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Surface Patterning and Adhesion of Neuroblastoma X Glioma (NG108-15) Cells

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Abstract. **A variable height flow cell was used to measure the adhesion properties of the neural cell line of neuroblastoma X glioma (NG108-15) cells cultured on substrates of organosilane self-assembled monolayers (SAMs). The SAMs tested in this study were 13F, 15F, PEG 550, OTS, DETA and APTS. Utilizing deep UV lithography, patterning of the SAMs create three regions for cell attachment; the original SAM, the backfilled SAM, and the interface between the two. Upon plating, the cell soma show no preference for any of the three regions. One exception was on PEG 550, which was found to resist cell adhesion upon normal plating conditions. The cell processes of the NG108-15 cells show a preference for growth at the interface between two patterned surfaces. A factor of three increase in adhesive properties was found for the patterned surfaces over an uncoated glass surface. Design rules of a single whole cell biosensor using the NG108-15 cells can be developed based on these findings.**

Key Words. **cell adhesion, flow chamber, NG108-15 cells, self-assembled monolayers, SAMs, patterning**

Introduction

Current threats of biological warfare agents warrant new sensors to detect the presence of bio-toxins targeted for humans. Living cell-based sensors will undoubtedly play a role in the future detection of unknown biohazards and determination of a substance's toxicity towards the human species, even when the substance is unknown. Individual cells can act as functional sensors, where alterations in the action potentials based on receptor sites and ion channels of the cells indicate the presence of an identified or unidentified biohazard. Excitable cells (e.g., muscle, neurons and some cell lines) may function as the transducing element providing a small detectable electric field as the cell membrane spontaneously fires (Geddes, 1972; Thomas, 1972). The neural cell line of neuroblastoma X glioma (NG108-15) is one likely candidate to be used as a cell based biosensor because it is a clonal cell line, which has good longevity in culture and can be chemically stimulated to produce continuous action potentials.

Cell based sensors require that the cells be placed in a location appropriate for the recording apparatus. For instance, when using a planar array of metallic microelectrodes, the cells need to be positioned in close proximity or on the electrode pads to sense the electric field (Thomas et al., 1972). Controlling cell placement can be accomplished by several techniques. Clark et al. (1987, 1990 and 1991) have used grooves micromachined in the surface of silicon to control cell position. Surface chemistry methods have also been used to control cell placement. By modifying the surface with silanes of different functional groups, cells can be grown in patterns based on their affinity for one functional group over another (i.e., hydrophobic vs. hydrophilic end-groups). For example, microcontact printing may be used to produce reproducible patterns for cell culture growth by forming a self-assembled monolayer using an elastomeric stamp on a flat substrate (Xia et al., 1996). However, alignment of these stamp patterns with prebuilt electrode arrays has yet to be addressed. Orthogonal self-assembly modification of surfaces using trimethoxy silylpropyldiethylenetriamine (DETA) and 3-aminopropyltrimethoxysilane (APTS) have been shown to promote cell attachment and growth (Stenger et al., 1993). Conversely, the self-assembled monolayers (SAMs) polyethylene glycol (PEG), octadecyltrichlorosilane (OTS), 1H,1H,2H,2H, perfluorooctyltrichlorosilane (13F), and perfluorodecyldimethylchlorosilane (15F) have been known to hinder cell attachment and growth for certain cell types (Klinefeld et al., 1988, Dulcey et al., 1991). Patterning of similar SAMs on gold surfaces has been established as a means to control cell growth with respect to an electrode material (Lopez et al., 1993). Reproducible surface patterns can also be achieved by using UV-lithography, as was first done by Dulcey et al. (1991). This technique can address alignment problems of patterns with electrode arrays

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by utilizing mask aligners, which are common to most fabrication laboratories.

An understanding of the cell/surface interactions is necessary to adequately develop a biosensor using whole cells as the sensing element. Control of the cell with respect to an electrode is imperative to obtaining a usable signal based on the vitality of the cell. Cell patterning is crucial for control of a cell with respect to an electrical interface such as a microelectrode array. The electrode array itself needs to be designed with the characteristics of the cell in mind.

In this work, we utilize UV-patterning of self-assembled monolayers (SAMs) to control cell growth of NG108-15 cells. Organosilane SAMs are very versatile allowing for a variety of surface functionalities and surface free energies (Ulman, 1991). While cell counts can provide a rough measure of the health of a culture, they do not address the issue of cell adhesion, which provides a better understanding of the cell/surface interface and the interactions of the membrane proteins with the surface. The cells adhesive properties relating to the individual surface were investigated using a variable shear stress flow chamber. The utility and longevity of the NG108-15 cells as a biosensor element will be discussed in the following text.

Experimental

Organosilane precursors and other reagents

Six different organosilanes were used in these experiments. DETA and APTS were obtained from United Chemical Technologies (Bristol, PA). A methoxylated PEG silane with an average molecular weight of 550 g/mole (PEG-550) was obtained from Shearwater Polymers (Huntsville, AL). OTS, 13F and 15F were obtained from PCR, Inc. (Gainesville, FL). The silanes were used as received without further purification and were stored in a nitrogen atmosphere dry box (MBraun from Innovative Technologies, Inc.). Anhydrous methanol and toluene (Sure/Seal grade) were obtained from Aldrich (Milwaukee, WI) and used as received. The HCl, H_2SO_4, and glacial acetic acid were technical grade and the acetone, methanol, and toluene were all of HPLC grade and were purchased from Fisher Scientific (Pittsburgh, PA). SAM films were prepared on the benchtop under ambient atmosphere conditions. The substrates used in these experiments were No. 1 Thomas Red Label Micro Cover Glasses (22 × 22 cm) (Thomas Scientific, Swedesboro, NJ) and Fisherbrand (Fisher Scientific) precleaned plain microscope slides (3 × 1 × 1 mm).

SAM film formation

Substrate cleaning procedure. The glass substrates were cleaned by immersion in 1 : 1 HCl : Methanol for 15 minutes, rinsed three times in deionized ultra filtered (DIUF) water from an 18 MΩcm Barnstead (Dubuque, IA) Nanopure filtration system, soaked in concentrated sulfuric acid for 30 minutes and rinsed three times with DIUF. The substrates were boiled in DIUF water for 30 minutes, rinsed two times in acetone, and dried in an oven at 110°C for 20 minutes. SAM formation was accomplished by two different methods: a toluene preparation and a methanol preparation. (See Table 1 for more details.)

Toluene preparation method. Upon cooling, the clean substrates were immersed into a 0.1% (v/v) aminosilane/toluene solution (DETA, APTS, or 13F) and heated to 80–100°C for 30 minutes. The substrates were immediately immersed in fresh dry toluene to minimize contact with any moisture present in the air, rinsed 3 more times with toluene, and placed into a boiling toluene rinse for 30 minutes. The surface modified substrates were then baked in an oven at 110 °C for 2 hours.

Methanol preparation method. Methoxy silanes (e.g., DETA and APTS) were prepared on glass microscope slides by an acetic methanol preparation. The cleaned slides were immersed in a silane solution containing 1 mM acetic acid in methanol and 5% DIUF water for 15 minutes (see Table 1 for concentration of the silane). Finally, the slides were rinsed three times in methanol,

Table 1. *Reaction conditions and water contact angle measurement for self-assembled monolayers*

$Silane_{(prep\ method)}$	Silane concentration (M)	Reaction time (minutes)	Cure time at 110°C	Contact angle (adv, rec)
$13F_{(toluene)}$	3.3×10^{-3}	30 at room temp	15 minutes	(116,109)
$15F_{(toluene)}$	1.4×10^{-2}	60 at room temp	15 minutes	(90,75)
$PEG\ 550_{(toluene)}$	4.8×10^{-5}	60 at 100°C	15 minutes	(35,32)
$OTS_{(toluene)}$	1.2×10	60 at room temp	15 minutes	(99,95)
$DETA_{(toluene)}$	3.11×10^{-3}	60 at 100°C	2 hours	(43,25)
$APTS_{(toluene)}$	4.5×10^{-3}	60 at 100°C	2 hours	(50,23)
$DETA_{(MeoH)}$	3.9×10^{-2}	15 at room temp	15 minutes	(33,15)
$APTS_{(MeoH)}$	5.7×10^{-2}	15 at room temp	15 minutes	(38,15)

twice in acetone, and cured in an oven at 110 °C for 15 minutes.

Contact angle measurements

Contact angle measurements were taken on a NRL Contact Angle Goniometer Model 100-00 (Ramé Hart, Inc.). A drop of DIUF water was controlled via a micropipetter. The angle at which the water contacts the surface was measured by advancing and receding the drop. An average of three measurements of both the advancing and receding contact angles were taken across the surface. Table 1 shows values for all surfaces prepared.

Deep UV irradiation and patterning

Aminosilane treated surfaces were patterned by exposure to 193 nm deep UV radiation using a Lambda LPX210 Ar/F excimer laser customized with a beam homogenizer (Exitech, TecOptics, Merrick, NY). The beam homogenizer lens has an array of 36 elements to give $\pm$ 5% homogeneity. The laser was used in constant wavelength mode (18.4 kV) with a 30 Hz repetition rate. Patterns were created by positioning the aminosilane treated surface tightly against a 3 in $\times$ 3 in fused silica mask containing the desired geometrical features. A 10 J/cm^2 laser dose was required to remove the cytophilic DETA or APTS. The ablated areas contained surface silanol end-groups that were either backfilled with a subsequent silane following procedures described above or left as an uncoated surface.

Cell culture

SAM modified substrates were soaked in ethanol for sterilization then dried under aseptic conditions before plating with NG108-15 cells. Cells were grown in culture using Dulbecco's Modified Eagle's Medium (DMEM, Life Technologies, Gaithersburg, MD) and supplemented with either N2 supplement (Life Technologies) and 25 mM HEPES (Sigma Chemical Co., St. Louis, MO) with the pH adjusted to 7.4 for differentiated cultures or 10% fetal bovine serum (FBS, Life Technologies) and 1% HAT solution (0.1 mM hypoxantine, 0.4 mM aminopterin, 0.16 mM thymidine, all purchased from Life Technologies) for dividing cultures. DMEM supplemented with 25 mM HEPES was used during all adhesion experiments at room temperature (approximately 21°C).

Flow cell

The flow cell was developed as a tool to measure the adhesion of a cell to the surface. A flow chamber was fabricated following the design of Burmeister et al. (1996). Figure 1 shows an example of the flow cell from a side view. The flow chamber was built with a variable height to provide an increasingly stronger laminar flow shear stress along the length of flow. Fluid flow (5 to 105 ml/min) was obtained using a Harvard Apparatus (South Natick, MA) PHD 2000 syringe pump. Equation (1) can be used to calculate the shear stress (τ_w):

$$\tau_w = \frac{6\mu Q}{wh^2}, \text{g/cm} \cdot \text{s}^2 = \text{dynes/cm}^2 \qquad (1)$$

where Q is the flow rate in cm^3/s, μ is the fluid viscosity in g/cm · s, w and h are the chamber width and height in cm, respectively. The chamber height varied from 129 μm to 300 μm, yielding shear stresses of up to 200 dynes/cm^2. Since the microscope objective has a nonzero diameter, a variance in shear stress is introduced that amounts to approximately $\pm$ 6% at maximum shear stresses (lower shear stresses have smaller variances). An Olympus CK2 (Olympus America Inc., Melville, NY) upright microscope was used to monitor and count cell attachment.

Flow cell experiments in a variable shear stress chamber can be done in one of two ways. In the first method, the cells are exposed to a high, constant flow for a set period of time. After flow has ceased, the cells are counted as a function of distance down the length of the chamber; a shear stress can be calculated for each microscope field of view along the length of flow to give cell number vs. shear stress. Counting the cells while still in the chamber and on the microscope stage can be difficult without an accurate XY stage controller and can get tedious. It is best to fix the cells and then count them with the aid of imaging software. However, fixing the cells in the chamber will foul the instrument and removing the glass slide from the chamber often disturbs the remaining cells. We prefer another method for the flow cell experiments in which the cells are exposed to

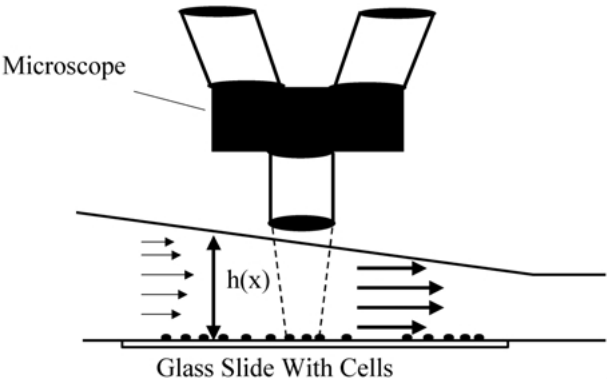

Fig. 1. *Schematic of variable height flow cell (cultured cells on a glass slide on bottom) and microscope to view field of cells. Arrows indicate relative magnitude of laminar flow velocity vs. cell height $h(x)$.*

short periods of progressively increasing flow. A representative field of view is picked with a microscope and the cells in that field are enumerated at the start and then again after each successive flow event. A cartoon of the experimental setup is shown in Figure 1. For example, a typical experiment would begin with an initial cell count, then the cells are exposed to flow at a rate of 5 ml/min. for 5 seconds, the remaining cells are counted and then the procedure is repeated using increasing increments of 5 ml/min., or until either a maximum pump flow rate (105 ml/min. for these data) is reached or there are no cells remaining in the field of view. The syringe pump establishes an equilibrium flow in less than one second, therefore the time interval of 5 seconds in these experiments is sufficient to allow for reproducible results. The strength of this method involves observing the same exact culture specimen before and after each increasing flow rate or shear stress.

Results and Discussion

Unpatterned surfaces

Silane based self-assembled monolayers were used to modify substrates for determination of the functional group dependence of NG108-15 cell growth. With one exception, a PEG silane (MW 550) SAM, the cells adhered well to and differentiated on a wide variety of surfaces displaying differences in both functional groups and wetting characteristics. Surfaces modified with PEG functional groups have previously been shown to prevent protein adsorption (Prime and Whitesides, 1991) and cell adhesion (Lopez et al., 1993). Independent of wetting properties, all other surfaces (polystyrene, 13F, DETA, and APTS) show the same relative ability to support cell growth, as shown in Table 2. For instance, 13F is a very hydrophobic surface quite similar to Teflon for its surface wetting properties. This surface shows cell counts on days 4, 10, and 20 similar to cultures grown on DETA, which is a relatively hydrophilic surface comprised of primary and secondary amines which may be charged depending on pH of solution. It has been reported that charged amine containing hydrophilic SAM surfaces such as DETA promote somal (cell body) adhesion and the maintenance of discrete processes for primary embryonic hippocampal neurons whereas aromatic aminosilane SAM surfaces were found to be detrimental to adhesion and neurite outgrowth (Stenger et al., 1993). In contrast, somal plating and survival of the NG108-15 cells are unaffected by large changes in the charge and hydrophobicity of the surface.

Shown above in Table 2 are the results of one cell plating experiment. These experiments do not represent adhesion of a cell on a surface. To determine adhesion of a cell culture on a surface, we have utilized a variable height flow cell as discussed in the experimental section. Briefly, a representative field of view is picked with a microscope and the cells in that field are enumerated at the start and then again after each successive flow event while increasing the fluid velocity after each cell count. Figure 2 shows the difference in adhesion properties of NG108-15 cells plated on a hydrophobic 13F and a hydrophilic DETA SAM. The number of cells detached, reported as a percentage of the initial number of cells, is plotted against the shear stress that the fluid flow exerts on the cells. The data was fit with a polynomial regression of *n*th order. A 95% confidence level was calculated and plotted for each fit. At percent detachment

Table 2. *NG108-15 cell counts for four different surfaces on three different days in culture. Cell counts represent a single experiment with an average count of eight areas per coverslip. Cells were plated at an initial plating density of 3.7 × 10³ cells/cm²*

		$\times 10^2$ cells/cm^2	
Surface	Day 4	Day 10	Day 20
Polystyrene	9.0	5.5	2.0
13F	10.0	5.0	3.0
DETA	8.0	4.8	3.0
APTS	8.3	4.5	2.0
PEG 550	0	0	0

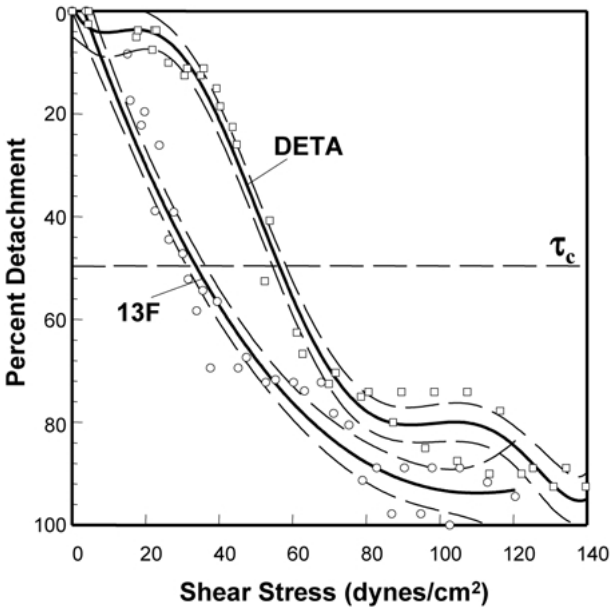

Fig. 2. *Percent detachment vs. shear stress for NG108-15 cells plated on either 13F (circles) or DETA (squares) surface. The data was fit with a 3rd and 6th order regression for 13F and DETA, respectively (thickest lines) and a 95% confidence interval (dashed thinner lines).*

values below 10% and above 80%, the confidence level broadens and values are not meaningful. However, between 10% and 80% detachment, reasonable values of shear stress can be determined from the data. Examination of the critical shear stress, τ_c, defined by Burmeister et al. (1996) as the applied wall shear stress for which 50% of the cells remain adherent, shows DETA as the more adhesive surface. DETA has a τ_c of 57 dynes/cm^2 as compared to 13F which has a τ_c of 35 dynes/cm^2.

While it is expected that different cell types will exhibit different adhesion characteristics, we can compare the τ_c for NG108-15 cells on DETA and 13F to that of other cell types. NG108-15 cells plated on DETA and 13F unpatterned surfaces exhibit critical shear stress values on the low end of what has been thus far reported in the literature. Rezania et al. (1997) found that primary bone cells incubated for both 20 minutes and 2 hours had τ_c values of approximately 50 dynes/cm^2 and 60 dynes/cm^2, respectively. Fibroblast cell cultures incubated for 2 hours exhibit a τ_c as large as 350 dynes/cm^2 (van Kooten et al., 1992). van Kooten et al. (1994) also found that human vascular endothelial cell cultures (1 hour incubation) lost 50% of the cells at a shear stress of 176 dynes/cm^2 while 50% of adult saphenous vein endothelial cells (3 hours incubation) weren't lost until a shear stress of 264 dynes/cm^2 was applied. NG108-15 cells have lower values than these literature references due to the fact that nerve cells are highly specialized for signal conduction and thus lack components geared toward planar adhesion. For instance, fibroblasts are a connective tissue cell type that secrete extracellular matrix components to enhance adhesion. Endothelial cells are specialized for lining blood vessels and, thus, would be expected to form strong, confluent single cell layers. Bone cells, on the other hand are more structural in nature and probably require a 3-dimensional matrix for optimal adhesion; not surprisingly the literature stated that bone cells have a lower adhesive strength like the NG108 cells.

Patterned surfaces

UV-lithography was used to fabricate patterns of self-assembled monolayers in a line/space geometry on glass substrates. The patterns examined were constructed from surfaces starting with either DETA or APTS. Line patterns of various dimensions from 105 to 1.5 μm were fabricated. Upon plating NG108-15 cells on these substrates, cell soma placement and cell process outgrowth were monitored. The NG108-15 cell soma will attach on many different SAM coated surfaces with a variety of wetting properties and functional groups, with the exception of PEG surfaces and other surfaces which inhibit protein adsorption (Bohanon et al., 1996). This was shown in the unpatterned surface experiments.

Photomicrographs of typical cell cultures of both patterned and unpatterned surfaces are shown in Figures 3 and 4. An unpatterned surface is shown in Figure 3 of APTS with NG108-15 cells at day 22 in culture. The cells were plated in serum free DMEM with 0.25 mM HEPES. Arrow A points to a cell soma that has attached to the APTS glass coated surface and extended processes

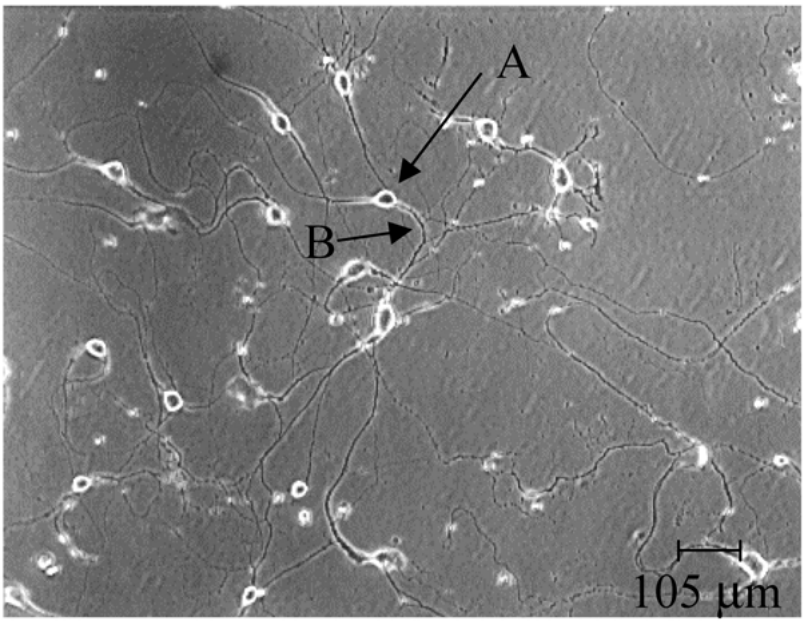

Fig. 3. *Day 22 of NG108-15 cell culture on unpatterned APTS. Arrow A indicates the cell soma and Arrow B indicate the cell process.*

(indicated by arrow B). The cell processes have extended in a random fashion.

Figure 4A shows NG108-15 cells growing on a patterned surface of APTS (32 μm) with no backfill (105 μm) at day 22 in culture. The cell soma appears unaffected by the pattern, attaching to either surface in a random order. However, the processes follow the interface of these two surfaces as they differentiate. The processes either prefer to contact both surfaces simultaneously or either surface for any duration of process outgrowth. As a visual test for pattern structure and integrity, a technique utilizing a catalytic Pd solution followed by a Ni-based reagent is employed. This technique labels nitrogen-containing SAMs with a thick nickel layer that is visible to the naked eye (Dressick et al., 1994; Kapur et al., 1996). Figure 4B shows a metallized DETA SAM pattern (the metallized amine containing SAM appears as the brighter and thinner lines). The metallized line space pattern in Figure 4B can be directly compared to the cell pattern of Figure 4A showing the cell attachment and differentiation defining the pattern produced for this surface.

NG108-15 cells will extend processes on most surfaces, the processes prefer growing at the interface on patterned surfaces (i.e., the region between the two surface modifications). The cell soma show no preference for the interface regions as do the processes. Other cell types exhibit different behavior. For example,

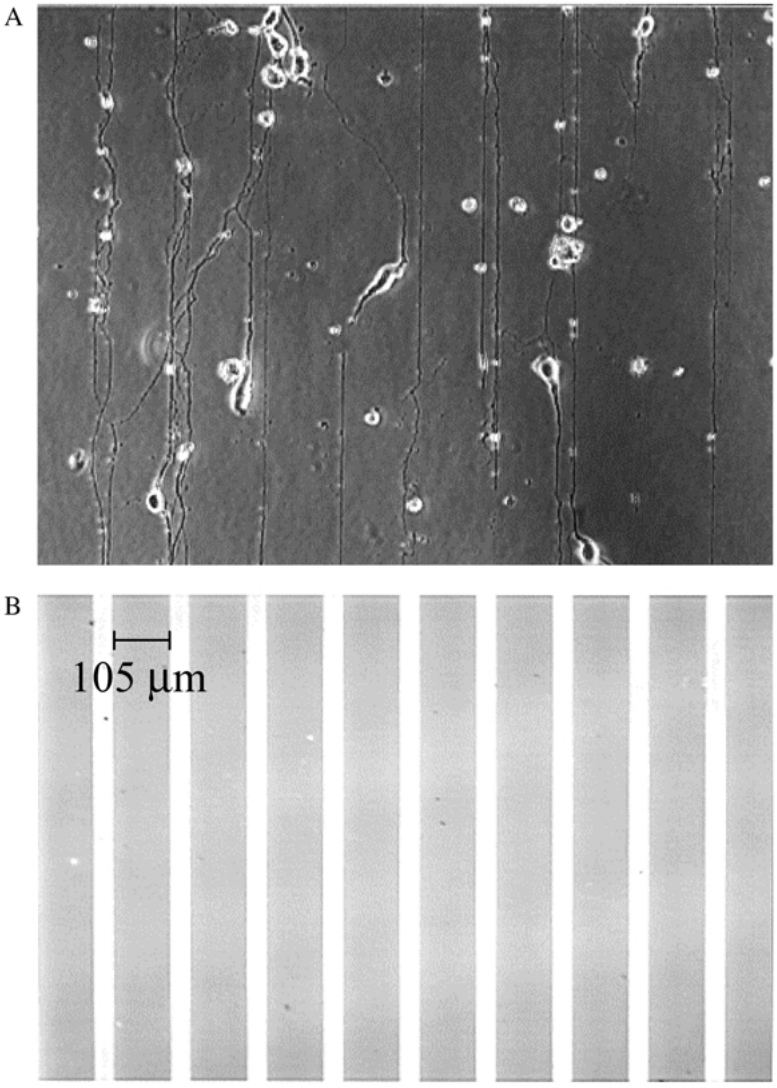

Fig. 4. *(A) Day 22 of NG108-15 cell culture on patterned APTS with no backfill. (B) Line/space pattern made visible by metallization technique. Amine-containing SAMs appear as thin bright lines in both A and B.*

hippocampal cells will attach on DETA preferentially over other surfaces such as 13F, with cell processes only extending on the DETA surface (Ravenscroft et al., 1998).

Cultures of patterned NG108-15 cells were photographed at day 50 in culture (data not shown). The cells are still viable at these prolonged times in culture and still show pattern fidelity. However, some loss in cell population has occurred at these prolonged times. These data have promising results for long term applications of cell patterning for device applications, however, cell viability was not examined.

Figure 5 compares the cell adhesion for NG108-15 cells on uncoated glass, APTS, and patterned APTS surfaces with no backfill. The τ_c for the uncoated and unpatterned APTS data is similar to the data for the 13F and DETA unpatterned cases from Figure 2 with an average value of 38 and 45 dynes/cm^2, respectively. The APTS patterned surface with no backfill has a greater τ_c with an average value of 91 dynes/cm^2. This result shows a combination of these surfaces increases the cell adhesion by allowing the cells to extend processes at the interface. The differences in cell adhesion are even greater when examining the data at larger percent detachments. The uncoated and APTS unpatterned cell cultures detached as an exponential decay, while those on patterned surfaces were removed in a more linear fashion. At 70% detachment, the uncoated surface

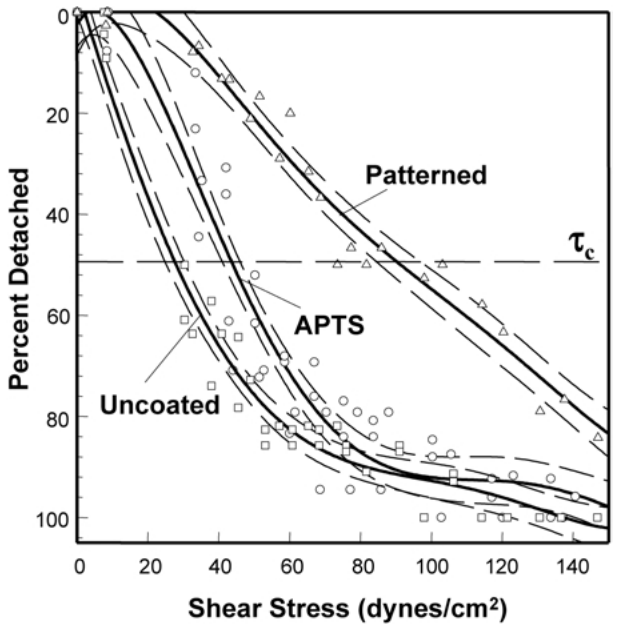

Fig. 5. *Percent detachment vs. shear stress for NG108-15 cells cultured on three different surfaces: uncoated (squares), APTS (circles), and patterned APTS (triangles) with no backfill. The data was fit with a 5th order regression (thickest lines) and a 95% confidence interval (thinner lines).*

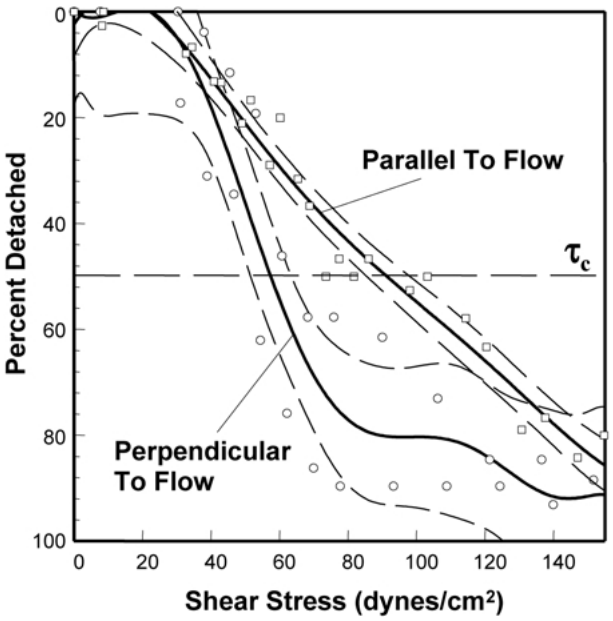

Fig. 6. *Percent detachment vs. shear stress for NG108-15 cells APTS patterns parallel (squares) and perpendicular (circles) to flow. The data was fit with a 8th and 5th order regression for perpendicular and parallel patterns, respectively (thickest lines) and a 95% confidence interval (thinner lines).*

showed critical shear stress values of ~ 45 dynes/cm^2 while APTS unpatterned surface showed values close to that of ~ 55 dynes/cm^2. The APTS patterned surfaces showed well over twice the cell adhesion; 70% of the cells detached at a shear stress of ~ 120 dynes/cm^2. The patterns for this data were aligned parallel to the flow.

It was anticipated that aligning the cells perpendicular to the flow would produce results different from those aligned parallel to the flow. Cells aligned parallel to flow have a smaller percent of the cell, which experiences the fluid forces head-on. Cells on the perpendicular pattern have a larger percent of the cell exposed to the liquid flow and are thus less challenged by the fluid forces for the entire cell.

Figure 6 shows the difference in adhesion of cells growing on patterned surfaces parallel and perpendicular to flow (note that Figures 5 and 6 both contain the same APTS parallel patterned data). While the perpendicular pattern flow data shows a larger 95% confidence region than the parallel data, it is clear that the parallel patterns resulted in greater cell adhesion than the perpendicular patterns. The shear stress for these data at a 70% detached value was ~ 60 dynes/cm^2 for the perpendicular patterns and ~ 100 dynes/cm^2 for the parallel patterns. Adhesion values for both patterned cases are greater than for the unpatterned cases, showing that not only can homogeneous surface properties effect cell adhesion but heterogeneous patterns can as well.

Conclusions

Based on surface modifications for cell placement and biocompatibility, SAMs are a very well characterized and reproducible surface that will lead to a better understanding of cell survival and longevity *in vitro*. SAMs provide a means to understand cell/surface interactions where the surface functional groups and wetting characteristics can be systematically varied. The flow cell provides an easy experimental means to determine adhesion characteristics of cells on these different surfaces. The adhesion of a cell population on a surface provides information about the membrane proteins interacting with that surface. A cell can exhibit more than one protein or a protein can have multiple interactions with different functional groups or wetting properties. Cell soma do not plate on PEG surfaces but will make good patterns when used as a backfill. Interactions with multiple surfaces may cause stronger interactions than that of a single surface alone. As we understand more about these surface interactions, better control in patterning and placement can be achieved. For example, when using NG108-15 cells, patterns can be a valuable tool for guiding the processes to electrodes and orienting them in a fluid flow stream providing a more robust system to exchange fluids while maintaining adhesion. These data show that this can be achieved by exploiting the processes' affinity for the interfacial regions.

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