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Growth of Endothelial Cells on Microfabricated Silicon Nitride Membranes for an *In Vitro* Model of the Blood-brain Barrier

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Abstract The blood-brain barrier (BBB) is composed of the brain capillaries, which are lined by endothelial cells displaying extremely tight intercellular junctions. Several attempts at creating an *in vitro* model of the BBB have been met with moderate success as brain capillary endothelial cells lose their barrier properties when isolated in cell culture. This may be due to a lack of recreation of the *in vivo* endothelial cellular environment in these models, including nearly constant contact with astrocyte foot processes. This work is motivated by the hypothesis that growing endothelial cells on one side of an ultra-thin, highly porous membrane and differentiating astrocyte or astrogliomal cells on the opposite side will lead to a higher degree of interaction between the two cell types and therefore to an improved model. Here we describe our initial efforts towards testing this hypothesis including a procedure for membrane fabrication and methods for culturing endothelial cells on these membranes. We have fabricated a 1 μm thick, 2.0 μm pore size, and ~55% porous membrane with a very narrow pore size distribution from low-stress silicon nitride (SiN) utilizing techniques from the microelectronics industry. We have developed a base, acid, autoclave routine that prepares the membranes for cell culture both by cleaning residual fabrication chemicals from the surface and by increasing the hydrophilicity of the membranes (confirmed by contact angle measurements). Gelatin, fibronectin, and a 50/50 mixture of the two proteins were evaluated as potential basement membrane protein treatments prior to membrane cell seeding. All three treatments support adequate attachment and growth on the membranes compared to the control.

Keywords: low-stress silicon nitride membrane, blood-brain barrier, endothelial cells, microfabricated membrane, fibronectin, gelatin

INTRODUCTION

The blood-brain barrier (BBB), located at the brain capillaries, provides a protective and controlled environment for brain chemical processes. Specialized endothelial cells lining the brain capillaries exhibit extremely tight intercellular junctions and form a cellular barrier essentially impermeable to water-soluble molecules lacking a specific transporter. In the brain, capillary endothelial cells have intimate interaction with foot processes from astrocytes, a type of microglial cell. There is evidence that this interaction plays a major role in the differentiation of brain capillary endothelial cells [1-3].

The ability to accurately predict brain permeability of potential therapeutics is important, both for developing drugs to treat ailments within the brain as well as protecting the brain from potential neurotoxicants. Numerous cell culture models of the blood-brain barrier have been developed to avoid some of the technical difficulties, ethical considerations, and cross-species extrapolations associated with BBB permeability measurements made on animals (see for review [3-5]). Cell culture inserts are used in a majority of these models. Unfortunately, as cultured BBB endothelial cells tend to lose their differentiated characteristics, most *in vitro* models are marginal replicas. This shortcoming is likely due to the inability to recreate in the models certain environmental, mechanical, and chemical cues that exist in the brain. For instance, an increase in model barrier tightness has been observed when the endothelial cells are cultured with astrocyte or astroglioma cells. Further increase has been observed by the addition of a membrane-permeable cAMP analog and a phosphodiesterase inhibitor along with the astrocytes [6,8]. Some studies indicate that direct contact between endothelial cells and astrocyte/astrogliomal cells, achieved by growing of the two cell types on opposing sides of the insert membrane, is essential in restoring the barrier [1,9]. However, the physical dimensions of the membranes used in culture insert models do not promote a high degree of interaction between two cell types grown on opposite sides. Cell culture insert membranes are track-etched which results in a wide pore size distribution, a random arrangement of pores with a maximum porosity of 15%, limited pore size

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selection (0.4 μm, 1.0 μm, and 3.0 μm), and a thickness of 10 μm.

The driving force for this work is the hypothesis that increasing the amount of direct contact between endothelial cells and astrocytes while maintaining two distinct cell layers will lead to a more differentiated and appropriate BBB model. In this report, we describe the initial efforts towards testing this hypothesis through fabrication of ultra-thin, high porosity membranes from SiN. We also present techniques for culturing endothelial cells on these fabricated membranes. We believe the attributes of the fabricated SiN membranes will allow a higher degree of interaction between endothelial cells and astrocytes/astrogliomal cells grown on opposite sides than commercial membrane inserts. However, no data from endothelial/ astrocyte co-cultures on the microfabricated membranes is presented in this paper.

MATERIALS AND METHODS

Materials

The following materials were purchased from the indicated vendors: standard 100 mm, <100> oriented, 480 μm thick silicon wafers from Virginia Semiconductor (Fredericksburg, VA, USA); Shipley 1827 and 1813 photoresists from Shipley Corporation, a subsidiary of Rohm & Haas (Philadelphia, PA, USA); 29% ammonium hydroxide, 45% potassium hydroxide, 6-well culture plates, and 25 cm^2 culture flasks from Fisher Scientific (Pittsburgh, PA, USA); 30% hydrogen peroxide, heparin, and phosphate buffered saline (PBS) tablets from Sigma (St. Louis, MO, USA); 14% sulfuric acid from LabChem (Pittsburgh, PA, USA), 1 mm thick clear silicone sheets from Grace Bio Labs (Bend, OR, USA); M199 powder medium, fetal bovine serum (FBS), 100× insulin-transferrin-selenium (ITS), and trypsin/EDTA from Invitrogen (Carlsbad, CA, USA); endothelial cell attachment factor from Cascade Biologics (Portland, OR, USA); human plasma fibronectin from Chemicon (Temecula, CA, USA); AZ MIF 300 developer from Clariant (Sommerville, NJ, USA); Microprime Primer P-20 from MicroSi (Phoenix, AZ, USA).

Fabrication of Silicon Nitride Membranes

SiN membranes were fabricated similar to the procedure outlined in Kuiper *et al.* [10]. First, wafers were prepared with a standard Radio Corporation of America (RCA) clean. Then 1 μm of SiN was thermally grown by low pressure chemical vapor deposition on standard 100 mm, 480 μm thick, <100> oriented, single side polished silicon wafers. SiN thickness was verified with ellipsometry by a 5 point measurement on two wafers.

The goal of the next few steps was to lithographically pattern on the back side of the wafer the chip divisions and the series of openings which would form the membranes. Initially, the back side of the wafer was primed with P-20 to improve photoresist adhesion. Shipley 1827 photoresist was spun on the back side 3.2 μm thick, and the wafer was pre-baked for 120 s on a hot plate set at 115°C. The photoresist was exposed using an HTG System III contact aligner. The mask was designed to divide the wafer into 24 chips, each 15 mm × 15 mm centered around a single 1,500 μm × 1,500 μm square. Dice lines 150 μm wide by 14.7 mm long were made at each side of the chips to facilitate wafer division after fabrication. The photoresist was developed in MIF300 and post-baked at 115°C for 60 s. The SiN was etched to the silicon underneath using a PlasmaTherm 72 (Unaxis, Pfaffikon, Switzerland). The wafer was placed in 15% (w/v) KOH in water at 85°C with 100 rpm magnetic stirring for approximately 9 h until the exposed square openings were etched to the SiN film on the top side. As KOH selectively etches the <100> plane, the side-walls of the wells make a 54.5° angle with the wafer surface and the final dimensions of the SiN windows on the top side were 800 μm × 800 μm × 1 μm thick. We will refer to the volume etched below the membrane as the membrane well. After the KOH etch, the wafers were rinsed 4 times in distilled water.

Outlined below are the lithography steps to transfer an array of pores 2.0 μm in diameter and regularly spaced 2.4 μm apart into the SiN windows. The top side of the wafer was first primed with P-20, and Shipley 1813 photoresist was then spun to 1.4 μm thick. A vacuumless chuck with clips was used in the wafer spinner to avoid breaking the SiN windows. The resist was pre-baked for 60 s at 115°C prior to exposure of the pore array with a GCA-6300 5× g-line stepper. The overall dimensions of the exposed pore array was large in comparison to the 800 μm × 800 μm squares which allowed for coarse alignment with the back side pattern. The resist was developed in MIF 300 and post-baked for 60 s at 115°C. The pores were etched through the nitride with the PlasmaTherm 72. Residual resist was removed by acetone and then isopropanol. The wafer was manually cleaved into chips along the dice lines. Pore size was confirmed by using a Zeiss 982 scanning electron microscope.

Membrane Cleaning and Sterilization

We developed a cleaning and sterilization routine based loosely on standard cleaning procedures used in the microelectronics industry. The purposes of this routine were to remove any residual chemicals remaining from fabrication, increase the hydrophilicity of the SiN surface, and decrease trapping of air bubbles in the membrane wells. Chips were first put in 4% ammonium hydroxide with 4.3% hydrogen peroxide at 80°C for 5 min to oxidize organic films and complex heavy metals. After 2 washes in distilled water, chips were placed in a 12.5% sulfuric acid with 4.3% hydrogen peroxide solution at 80°C for 5 min to strip residual organics and form a more hydrophilic oxide layer. One mm thick cell culture grade silicone was cut to 15 mm by 15 mm with a 10 mm by 10 mm square removed from the center to form a gasket. The silicone gasket was treated in the same

sulfuric acid/ hydrogen peroxide solution for 5 min each side to increase hydrophilicity. Chips and silicone gaskets were put through a series of 6 rinses in distilled water by moving the pieces from bath to bath. Membranes were allowed to air dry and the silicone gasket was placed on the top side of the chip to form a 1 cm^2 well centered on the 800 μm square SiN membrane. We will refer to the volume inside the silicone gasket as the chip well. The assembly was placed gasket side down in 20 mL distilled water in a Petri dish and autoclaved in the liquid cycle for 15 min at 121°C.

Cell Culture Maintainance

Immortalized human brain capillary endothelial cells (SV-HCEC), passage 32, were kindly provided by A. Muruganandam of the National Research Council of Canada [11]. Cells were cultured in M199 medium with 10% FBS, 1× ITS, and heparin at 600 USP units/L. Every 3 to 4 days cells were either fed with fresh medium or split 1:10 if nearly confluent into an attachment factor coated T-flask.

Cell Seeding of Fabricated Membranes

Each chip was placed gasket up in 0.7 mL cell culture medium in a 6-well plate. 100 μL of either cell culture medium, endothelial cell attachment factor (0.1% sterile gelatin solution), 60 μg/mL human plasma fibronectin in PBS, or a 50/50 mixture of the previously mentioned attachment factor and fibronectin solutions was pipetted into the chip well. Each treatment was done on triplicate chips. For a control, a tissue culture 25 cm^2 polystyrene flask was treated with 1.5 mL attachment factor. The protein solutions sat on the membrane surfaces for 30 min at 37°C. While cells were being released from the cell culture flask with trypsin/ EDTA, the protein solutions were aspirated from the surfaces. The detached cells were resuspended in cell culture medium and plated at 100 μL cell suspension per membrane. Cell density by hemacytometer count was 2.0×10^4 cells/cm^2, including the polystyrene flask control. SV-HCEC cells were at passage 39. Medium volume in control flask was 5 mL. Cells were incubated at 37°C and 5% CO_2. At 3 h, 0.8 mL cell culture medium was added to bring the total volume to 1.6 mL per well. Pictures of the cells on the fabricated membranes were taken using a phase contrast microscope and Nikon CoolPix990 digital camera. In order to monitor cell attachment and growth, the number of cells per membrane was manually counted from laser printed digital photographs taken 3, 24, and 48 h post seeding.

Contact Angle Measurements

The contact angles of 1 μm SiN on silicon wafers after the base wash, acid wash, autoclaving in water, and protein treatments mentioned earlier were measured using the sessile-drop method with a goniometer. The samples were rinsed 1 time in distilled water after all treatments except autoclaving in water. Triplicate drops of 4 μL of chromatography grade water were made on each sample and allowed to rest for 2 min after removing syringe needle. Contact angle was measured on both sides of each drop. Each treatment was done in duplicate.

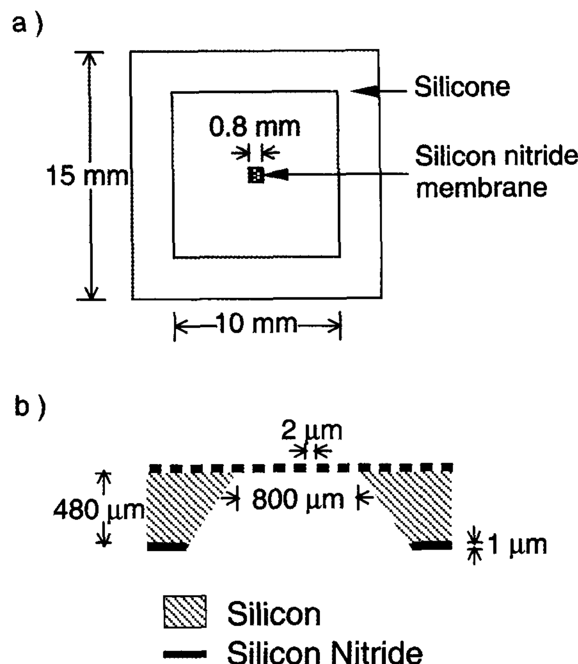

Fig. 1. Diagram of chip and membrane layout. a.) Top view of chip and gasket assembly. b.) Side view of SiN wafer after fabrication process (not to scale).

RESULTS AND DISCUSSION

SiN membranes were fabricated at approximately 90% yield of chips with intact membranes by careful attention to wafer handling and cleanliness (especially during lithography). SiN thickness was measured to be 1.01 ± 0.03 μm by ellipsometry. Shown in Fig. 1 is a schematic of the top view of the chip, membrane, and gasket as well as a side cut-away view of the fabricated membrane. Due to imperfect alignment of the mask pattern with the wafer crystal plane, underetch occurred resulting in some membranes being slightly larger than the target size of 800 μm × 800 μm. The fabrication scheme allowed for good control of the nitride thickness, pore size, and membrane dimensions. Also, by patterning the pores after etching through the back side, the size and number of membranes and chips could be changed by making just one new mask for the backside exposure. Scanning electron microscopy (SEM) images of the membrane surfaces showed a very narrow pore size distribution as well as regular pore spacing. Displayed in Fig. 2 is an SEM image of one such membrane. The size of the pores for the growth study were approximately 2.0 μm in diameter, spaced at 2.4 μm, for a membrane porosity of

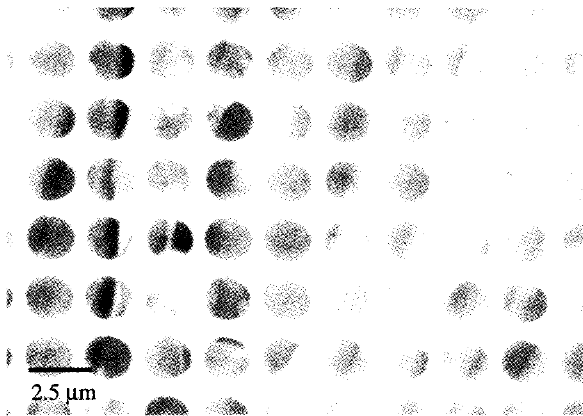

Fig. 2. SEM of fabricated membrane surface (viewed in white). Striations in background were due to the mounting stub. Pore diameter was measured to be 2.0 μm.

Table 1. Contact angle measurements

Treatment	Contact angle
Ammonium hydroxide with hydrogen peroxide	54.9 ± 4.1
Sulfuric acid with hydrogen peroxide	48.0 ± 3.0
Autoclave 15 min in distilled water	41.9 ± 4.6
Attachment factor	43.6 ± 2.5
Attachment factor/ Fibronectin	78.4 ± 6.3
Fibronectin	83.5 ± 5.8

Contact angles are reported as the average measurement ± one standard deviation. Each value is the average of triplicate measurements taken on duplicate samples.

54%.

Contact angle measurements are given in Table 1. As the contact angle is the angle a water droplet makes with the surface, a smaller angle indicates a more hydrophilic surface. The values reported here are within the range of previously published results for low-stress silicon nitride [12]. Angles from subsequent acid, base, and autoclave steps were found to be statistically different by the one-tailed Student's t-test with a confidence interval of 95%. By utilizing the cleaning routine, a decrease in hydrophobicity and removal of air bubbles from the membrane wells was achieved. Adsorption of 0.1% gelatin (attachment factor) did not have a significant effect on the surface hydrophilicity. However, the large increase in contact angle after application of fibronectin to the surface indicates protein adsorption. The contact angle for the 50/50 mixture was closer to that of the fibronectin coated surface. This observed increase is in agreement with measurements of contact angle for fibronectin on titanium surfaces [13].

Fibronectin and gelatin were chosen for this study based on unpublished results from a previous study conducted in our lab comparing type I collagen, gelatin, Matri-gel, and fibronectin. We found that growth rates on all proteins were similar when cell densities were similar, but fibronectin promoted a significant increase in initial cell attachment. This initial study was done at seeding densities below what will be used for BBB co-culture model studies. Reported here are the results of a follow-up study done at more relevant seeding densities. The proteins selected for this study were fibronectin, as it promoted higher initial cell attachment, and gelatin, as it is traditionally used in endothelial cell culture. A mixture of the two proteins was used to investigate possible synergistic effects, possibly by orienting fibronectin so the cell binding sites were more available to approaching cells. Type I collagen and Matri-gel were not considered as cell behavior on these treatments was nearly identical to gelatin and gelatin is easier to work with and less expensive.

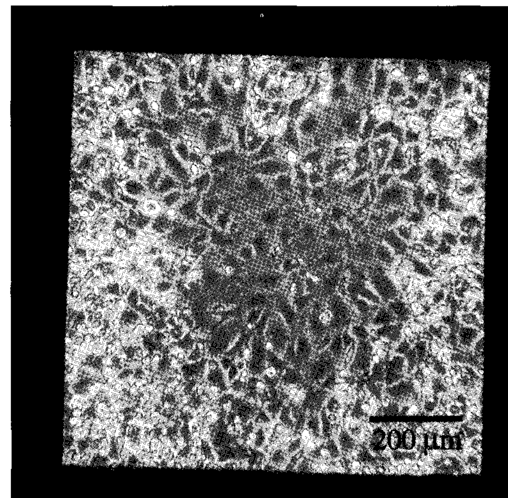

Fig. 3. SV-HCEC seeded on fibronectin and gelatin (50/50) treated membrane, pictured 3 h post seeding.

Fig. 3 is a photograph typical of the protein coated membranes 3 h post cell seeding. At this time, some cells appeared rounded indicating they were not yet attached; these rounded cells were not counted in the comparison. After 24 and 48 h, the frequency of these rounded cells was far less. The results of the basement membrane coating effect on endothelial cell attachment and proliferation are shown in Fig. 4. Interestingly, the tissue treated polystyrene with gelatin had the lowest cell count, even below the seeding density. This decrease was most likely due to a portion of the cells not attaching to the surface and subsequently proliferating. On the other hand, the initial cell densities on the fabricated membranes were higher than the initial seeding density. We believe this was due to a repelling of the cells by the silicon gasket to the center of the chip and to the formation of a meniscus in the chip well. The meniscus had greater height, and therefore cell number, at the center of the chip above the membrane. Cell shape and morphology qualitatively look the same on the fabricated membranes and tissue cultured polystyrene, except that

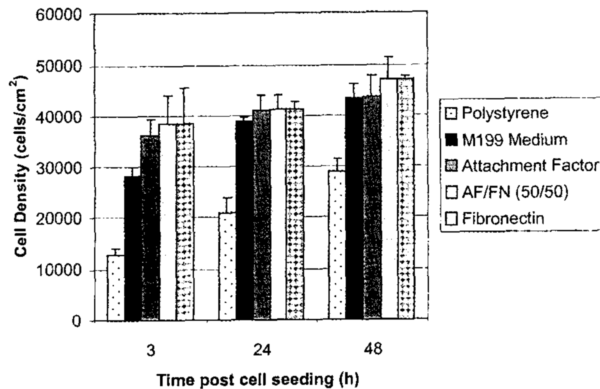

Fig. 4. Effect of basement membrane treatment on cell attachment and growth on fabricated membranes. Gelatin coated polystyrene flask and non-coated SiN membranes serve as controls. Cell density determined by manually counting the number of cells per membrane from a digital picture taken of a phase contrast microscope image and dividing by membrane surface area. Error bars are plus one standard deviation (n=3 except for fibronectin n=2).

the cells are slightly larger on the polystyrene. This difference may be attributed to a lower cell density on the polystyrene. We also observed that the basement membrane treated fabricated membranes had significantly higher cell attachment compared to the medium treated membranes. However, there were no statistically significant differences between the gelatin, fibronectin, and 50/50 mixture treatments with regards to cell attachment and proliferation. It was not possible to compare cell proliferation rates between treated and untreated membranes in this study as the cells were plated near confluency on the fabricated membranes and displayed contact inhibition. Cell density on the protein treated fabricated membranes essentially reached a plateau near 40,000 cells/cm^2 at 3 h. Cells on the medium treated membrane reached a density nearly equivalent to the protein treated membranes after 24 h, but the polystyrene flask density never reached those of the fabricated membranes within the 48 h study.

One aspect not considered in this study is the effect of basement membrane proteins on the barrier properties of the endothelial cells. An increase in barrier tightness of low resistance endothelial cell monolayers when cultured on laminin, type IV collagen, and fibronectin versus rat tail (type I) collagen was previously reported [14]. However, no difference between the treatments was observed when the endothelial cells had a higher intrinsic resistance. The SV-HCECs are low resistance endothelial cells. Because gelatin is a hydrolyzed form of collagen, it is difficult to predict where it fits in the previous study. Initial studies conducted in our lab showed a higher transendothelial electrical resistance (a measure of barrier tightness) for cells cultured on pure fibronectin compared to the other coatings tested. Because fibronectin promoted higher initial cell attachment (although not observed at the cell densities used in this study), supported adequate cell proliferation, and possibly contributed to the barrier properties of endothelial cells, it appears to be the best candidate for treatment of the fabricated membranes of those we have screened. However, the BBB system we intend to test includes a second cell type and is therefore more complicated than this single cell model. We did not consider the effect of basement membrane proteins on the growth of the astrogliomal cell line we will use for future studies because the line is very robust compared to the SV-HCEC line. Thus far, the astrogliomal cells have grown adequately on all membranes tested regardless of protein treatment.

To our knowledge, this was the first study of cell growth on microfabricated SiN membranes with high porosity. Mibrofabricated SiN membranes have been previously been used in biological applications as cell filters [10,15]. Fibroblast growth on SiN films on silicon has also been studied [12]. In the fibroblast study they found higher cell attachment to RGD (a cell binding amino acid sequence on fibronectin) absorbed silicon as compared to SiN. They did not compare fibroblast growth on RGD absorbed SiN versus non-treated SiN.

The dimensions of our membranes used for this study did not technically fall within the range of nanobiotechnology. Membranes hundreds of nanometers in thickness could be more advantageous for promoting interaction between cell types on opposing sides. However, as changing the membrane thickness reduces the strength of the membrane, this approach is not very practical. We are interested in reducing the pore size to 600 nm. Initial studies of astrogliomal cell growth on the fabricated membranes have indicated that pore diameters in the hundreds of nanometers range may be necessary to maintain two distinct cell layers on opposite sides of the membranes. We believe it will be necessary to maintain two distinct cell layers such that the astrocytes or astrogliomal cells will not be able to grow through the membrane and disrupt the endothelial monolayer on the opposite side. We aim to fabricate membranes with smaller pores by using a stepper with greater image reduction and by reducing the exposure time for the pore array.

CONCLUSION

In this study, we have demonstrated the fabrication of ultra-thin, high porosity membranes from SiN using microelectronic techniques. These membranes will form the essential component of a new *in vitro* model of the BBB. Our fabrication scheme allowed for good control of membrane thickness, pore size, and pore distribution. We have developed a pre-treatment routine that increased wettability of our membranes. Gelatin and fibronectin were evaluated as basement membrane treatments for the fabricated membranes prior to cell seeding. We observed an increase in cell density on the fabricated membranes in comparison to polystyrene due to concentrating effects of the silicone gasket. Both the proteins alone and a mixture

of the two promoted adequate cell growth and attachment. Fibronectin may prove to have the most desirable properties for future work on the BBB model because it promoted high cell adhesion and may increase the tightness of endothelial intercellular junctions.

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