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Microfabrication of high-resolution porous membranes for cell culture

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ABSTRACT

Microporous membranes are widely utilized in cell biology to study cell–cell signaling and cell migration. However, the thickness and low porosity of commercial track-etched membranes limit the quality of cell imaging and the degree of cell–cell contact that can be achieved on such devices. We employ photolithography-based microfabrication to achieve porous membranes with pore diameter as small as 0.9 μm, up to 40% porosity, and less than 5% variation in pore size. Through the use of a soap release layer, membranes as thin as 1 μm can be achieved. The thin membranes minimally disrupt contrast enhancement optics, thus allowing good quality imaging of unlabeled cells under white light, unlike commercial membranes. In addition, the polymer membrane materials display low autofluorescence even after patterning, facilitating high quality fluorescence microscopy. Finally, confocal imaging suggests that substantial cell–cell contact is possible through the pores of these thin membranes. This membrane technology can enhance existing uses of porous membranes in cell biology as well as enable new types of experiments.

1. Introduction

Membranes with pore sizes on the order of 1–10 μm are useful in cell biology for studying cell–cell signaling [1], cell migration [2,3], and cell filtration [4]. A common configuration is to suspend a population of cells on a porous membrane about a millimeter above the bottom of a tissue culture well. Soluble paracrine factors can then diffuse through the membrane pores to facilitate communication between the suspended population and cells cultured on the bottom of the well. Cell migration through the membrane is typically blocked for pore diameters around 1 μm, allowing two cell populations to interact while remaining unmixed [1,5]. Lack of mixing is important for studies in which each population needs to be analyzed separately, for example to assay cell-specific changes in gene or protein expression. Alternatively, pore diameters of roughly 10 μm are utilized for studies of cell migration through the membrane [1].

Commercial cell culture membranes are typically made from polyethylene terephthalate (PET) or polycarbonate (PC) by using a track-etching process [6,7]. High-energy particles from radioactive decay leave a trail of damage as they fly through the polymer, and the damaged material is later removed by chemical etching to create long and narrow pores. While pore diameter is well controlled in this process, the placement of pores is random. This leads to several disadvantages: local pore density is not uniform, and membrane porosity must be kept fairly low in order to minimize the overlapping of pores (Fig. 1). Additionally, commercially available track-etched membranes have thicknesses of around 10 μm [8]. While such membranes are well suited for transmitting diffusible paracrine signals, the same is not true for juxtacrine cell–cell signaling, which requires direct membrane-to-membrane contact between cells. If small pores are utilized to block migration, cells on opposite sides of the membrane must reach through long and narrow tunnels in order to contact one another [5,9]. Juxtacrine signaling could be considerably improved with membranes that are significantly thinner and higher in porosity [8].

Porous membranes for cell culture have also been produced by microfabrication. This approach can achieve similar pore sizes to track etching, but pores can be placed precisely rather than randomly, thus allowing high porosity without pore overlap. Electron beam lithography has been utilized to produce silicon–nitride membranes with pore sizes down to 0.3 μm and 0.5 μm in thickness [8]. While these dimensions are excellent, the cost of semiconductor processing is high, and electron beam lithography is a serial writing process, making the production of large-area membranes impractical (<1 mm^2 in the cited work). Photolithographic patterning of polymer materials enables cheaper and larger-area membranes. For example, membranes have been fabricated in poly(para-xylylene) polymers (Parylene) [4] or polydimethylsiloxane (PDMS) [10] at sizes on the order of 1 cm^2, which is comparable in area to commercial cell culture membranes and holds adequate cell numbers for standard protein and nucleic acid quantification assays. However, the minimum achievable dimensions are not as small as with electron beam

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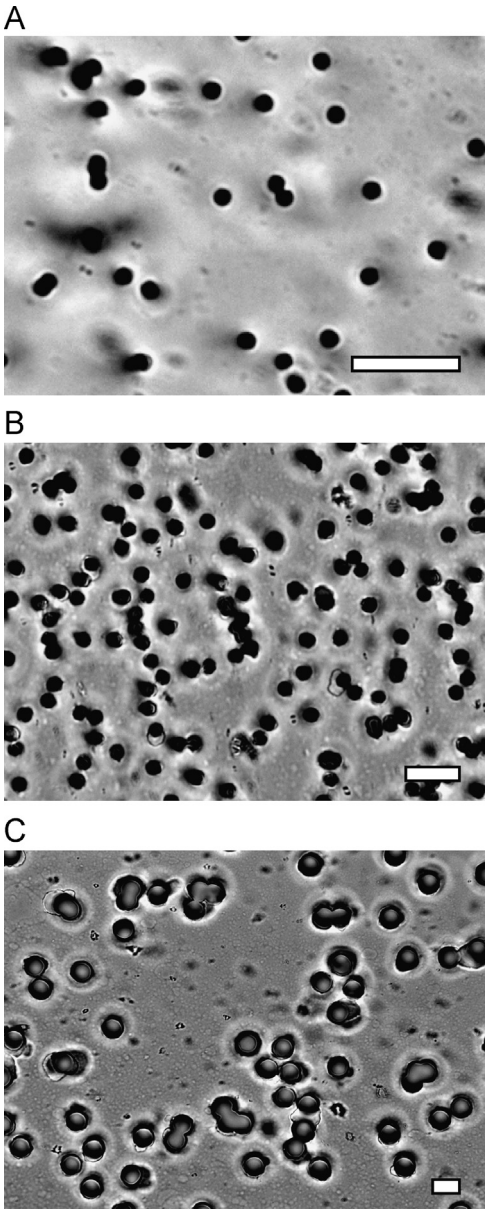

Fig. 1. Commercial porous cell-culture membranes (PET) imaged by 60× optical microscopy. Imperfections include regional variations in pore density and fused pores with larger-than-desired pore diameter. (A) 1-μm pores, 1.6% porosity. (B) 3-μm pores, 14% porosity. (C) 8-μm pores, 10% porosity. All scale bars are 10 μm.

lithography, with typical pore sizes on the order of 10 μm and thicknesses of roughly 10 μm. Recently, a high-resolution porous membrane process was reported in a photocrosslinkable polymer known as SU-8 [11]. Minimum pore size and membrane thickness were quoted as <1 μm; however, the presented data only showed pores down to ~ 4 μm. Also, the variability of the pores was not discussed but appeared to be considerable in the images provided.

Cell culture membranes should facilitate clear imaging by both white light and fluorescence microscopy. Although commercial PET and PC membranes are fairly transparent, imaging of unlabeled cells by white light microscopy is poor. The thick and inhomogeneous membranes appear to disrupt the effectiveness of contrast enhancement optics such as phase contrast, Hoffman modulation, or differential interference contrast, suggesting that a thinner membrane could be advantageous. Fluorescent imaging of cells on PC and PET membranes is much better than white light imaging, but it is still important to minimize autofluorescence in order to reduce background noise. For microfabricated membranes, it has been shown that Parylene HT is superior to Parylene C in terms of autofluorescence [12]. Additionally, the photocrosslinkable polymer 1002F was developed specifically to provide a low-autofluorescence alternative to SU-8 [13].

In this work, we investigate the resolution limits of photolithographically defined polymer cell culture membranes. Two different fabrication processes are studied: a positive patterning process in which pores are etched out of a sheet of Parylene HT, and a negative patterning process in which 1002F is crosslinked around a pore pattern. We introduce the use of a soap release layer, which allows delicate membranes to be floated off gently from a support substrate. Thus, we achieve simple and low cost fabrication of membranes down to 1 μm in pore size and thickness, up to 40% porosity, and less than 5% variation in pore size. We demonstrate the culture of mammalian cells on these microfabricated membranes and show that the cells can be imaged clearly by both white light and fluorescence microscopy.

2. Experimental

2.1. Materials

Cell culture membranes of polyethylene terephthalate (PET, Millicell Cell Culture Inserts, Millipore) and polycarbonate (PC, Transwell Inserts, Corning) were purchased commercially. Microposit S1808 positive photoresist and Microposit MF-319 developer were purchased from MicroChem. EPON 1002F resin (phenol, 4,4′-(1-methylethylidene)bis-, polymer with 2,2′-((1-methylethylidene)bis (cyclohexane-4,1-diyloxymethylene))bisoxirane) was obtained from Miller-Stephenson. UVI-6976 photoinitiator (triarylsulfonium hexafluoroantimonate salts in propylene carbonate) was purchased from Dow Chemical. Propylene glycol methyl ether acetate (PGMEA) and γ-butyrolactone (GBL) were acquired from Sigma Aldrich.

2.2. Preparation of photomasks

Photomasks were designed using the open source Kic layout editor (version 2.4.b, www.wrcad.com/freestuff.html) and printed at Photronics by electron beam lithography on 125-mm chromium–quartz substrates. Due to the differences in photoresist polarity, a brightfield mask was used for Parylene patterning, and the inverse darkfield mask was used for 1002F patterning. The mask features were scaled for 1:1 exposure.

The mask contained 16 membrane designs on one 100-mm wafer. Each of the 16 membranes consisted of different pore sizes (0.8 μm, 1.2 μm, 2 μm, and 4 μm) and porosities (5% to 40%). Porosity was calculated by dividing the area of the holes by the total area on the membrane. Since the computer-aided design (CAD) software and mask printer utilized Manhattan geometry (rectangular patterns), the pores were drawn as overlapping squares (Supplemental Fig. S1), which rounded out into circular shapes during the photolithography process. Membranes were patterned as 15-mm diameter circles with a 6.5×6.5 mm^2 porous region.

A lower-resolution dark field photomask (100-μm pores, 125-μm center-to-center spacing) was printed on Mylar film at Fine Line Imaging for patterning an optional structural support lattice in 1002F.

2.3. Fabrication of Parylene membranes

The following process flow is illustrated in Fig. 2A. Test-grade 100-mm silicon wafers (University Wafer) were first treated with 2% Micro-90 (International Products, Burlington, NJ) by spin