with reactive functional groups, such as amine and carboxylic acid, are relevant to the effort to find a good model for neuron-to-electrode contact because of the potential to covalently link to proteins. The specific functions of the terminal groups have been used to control surface properties and immobilize biomolecules on the monolayers, thus increasing the selectivity of protein adsorption. SAMs have been extensively used to control the adsorption of proteins and to promote the attachment of mammalian cells to planar surfaces.

Against this backdrop, this work investigated fundamental aspects of cell–surface interactions and electrical cell signal responses related to the improvement of neuron-to-electrode contact with the goal of building cell-based biosensor systems suitable for detecting changes in the cell membrane impedance characteristics. Of course, neuron-to-electrode attachment is also vitally important in the performance of deep stimulating electrodes used in the treatment of Parkinson’s disease and is a concern in the performance of prosthetic devices such as retinal and cochlear implants. The prerequisite for monitoring the electrical impedance of cells is to have a tight neuron-to-electrode interface. This involves electrode materials selection and design, surface chemical modification strategies (chemically modified electrodes using organosilanes, laminin, polylysine, fibronecin, and bioactive peptide chain), and optimization of cell culture conditions. The SAM-forming alkanethiol molecules, cysteamine (CA) and 11-amino-1-undecanethiol hydrochloride (11-AUT), were studied as linking adlayers for the covalent attachment of the ECM adhesion proteins: collagen, fibronecin, and laminin. Here, the bifunctional molecular properties of HS–C6H4–NH2 and HS–C11H22–NH2 allow the sulfur headgroup to bind to the gold surface while the ω-amino group could be used for the covalent immobilization of the several target proteins. Moreover, these SAMs contain the desired charge, polarization, or chemical functionalities that will effect intermolecular interactions such as van der Waals forces, hydrogen bonding, polar attractions, and hydrophobic interactions between the biological surface and the SAM.
In building such supramolecular structures on electrodes, it is vitally important to provide molecular and full characterization of the various steps involved in the assembly. Scanning tunneling microscopy (STM) and atomic force microscopy (AFM) are routinely employed to visualize surfaces of materials with the highest spatial resolution. Thus, molecular resolution images are now easily obtained via AFM, although STM has been used extensively for molecular imaging because of its capability of atomic and molecular resolution. In this work, we used tapping mode atomic force microscopy (TM-AFM) to visualize the surfaces of planar microelectrodes before and after surface modification and functionalization. Fluorescence tagging and subsequent imaging was used to confirm immobilization of proteins to the SAM-modified surfaces and to quantify immobilization. Dynamic contact angle (DCA) measurements were used to access the wetting characteristics of the bare gold, SAM-modified, and SAM-modified and protein-functionalized electrodes. Time-dependent alternating current (ac) impedance was used to evaluate the performance of the bare gold, SAM-modified, SAM-modified and protein-functionalized, and SAM-modified, protein-functionalized, and cell-bearing electrodes under cell culture conditions. Together, these techniques support the improved adhesion of PC-12 cells to the electrodes and confirm the improved impedance response as arising from improved neuron-to-electrode attachment.

Materials and Methods

Materials. Eight-well array cultureware (ECIS 8W1E) consisting of one active electrode (250-μm diameter) and one large area counter electrode (100 cm²) per well were purchased from Applied Biophysics (Troy, NY). Planar metal electrodes (PMES) of magnetron sputter-deposited gold (100 Å) on titanium tungsten (100 Å; 1 cm × 2 cm × 0.05 cm; PME-Au 118) and custom-prepared gold-coated 24 mm × 0.13 mm glass coverslips that were similarly magnetron sputter-coated on both sides were purchased from ABTECH Scientific, Inc. (Richmond, VA). CA, purchased from Sigma Chemical Co. (St. Louis, MO), along with 11-AUT, was used without further purification. All other chemicals and ECM proteins, collagen, fibronectin, and laminin, were obtained from Sigma Chemical Co. (St. Louis, MO). Water used was Milli-Q quality (Millipore, Bedford, MA).

Preparation of SAM-Modified Electrodes. The ECIS 8W1E cell culture arrays (illustrated in Figure 1) were functionalized with self-assembling CA and 11-AUT using conditions optimized for the formation of organized monolayers. The PME substrates were first prepared according to the surface cleaning instructions (parts A, B, and E) provided by ABTECH Scientific. The ECIS substrates were prepared by subsequent washing with sterilized Milli-Q water followed by UV sterilization. The PME substrates were prepared by transferring aliquots of deaerated (10 min with N₂) aqueous solutions of 2 mM CA or 11-AUT into the designated wells and allowed to adsorb for 3 h at room temperature. Following incubation, the solutions were removed by aspiration, and the electrodes were immediately and thoroughly rinsed with Milli-Q water. Typically, wells 1, 2, 5, and 6 were kept as controls (no surface modification), while wells 3 and 4 contained the CA-SAM and wells 7 and 8 contained the 11-AUT-SAM.

Fluorescein Labeling of CA- and 11-AUT-SAMs. To confirm the chemisorption of aminoalkanethiols onto the gold ECIS electrode surfaces, the amine-terminated CA- and 11-AUT-SAMS were functionalized by covalent coupling to fluorescein and visualized by confocal fluorescence imaging microscopy. The SAM-modified ECIS gold microelectrodes were functionalized with fluorescein (Scheme 1). The fluorescein coupling was achieved by using 6.5 and 2.13 μL of 10 mg/mL of FITC in 100% dimethylformamide (DMF) for the CA- and 11-AUT-SAMs, respectively, each in an equimolar solution of sodium bicarbonate buffer (50 mM at pH 8.5) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC; 50 mM), followed by continuous agitation in the dark at room temperature for 90 min. Finally, the electrodes were thoroughly washed with phosphate buffered saline (PBS) and stored in the dark at 20 °C until used for fluorescence microscopy.

Fluorescein Labeling of Laminin. Laminin was fluorescein-labeled with FITC according to instructions provided by Pierce.

Figure 1. Layout of the ECIS 8W1E chip consisting of 8 independently addressable micro-dot working electrodes, each 250 μm in diameter, with a large-area counter electrode. (Reprinted with permission from Applied Biophysics, Inc. Copyright 2004.)
bicarbonate buffer (pH 8.5) was incubated in 1.2

Briefly, a 1.0-mL laminin solution (1 mg/mL) in 50 mM sodium

protein-functionalized electrodes were stored in the dark at 20

laminin was thoroughly washed away with PBS, and the resulting

present) and protein functionalized by derivatization conditions

mention, wells 1, 2, 5, and 6 were not modified with SAMs.

Functionalization of Electrode Surfaces. Surface functionalization by derivatization with unlabeled ECM proteins (collagen, fibronectin, and laminin) and fluorescein-labeled laminin on both the CA- and the 11-AUT-SAM-modified and unmodified gold electrode surfaces was examined. This served to compare the performance of each of the separate proteins under cell culture conditions as well as compare the performance of proteins that were chemically coupled to SAM-modified gold electrodes with that of proteins that were directly adsorbed onto SAM-free gold electrodes. Dedicated eight-well plates, each comprising a combination of control electrodes and protein-functionalized electrodes, were used for each protein studied, and these were used in cell culture evaluations. To evaluate protein coverage and demonstrate protein coupling to these surfaces, separate eight-well plates were produced and functionalized by derivatization with FITC-labeled laminin. As mentioned, wells 1, 2, 5, and 6 were not modified with SAMs. Wells 1 and 5 were kept as dry controls (no surface modification), and wells 3 and 7 were FITC-labeled SAMs. Each of these wells (excluding wells 1, 3, 5, and 7) were then incubated under heterofunctional coupling conditions with 500 µL of 0.01 mg/mL fluorescein-labeled laminin via EDC and N-hydroxy succimide (NHS) and incubated for 2 h. This produced protein functionalization under adsorption conditions in wells 2 and 6 (no SAMs present) and protein functionalized by derivatization conditions in wells 4 and 8 (SAMs present). The unbound fluorescein-labeled laminin was thoroughly washed away with PBS, and the resulting protein-functionalized electrodes were stored in the dark at 20 °C until used for fluorescence microscopy.

Fluorescence and Optical Microscopy. For fluorescence microscopy, approximately 2 mM of CA and/or 11-AUT was chemisorbed to the appropriate gold electrodes followed by heterofunctional coupling of FITC-labeled laminin within the designated wells of eight-well-ECIS arrays that comprised an optically clear Lexan polycarbonate substrate. The fluorescence intensity of each 250-mm spot electrode was determined using a Packard Bioscience Biochip Technologies ScanArray Express with a blue (488-nm) internal laser with filters (514 nm) purchased from Perkin-Elmer, Inc. (Fremont, CA). Laser power and photomultiplier tube sensitivity were set at 80 and 70%, respectively. Images were acquired as single optical scan at 5-μm resolution. For all types of acquisitions, the intensity level of each microelectrode was measured by defining the spot and its diameter to optimize the dynamic range of the image. Quantitative data on the area of the fluorescently decorated microelectrode and the mean diameter of each microelectrode from the center of the microelectrode were obtained using the ScanArray Express software. Optical microscopy was performed with a Leica DM IRBE confocal inverted microscope purchased form Leica Mikroskopie und System GmbH (Wetzlar, Germany) with a Cohu 2200 Series charge-coupled device camera purchased from Cohu, Inc. (San Diego, CA). Images were converted to picture files.

TM-AFM. AFM measurements were made with a NanoScope IV multimode AFM fitted with a NanoScope IV controller and a Dimension CL scanner head (Veeco/Digital Instruments, Santa Barbara, CA). TM-AFM scans of the PMEs were performed in air with standard etched silicon cantilevers/tips (cantilever resonance frequency f₀ = 60–400 kHz). The free amplitude was kept constant for all experiments, and the amplitude damping (set point) ratio was adjusted to ~0.90. Sample (1 cm × 2 cm) PMEs were rinsed with Milli-Q water (18.2 MΩ) to remove any surface impurities prior to AFM.

DCA Measurements. Advancing and receding contact angles were measured on glass coverslips (24 mm × 60 mm × 0.13 mm; Corning no. 1; VWR Scientific) and were based on the Wilhelmy plate method. Coverslips were either flame-cleaned, cleaned with RCA I [1 part 30% H₂O₂:1 part concentrated H₄O₄:4 parts deionized (DI) water], cleaned and magnetron sputter-deposited with gold (both sides), and had the gold modified with CA- and 11-AUT-SAMs (both sides) or were gold-modified with CA- and 11-AUT-SAMs and then heterofunctionally coupled to laminin (both sides). The instrument used was a DCA analyzer (model

Scheme 1. Schematic Diagram of the CA and 11-AUT Surface Modification and Derivatization

- The left portion of the diagram shows the general structure of CA and 11-AUT, with –NH₂ representing the amine terminal used in the coupling of fluorescein. The right portion of the diagram illustrates the amine group of CA and 11-AUT being coupled to fluorescein via the isothiocyanate.
DCA-322; Cahn, CA) operating at 100 m/s. Wettability measurements were made using organic-free Milli-Q quality water (18.2 MΩ cm⁻¹; γ = 72.9 dyn/cm). The water was changed between samples to avoid possible cross-contamination during the course of DCA measurements. Surface contamination changes the apparent water surface tension and affects the force-distance curves (fdc's). Analysis of the water using flame-cleaned glass coverslips confirmed the absence of contamination in the water.

**PC-12 Cell Cultures.** The PC-12, rat pheochromocytoma cells (CRL 1721), were cultured in a T-50 culture flask in RPMI 1640 medium (Cellgro) supplemented with 25 mM N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid and 2 mM L-glutamine from Mediatech, Inc. (Herndon, VA), for 10–14 days. The medium also contained 10% fetal bovine serum, 8.5% horse serum, penicillin (100 U/mL), streptomycin sulfate (100 U/mL), fungizone (1.25 μg/mL), and 1% sodium pyruvate that were all obtained from Fisher Scientific (Malvern, PA). Cells were grown at 37 °C in a humidified atmosphere of 95% air and 5% CO₂ and subcultured every 2–3 days at 80% confluency. Cells were plated in the modified ECIS cultureware at a typical density of 2 × 10⁶ cells/mL in wells 2–7, with wells 1 and 8 containing the medium only followed by impedance interrogations for 32.4 h.

**Impedance Measurements.** Impedance measurements were performed using a model 1260 impedance/gain-phase analyzer (Schumberger Technologies, Huston, TX) that was interfaced through an HP 34970A data acquisition/switch unit (Agilent Technologies, Palo Alto, CA) that allowed multiplexed addressing of the eight separate electrodes of the array. Arrays were placed in a model 3546 incubator (Forma Scientific, Marietta, OH). Electrodes were subjected to a 4000-Hz interrogating sine wave of 50 mV p-p voltage. The impedance of each electrode was monitored over time.

**Measurement Procedures.** For any condition studied, the impedance of all eight dry, unmodified gold electrodes was first measured using 500 μL of fresh medium containing no serum. After the unmodified or bare impedance was obtained, the ECIS cultureware electrodes were thoroughly washed, subsequently modified with CA and 11-AUT, and then functionalized by conjugation to 0.01 mg/mL ECM protein in the designated wells in the presence of EDC and NHS. Impedance interrogation was then performed for about 2 h at 37 °C. The solution was subsequently removed by aspiration, and the cell culture wells were washed thoroughly before 500 μL of fresh medium without serum (that was prewarmed at 37 °C) was added to each well and further incubated at 37 °C for 30 min. Again, the medium was removed by aspiration, and 500 μL of cell suspension was added to the now modified and functionalized ECIS cultureware at a density of approximately 2 × 10⁶ cells/mL (as determined using a hemacytometer). Cells were added to wells 2–7 while wells 1 and 8 contained medium only. This was followed by impedance interrogation at 37 °C for 32.4 h. In addition, the affinity of PC-12 cells on laminin, fibronectin, and collagen on SAM-modified surfaces was examined to determine which protein leads to the optimal condition for the improvement of neuron-to-electrode contact. Results, expressed as mean ± standard deviation (SD), were calculated. Differences between the corresponding well groups from chip to chip were analyzed by Student’s t-test and ANOVA. Differences were considered significant for P < 0.05. The impedance profiles (50 mV p-p; 4 kHz) obtained from six separate dry ECIS 8W1E biochips were used to assess the chip-to-chip and within-chip electrode variability. Impedances divided into two groups with no significant differences within each group but with all “between-group” differences being significant. Shown in Table 1, the average real component of the impedance and

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**Scheme 2. Schematic Depiction of Laminin Labeling and SAM Surface Derivatization**

A (A) The upper portion shows a general conjugation reaction of FITC to laminin, with –NH₂ representing the amine side group of lysine residues used in the coupling of fluorescein. (B) The lower portion shows the chemical coupling of laminin through the –COOH Groups of glutamic acid residues to CA- and 11-AUT-SAMs via EDC:NHS.

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Table 1. Average Impedance and Calculated RMS Noise for the Separate Electrodes of an ECIS 8W1E Chip

<table>
<thead>
<tr>
<th></th>
<th>well no. 1</th>
<th>well no. 2</th>
<th>well no. 3</th>
<th>well no. 4</th>
<th>well no. 5</th>
<th>well no. 6</th>
<th>well no. 7</th>
<th>well no. 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average Z_{Re} (M\Omega)</td>
<td>2.38</td>
<td>5.29</td>
<td>4.75</td>
<td>6.06</td>
<td>2.64</td>
<td>4.10</td>
<td>5.54</td>
<td>5.15</td>
</tr>
<tr>
<td>RMS noise (M\Omega)</td>
<td>±0.02</td>
<td>±0.17</td>
<td>±0.18</td>
<td>±0.26</td>
<td>±0.03</td>
<td>±0.15</td>
<td>±0.30</td>
<td>±0.15</td>
</tr>
</tbody>
</table>

Table 2. DCA Measurements\(^a\) of Various Blank, Control, Modified, and Derivatized Substrates Performed in DI Water at 20 ± 1 °C

<table>
<thead>
<tr>
<th></th>
<th>RCA glass</th>
<th>Au</th>
<th>CA-SAM</th>
<th>11-AUT-SAM</th>
<th>L-Au</th>
<th>L-glass</th>
<th>L/CA-SAM</th>
<th>L/11-AUT-SAM</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\theta_a)</td>
<td>93 ± 1.5</td>
<td>69 ± 4.1</td>
<td>65 ± 1.8</td>
<td>65 ± 3.3</td>
<td>39 ± 0.3</td>
<td>63 ± 0.9</td>
<td>63 ± 3.7</td>
<td>63 ± 3.7</td>
</tr>
<tr>
<td>(\theta_r)</td>
<td>59 ± 0.5</td>
<td>9 ± 1.2</td>
<td>30 ± 0.5</td>
<td>9 ± 0</td>
<td>11 ± 0.6</td>
<td>8 ± 0.6</td>
<td>7 ± 0.2</td>
<td>7 ± 0.2</td>
</tr>
<tr>
<td>(\Delta \theta_{ar})</td>
<td>34 ± 1.0</td>
<td>60 ± 2.9</td>
<td>35 ± 1.3</td>
<td>56 ± 3.3</td>
<td>28 ± 0.3</td>
<td>55 ± 0.3</td>
<td>56 ± 3.5</td>
<td>56 ± 3.5</td>
</tr>
</tbody>
</table>

\(^a\) Average of triplicate cycles performed on triplicate samples.

Results and Discussion

SAM Formation and Characterization. Amineterminated alkanethiol gold functionalization was selected for the relative ease with which covalent attachment to the carboxylic acid functional groups of glutamic acid residues, widely abundant in most adhesion proteins, could be achieved. The impedance magnitude obtained from bare gold electrodes and those obtained from electrodes that were modified with CA- and 11-AUT-SAMS were examined. Impedances were obtained in the same cell culture media although from different wells. The effect of the alkanethiol surface modification via SAM formation did not produce a significant difference in the magnitude of the overall electrical network impedance. This likely indicates that both SAM-forming alkanethiols do not form fully contiguous passivating layers on the electrodes that would otherwise give rise to significant impedance changes. Such passivating layers are formed by C\(_{16}\) alkanethiols.\(^{46}\)

Direct fluorescent labeling of CA- and 11-AUT-SAMs achieved by direct reaction of FITC to the free amines of these SAMs gave image intensities that confirmed the disordered packing behavior of the CA-SAM. Shown in Figure 2 (images 3 and 7), these reveal intensities for the FITC-labeled 11-AUT-SAM. We achieved by direct reaction of FITC to the free amines of these SAMs and 11-AUT-SAMs. Fluorescence microscopy of unmodified substrate and fluorescein-labeled CA- and 11-AUT-SAMs incubated with fluorescein-labeled laminin. The fluorescein-labeled laminin was pretreated with EDC and then added to the SAM-modified ECIS culture wells followed by incubation at 37 °C for 2 h.

The advancing \(\theta_a\) and receding \(\theta_r\) contact angles of pure water at the various substrate surfaces are summarized in Table 2 and the corresponding representative data set corresponding to three dipping cycles or fdc's are shown in Figure 3. The RCA-cleaned and flame-dried glasses, as well as the bare gold substrate, all conform to expected values of advancing and receding contact angles.\(^{47}\)

The flame-dried glass and RCA-cleaned glass produced an almost perfect wetting situation with \(\theta_a\) and \(\theta_r\) being 0 and with the water surface tension of 72.9 and 72.1 dyn/cm, respectively. The advancing contact angles of CA- and 11-AUT-SAMS on gold are quite similar (69±4° and 65±2° in columns 4 and 5 of Table 2) but quite different from that of bare gold (93±2°) and vastly different from that of glass (0°). Of course, the SAM formation on the gold electrode is expected to result in the presentation of amine functionalities to the water–electrode interface. The difference between the advancing \(\theta_a\) and \(\theta_r\) contact angles is described as hysteresis.\(^{48}\) Hysteresis is mainly influenced by surface roughness, chemical heterogeneity, surface deformation, surface-configuration changes, and adsorption and desorption of the sample surface, all of which act as barriers.\(^{49}\) In general, it is a measure for the homogeneity of the surface. This difference is larger for the CA-SAM (60°) than the 11-AUT-SAM (35°), suggesting a more patchy distribution of CA-SAM islands on the gold electrode.

TM-AFM of the SAM-modified gold surfaces was utilized, and the resulting images were compared to bare magnetron sputter-deposited gold. Figure 4 shows sets of the high-resolution images (intensity left and topographic right) of a clean, sputter-deposited, gold PME (A) and similar electrodes that were modified with CA- (B) and 11-AUT- (C) SAMs. The topological features of these surfaces were visualized in striking detail. The planar...
A gold electrode has a mean roughness ($R_a$) of 0.95 nm and grain size of about 20 nm. The structural features of CA-SAMs are similar to those observed for 11-AUT-SAMs. Particularly, the dark areas that represent the depressions formed as a result of chemisorption of CA- or 11-AUT-SAMs. The amino groups in CA- or 11-AUT-SAMs contribute to the formation of a hydrogen-bonded network between CA molecules or 11-AUT molecules, thus, resulting in the formation of a rigid CA or 11-AUT monolayer. The CA-SAM has a slightly rougher surface with a $R_a$ of 1.0 nm, a modest increase of 6% in surface roughness compared to unmodified gold. The 11-AUT-SAM has a smoother surface with a $R_a$ of 0.78 nm. This represents a 22% decrease in surface roughness. It is expected that, as a result of increased secondary interactions and $-\text{CH}_2-$ packing, the 11-AUT forms a more coherent and contigu-

Figure 3. Typical DCA fdc’s for (A) RCA cleaned glass, (B) cleaned, sputter-deposited Au PME, (C) CA-SAM|Au, (D) 11-AUT-SAM|Au, (E) L|CA-SAM|Au, and (F) L|11-AUT-SAM|Au.

Figure 4. High-resolution TM-AFM morphology (height, left) and phase topographs (contrast, right) of prepped and modified gold surfaces (500 nm × 500 nm scan). (A) Cleaned sputter-deposited Au PME, (B) 11-AUT-SAM|Au and (C) CA-SAM|Au.
prior to cell culture. A linkage between the electrode and the protein. In this protein to the electrode and so give rise to a strong covalent and 11-AUT-SAM-modified electrode surfaces wherein thus, indicating that the proteins were simply physisorbed washing in our experiments with unmodified gold surfaces, been observations of loss of protein following subsequent ac current upon introduction of biomolecules or cells. A similar observation of changes in coverage of a small electrode to associated with L \( \text{CA-SAM} \) and 11-AUT-SAM layers. (A) L\([\text{CA-SAM}]\)Au and (B) L\([11-\text{AUT-SAM}]\)Au in the cell culture medium.

| Figure 5. Impedance profile of laminin (L)-derivatized gold electrodes coupled through CA- and 11-AUT-SAM layers. (A) \( [\text{CA-SAM}] \)Au and (B) \( [11-\text{AUT-SAM}] \)Au in the cell culture medium. |

mOUS SAM, thus, leading to a more homogeneous surface when compared to CA, although both appear not to be electrochemically passivating, and so this is consistent with a decrease in surface roughness. Many different features such as, size, shape, and distribution of depressions were also noticeably visible in the AFM topologies. These marked differences in the packing behavior of the twoSAM systems, where the smoother surface is ascribed to the more densely packed monolayer of 11-AUT, were, thus, the templates for attaching ECM proteins.

**Protein-Derivatized SAMs and Characterization.** The high incidence of carboxylic acid groups of ECM proteins suggests that they may be readily coupled to surface amines for immobilization. The most abundant amino acid residue in laminin, fibronectin, and collagen is glutamic acid (122/1000, 116/1000, and 102/1000, respectively) with its \(-\text{COOH}\) side group. There have been observations of loss of protein following subsequent washing in our experiments with unmodified gold surfaces, thus, indicating that the proteins were simply physisorbed to the surface of the electrode and may be washed away. This dynamic may be quite different in the case of CA- and 11-AUT-SAM-modified electrode surfaces wherein amine-terminated SAMs serve to irreversibly couple the protein to the electrode and give rise to a strong covalent linkage between the electrode and the protein. In this way, nonspecifically adsorbed protein may be washed away prior to cell culture.

| Electrical Impedance. | Impedance spectroscopy allows observation of changes in coverage of a small electrode to ac current upon introduction of biomolecules or cells. A measurable difference in the magnitude of the impedance was observed for the six wells (2–7) functionalized with CA-SAM and derivatized with laminin (L\([\text{CA-SAM}]\)Au). While a CA-SAM in the cell culture medium showed no difference from bare gold, the covalently coupled L\([\text{CA-SAM}]\)Au showed an increase in impedance magnitude that was about 390 \( \Omega \) \([2.51 \text{k}\Omega \text{ (L\([\text{CA-SAM}]\)Au from 2.12 k}\Omega \text{ (Au)}])\). A similar observation was made for all four wells (1, 2, 5, and 7) functionalized with L\([11-\text{AUT-SAM}]\)Au. While an 11-AUT-SAM in the cell culture medium showed no difference from bare gold, the covalently coupled L\([11-\text{AUT-SAM}]\)Au also showed an increase in impedance magnitude that was about 290 \( \Omega \) \([2.41 \text{k}\Omega \text{ (L\([\text{CA-SAM}]\)Au from 2.12 k}\Omega \text{ (Au)}])\). That the impedance magnitude should increase is consistent with a layer that limits ion transport. That the more disordered CA-SAM should effect a larger increase in impedance magnitude than its corresponding more ordered and 11-AUT-SAM when coupled to laminin suggests that the coupling of laminin to the CA-SAM may be accompanied by conformational changes in the protein that expose hydrophobic segments to the electrode surface and hydrophilic segments to the aqueous phase. This correlates the receding contact angles, \( \theta_r \), of the CA- and 11-AUT-SAMs, which are 9 and 30°, respectively.

Confocal Fluorescence Imaging Microscopy. Confocal fluorescence imaging microscopy of FITC-labeled laminin that was subsequently covalently coupled to the SAM according to Scheme 2 is shown in Figure 2 (wells 4 and 8). The fluorescence intensity of the L\([\text{CA-SAM}]\)Au was about twofold that of its 11-AUT counterpart, suggesting that more FITC-labeled laminin was successfully coupled to the more disordered and hydrophilic CA-SAM. Indeed, some nonspecific protein adsorption of FITC-labeled laminin did occur, and this was evaluated in wells 2 and 6. The fluorescence intensities of these untreated wells were 8637 and 8692 counts, respectively, suggesting that nonspecific laminin adsorption was, first, quite uniform and, second, only about 25–50% of the corresponding derivatized signal.

Contact Angle Analysis. The flame-dried and RCA-deaned glass, as well as the bare gold substrate, all conformed to expected values of advancing \( \theta_a \) and receding \( \theta_r \) contact angles. Flame-dried and RCA-deaned glass were completely wetted \( \theta_a = \theta_r = 0 \) yielding \( \gamma = 72.9 \) and 72.1 dyn/cm, respectively, \( \theta_a \) and \( \theta_r \) for pure water at the L\([\text{CA-SAM}]\)Au and L\([11-\text{AUT-SAM}]\)Au surfaces are shown in columns 8 and 9 of Table 2. \( \theta_a \) and \( \theta_r \) for pure water at laminin adsorbed on clean, bare gold and laminin adsorbed onto RCA-deaned borosilicate glass appear in columns 6 and 7. The corresponding three dipping cycles of representative fdc’s are shown in Figure 3. Protein adsorption dramatically alters the wetting characteristics of gold, particularly \( \theta_a \), which remains constant during the three cycles at about 9°. The change in \( \theta_r \) is profound, falling from 93° (Au) to an average of 65°. The net effect of protein adsorption onto gold is to make the surface more hydrophilic but also more heterogeneous. The effect of protein adsorption onto the RCA-deaned glass substrate is similarly profound. \( \Delta \theta_r \) changed from 0 to 28°. The net effect of protein adsorption onto glass is to make the surface more hydrophilic and likewise more heterogeneous.

The preceding illustrates the amphiphilic character of laminin, with the ability to adsorb via polar segmental interactions with highly wettable surfaces such as glass, and in so doing renders such a surface more hydrophobic. Likewise, laminin adsorbed onto gold is able to interact via its dispersive segments, leaving the more polar segments available for interaction with the aqueous phase.

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and rendering such a surface more hydrophilic. Protein coupling to the CA-SAM and 11-AUT-SAM resulted in contact angles that were similar to that found on laminin physisorbed onto bare gold. This suggests that the segmental interaction of laminin with the CA-SAM and 11-AUT-SAM may be similar to its interaction with gold, dispersive, yet quite unlike its interaction with glass. There was no discernible difference in the DCAs of the L\(_\text{CA-SAM}|\text{Au}\) and L\(_\text{11-AUT-SAM}|\text{Au}\) surfaces.

Topographical Imaging. To supplement observations made by DCA, TM-AFM measurements were made on laminin-derivatized SAM surfaces and on laminin adsorbed to cleaned, sputter-deposited gold electrode surfaces. Figure 6 shows the AFM morphology and topographs obtained for laminin adsorbed onto gold (A), laminin covalently immobilized to the CA-SAM supported on gold (B), and laminin covalently immobilized to the 11-AUT-SAM supported on gold (C). There was no discernible difference in the morphology or topology of these protein surfaces. The surface roughness values obtained were L\(_\text{CA-SAM}|\text{Au}\) \(R_\text{s} = 0.97\), L\(_\text{CA-SAM}|\text{Au}\) \(R_\text{a} = 1.00\), and L\(_\text{11-AUT-SAM}|\text{Au}\) \(R_\text{s} = 0.60\). Consistent with previous studies of laminin by transmission electron microscopy, the diameters of laminin molecules are on average about 75–85 nm.\(^{53}\) There is clear indication of protein immobilization in all cases as the observed feature size (ca. 120–200 nm) corresponds to dimers of laminin. However, the final protein topologies appear to be uninfluenced by the underlying materials chemistry or the manner of immobilization.

**Cell Culture on Protein-Derivatized SAMs and Characterization.** For studies involving the attachment of PC-12 cells, cells were plated on the MEA that was treated according to the modification and derivatization procedures detailed previously. Previous neuron-to-electrode studies suggest that the absorption of ECM proteins aids in the neuron-to-electrode interface.\(^7\) The cell’s interactions with these modified 250-\(\mu\)m diameter electrodes were used to assess the electrical quality of the neuron-to-electrode interface, because the neuron-to-electrode interface correlates with tight cellular coupling to the electrode. We have demonstrated, using fluorescence labeling techniques, that laminin could be bound and retained by covalent attachment to the amine terminal of the CA-SAM and 11-AUT-SAM layers formed on gold microelectrodes. We have also demonstrated that aminolkanethiol surface modification aids in uniform immobilization of laminin to gold electrodes. We next evaluated the quality of the NESA of PC-12 cells cultured on the surface-modified electrodes using electrical impedance spectroscopy. PC-12 cells are inherently poorly adherent cells and require an adhesion-promoting protein to adhere and proliferate on substrates. This presents an opportunity to investigate the covalent immobilization of laminin to the surface of the Au electrodes to yield conditions that allow for tighter neuron-to-electrode contact. The impedance analysis was used to determine the optimum surface for monitoring changes in the membrane impedance of the cells.

Figure 7 displays the normalized, time-dependent profiles of the impedance magnitude for PC-12 cells cultured on (A) unmodified, cleaned, sputter-deposited gold electrodes onto which laminin was physisorbed and on the modified and derivatized gold electrode surfaces (B) L\(_\text{CA-SAM}\) and (C) L\(_\text{11-AUT-SAM}\). These profiles arise from interrogation via an impressed 50 mV p–t–p sinusoidal ac voltage at 4 kHz. Temporal profiles are the average of \(n = 6\) electrodes and are represented by the mean and \(\pm\) SD. Included in the profiles are the responses of two blank electrodes that did not contain PC-12 cells. The impedance profile for a cleaned, sputter-deposited, gold electrode that bore physisorbed laminin shows a noisy, erratic response with an early rapid increase in impedance for select wells that achieved a modest maximum of about 1.14 \(\pm\) 0.05 \(\Omega\) after 4.5 h. Beyond this early change (2–3 h), the longer-term impedance is indistinguishable from that of the controls, indicating that PC-12 cells did

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**Figure 6.** High-resolution TM-AFM morphology (height, left) and phase topographs (contrast, right) of prepped, modified, and derivatized gold surfaces (500 nm \(\times\) 500 nm scan). (A) Laminin physisorbed onto cleaned, sputter-deposited Au PME. (B) Laminin covalently immobilized to a CA-SAM|Au. (C) Laminin covalently immobilized to an 11-AUT-SAM|Au.

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not establish a tight electrical contact with the gold microelectrode.

The impedance profile for the L\textsubscript{CA-SAM}\textsubscript{Au} modified and derivatized gold microelectrodes displayed uniform, highly reproducible, maximum normalized impedance values of about $1.54 \pm 0.07$ $\Omega$ after 28.6 h. Moreover, the impedance rose in a sustained manner and was highly consistent within all electrode-bearing wells examined as evidenced by the tight SD ($n = 6$). This value was substantially higher than those observed for the interaction of cells on laminin physisorbed onto unmodified surfaces ($1.14 \pm 0.05$ $\Omega$) and also higher than values observed for interactions of cells on L\textsubscript{11-AUT-SAM}\textsubscript{Au} modified surfaces, which had a response maximum of $1.22 \pm 0.14$ $\Omega$ after 31 h. The impedance profiles resulting from L\textsubscript{11-AUT-SAM}\textsubscript{Au} modified and derivatized electrodes rose in a sustained manner but were highly inconsistent from well to well. It is noteworthy that both the L\textsubscript{CA-SAM} and L\textsubscript{11-AUT-SAM} produced similar sustained impedance profiles achieving maximal impedance at about the same time (28.6 and 31 h, respectively) but of different impedance magnitudes. We believe these represent PC-12 growth curves on the way to confluence and the magnitudes reflect the quality of the neuron-to-electrode attachment.

The neuron-to-electrode interface, as judged from impedance measurements, was quite poor in the case of laminin-treated, unmodified gold electrodes and did not suggest tight cellular coupling to the electrode. Thus, the L\textsubscript{CA-SAM}\textsubscript{Au} surface proved to be the best surface with which to improve cell-to-electrode contact as judged from the significant increase in normalized impedance compared to those for L\textsubscript{11-AUT-SAM}\textsubscript{Au} and laminin-adsorbed gold electrodes (L\textsubscript{Au}). Physisorption of laminin alone appears inadequate to permit electrically tight PC-12 cell interaction with the substrate electrode, but surface modification by either the L\textsubscript{CA-SAM}\textsubscript{Au} or the L\textsubscript{11-AUT-SAM}\textsubscript{Au} enhances PC-12 cell-to-electrode electrical impedance significantly. The L\textsubscript{11-AUT-SAM}\textsubscript{Au} surface proved to also enhance PC-12 cell-to-electrode electrical surface attachment; however, the electrical impedance response ($1.22 \pm 0.14$ $\Omega$ after 31 h) was not as appreciable as that observed with L\textsubscript{CA-SAM}\textsubscript{Au} surface ($1.54 \pm 0.07$ $\Omega$ after 28.6 h).

Figure 7. Time-dependent profile of the impedance magnitude of the eight electrodes of an ECIS 8W1E chip cultured with PC-12 cells. (A) Laminin physisorbed onto cleaned, sputter-deposited Au PME. (B) Laminin covalently immobilized to a CA-SAM\textsubscript{Au}. (C) Laminin covalently immobilized to an 11-AUT-SAM\textsubscript{Au}. Electrodes were incubated with $1.9 \times 10^6$ PC-12 cells/mL for 32.4 h. Data represent the average and SD of normalized impedances of six separate experiments.

Differences were considered significant for $P < 0.05$. For the neuron-to-electrode surface attachment (NESA), the Tukey multiple comparison test showed that the improvement of NESA on the L\textsubscript{CA-SAM}\textsubscript{Au} surface was significantly different from that of L\textsubscript{Au} and L\textsubscript{11-AUT-SAM}\textsubscript{Au}, while L\textsubscript{Au} and L\textsubscript{11-AUT-SAM}\textsubscript{Au} were not significantly different from each other in enhancing NESA.

It is not clear why the L\textsubscript{CA-SAM}\textsubscript{Au} system, assembled from the more disordered CA-SAM, reproducingly shows more dramatic changes in time-dependent impedance during the proliferation of PC-12 cells. While this seems to originate with the orientation (availability of amino groups at the monolayer interface) and the length of the aminoaikanethiol chains, the consequence seems to be more dense and larger laminin aggregates on the electrode surface. Cell attachment on laminin-treated, unmodified gold microelectrode surfaces resulted in the lowest impedance response, which we have indicated earlier to be due to the nonuniformity of the protein layer but may also be due to the mobility of this protein layer. It is important to note that PC-12 cells will only adhere to surfaces that have been pretreated with adhesion protein; otherwise, they remain in suspension. The quality of the cell-to-electrode surface attachment is, therefore, governed, in this instance, by the tightness of cell-to-cell and cell-to-electrode interactions. These appear to be favored by large aggregates of adhesion proteins such as laminin. Anchoring the protein to the electrode also anchors the cells. The shorter, though more disordered CA-SAM, establishes tighter electrical intimacy between the cells and the electrode. In the absence of a chemisorbed anchoring means such as an aminoaikanethiol, cells may in fact move these proteins with them as they become mobile on the solid surfaces.

Having established the CA-SAM as the principal surface modification layer for subsequent derivatization with laminin, we undertook an evaluation of the quality of cell-to-electrode surface attachment of PC-12 cells cultured
on laminin, fibronectin, and collagen derivatized CA-SAM layers. This allowed us to evaluate the most appropriate protein that aids in the improvement of NESA. Figure 8 shows the impedance response of PC-12 cells cultured on the three protein-derivatized, CA-SAM-modified, gold microelectrodes as well as a control microelectrode of bare gold. A maximum normalized impedance value of 1.94 for laminin immobilized to CA-SAMs was observed. This value is substantially higher than those observed for the interaction of cells on fibronectin, collagen-treated CA-SAM-modified surfaces, and unmodified electrodes (1.40 ± 0.02, 1.44 ± 0.04, and 1.14 ± 0.08, respectively). Figure 9 shows the images of confluent layers of PC-12 cells grown on bare gold electrodes and on laminin, collagen, and fibronectin derivatized, CA-SAM-modified gold electrodes. The inset shows the unique morphology adopted by these cells on these different surfaces. It is clear that, on fibronectin, PC-12 cells develop a spindle-like morphology and extend processes on the electrode. Upon changing of the culture medium following impedance interrogation, significant numbers of cells were lost in the case of unmodified electrode surfaces but very few cells were lost in the case of SAM-modified, laminin-derivatized gold microelectrode surfaces. Moreover, a significant number of cells remained in place when cultured on SAM-modified, laminin-derivatized gold microelectrode surfaces. The adsorption of laminin on unmodified surfaces followed by plating of cells is consistent with previous work.54

To further demonstrate that the observed impedance signals were due to neuron-to-electrode contacts, in a separate set of experiments cultured PC-12 cells were treated with 0.1% sodium hypochlorite solution. Immediately following treatment, the normalized impedance catastrophically and precipitously decreased to values that were below the baseline value of “medium-only” wells (Figure 10). This occurred with PC-12 cells cultured within all electrode-bearing wells and cultured under all surface modification and derivatization conditions. Thus, it was

clearly shown that the observed increases in the electrical impedance profile of PC-12 cells was in fact due to the electrical contribution of intact, viable neuron-to-electrode contacts formed between cells and electrodes.

Conclusions

We have focused on the development of a cellular measurement system exemplified by the monitoring of the electrical impedance of PC-12 cells cultured on gold MEAs. Electrode arrays utilizing alkanethiol surface modification and protein derivatization techniques were developed to serve as the substrate for the culture of cell types that are dependent on attachment for confluency and proliferation. Fluorescence microscopy, AFM, and DCA measurements were used to confirm attachment of derivatizing proteins, changes in surface roughness and topology, and changes in water wettability attendant to the several steps in the construction of the supramolecular cellular assembly.

The results of the present studies are consistent with the following major conclusions. Surface modification of gold microelectrodes with alkanethiols dramatically changes the surface morphology, with CA promoting an increase in surface roughness of 22% and 11-AUT promoting a modest increase in smoothness by 6%. Such SAM modification promotes the availability of surface amine functional groups, confirmed by direct FITC labeling using heterobifunctional cross-linking with fluorescently labeled protein. This also renders the gold electrode wettable, confirmed by DCA measurements. Subsequent protein immobilization via covalent derivatization does not contribute significantly to the observed electrochemical impedance of gold microelectrodes when placed in the culture medium. There is a modest differential contribution with the L[CA-SAM]Au conferring modestly larger impedance than the L[11-AUT-SAM]Au. The surface topology following protein adsorption to gold or derivatization of SAMs is not significantly different. However, surface modification and derivatization contributes immensely to the establishment of an electrically tight layer of cultured PC-12 cells when grown on these electrodes.

We conclude that cells interacting with adhesion proteins anchored via chemisorbed SAM layers form tight neuron-to-electrode and tight cell-to-cell contacts by virtue of the covalent attachment of the protein layer to the electrode. This we believe is the result of receptor-mediated increased mean residence time of cells on the electrode. Thus, promoting covalent immobilization of laminin to gold microelectrodes enhances neuron-to-electrode contact of PC-12 cells. The converse, observed on physisorbed protein layers, is that cells likely similarly attach to these proteins but do not establish tight neuron-to-electrode and tight cell-to-cell contacts. We further infer that cells that become attached to the covalently immobilized proteins also become immobilized and remain anchored over the time scale of these experiments. On the other hand, cells that become attached to the physisorbed proteins continue their motility, exercising motility forces that remove these proteins from the electrode and do not lead to tight neuron-to-electrode and tight cell-to-cell contacts. We find that laminin, when compared to collagen and fibronectin, is best able to establish the strong attachment of PC-12 cells to gold microelectrodes. We find also that the more disordered SAM, CA, when compared to the more ordered 11-AUT, supports better electrical impedance performance of the PC-12 cells.

The observed stability of the impedimetric response of PC-12 cells cultured on laminin-derivatized CA-SAMs offers a promising approach for the development and application in cell-based sensing. An excellent area of application is that of microfluidic cell-based sensors with well-positioned recording sites for cell, virus, and bacterial adhesion. With such stable, reproducible systems now in place we will address the challenges of the frequency dependence of cellular impedance as well as the cytotoxic responses as we move toward the development of an impedimetric biosensor system as an alternative to the use of whole animals.

Acknowledgment. This work was supported by the Center for Biosensors, Bioelectronics and Biochip (C3B). The authors gratefully acknowledge the expertise of Umit Makal for assistance with the AFM and Dr. Phillippe Lam for programming assistance with the impedance analyzer.