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Emerging trends in precision fabrication of microapertures to support suspended lipid membranes for sensors, sequencing, and beyond

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Abstract

Suspended lipid membranes, also called black lipid membranes (BLMs), are an important model system that approximates the lipid bilayer environment of cell membranes. Increasingly, BLMs are utilized in sensing strategies that harness high sensitivity measurements of ion flux across the membrane, typically facilitated by ion channel proteins. BLMs are suspended across microapertures that connect two otherwise isolated fluidic compartments, and the precision fabrication of such microapertures can contribute to the stability and performance of the resulting BLM. Here, we highlight two emerging trends in the precision fabrication of microapertures for BLM formation: microfabrication in silicon-based thin film substrates, and microfabrication in the negative photoresist material SU-8. Four unique fabrication strategies are outlined, and we project the impact that these microfabrication strategies will have for BLM-integrated bioanalytical technologies.

Keywords

Black lipid membranes; microfabrication; planar apertures

Introduction

Biological function is modulated by transduction of information from the extracellular domain to the intracellular domain by transmembrane proteins. Ion channels provide regulated, ion-specific transport and serve as key transducers of cellular signaling pathways by modulating ionic gradients across biological membranes. Ligand gated ion channels are particularly important in cellular signaling and provide key points of intervention for pharmacological and physiological modulation of cell function [1, 2]. Ion channels also provide key targets for chemical and biological toxins [3, 4]. Due to the natural distribution of ion channels, and the corresponding ligand diversity, ion channels have been explored as sensors for hormones, neurotransmitters and other natural and synthetic targets [5–7]. Additionally, ion channels play a key role in the development of next generation single molecule DNA sequencing [8–10].

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Ion channel derived-sensors typically require the geometric and electrical isolation of a membrane, typically obtained from a fraction, or the entirety, of a cell membrane expressing the ion channel, across an aperture interface. A high resistance electrical seal between the aperture and the membrane enables high sensitivity measurement of ion flux across the membrane down to the single ion channel level. Upon pore opening or closure via ligand or voltage-induced conformational change or direct pore blockage by analyte binding, changes in ion flux are measured, allowing a quantitative correlation of ion channel activity with ligand binding or ion channel inhibition. Importantly, sensing in this manner does not require labeling of the ligand or inhibitor.

A key impediment to the development of ion channel-functionalized sensors is the requirement for a stable lipid bilayer environment to support ion channel function. While cell membranes facilitate the use of natural ion channels, the need to form high electrical resistance seals on living cells in a high precision, microscopic environment, and the limited longevity of these seals, limit the wide spread utilization of this approach. Additionally, cell membranes are highly heterogeneous environments and the electrophysiological measurement of ion channel activity results from measurement of net ion flux across the membrane, introducing substantial background and interferences.

To provide a more homogenous environment, artificial lipid bilayer systems have been developed. The most important of these for ion channel studies are suspended lipid bilayers, also called black lipid membranes (BLMs) [11, 12]. BLMs span an aperture that connects two otherwise isolated fluidic compartments (Figure 1), allowing fluidic and electronic access to both faces of the membrane, in a manner similar to traditional cellular patch clamp methodologies. The largest success of BLMs, to date, is the demonstrated utility in ion channel-based nanopore DNA sequencing [8–10]. Often, large diameter apertures, and thus large area BLMs, have been utilized, though smaller diameter apertures have been demonstrated to improve bilayer performance [13, 14]. Unfortunately, formation of BLMs is often a tedious, manual process that is slow and, in many instances, poorly reproducible. BLM lifetimes are typically on the order of hours, thus BLM-based sensing assays require membrane/sensor formation immediately prior to use, and newly formed sensors require characterization before reliable analytical measurements can be made. These limitations motivate the formation of BLMs within automated microsystems, which require planar microapertures that are readily integrated with existing microfabrication processes.

Effective planar aperture platforms must meet several criteria. Typical single channel ion currents are on the order of a few picoamperes, thus the aperture substrate material must offer high dielectric performance to minimize current leakage that contributes to the background in ion current measurements. Planar apertures for robust sensor systems should be fabricated with high precision at dimensions in the low micrometer regime, since BLM stability improves with decreasing aperture diameter. Furthermore, to achieve small diameter apertures, thin substrate materials are required to ensure contact between the two lipid membrane leaflets (Figure 1). Interactions between the membrane and the aperture can act to further stabilize BLMs and reduce current leakage; therefore, consideration of aperture surface chemistry is critical, with low surface energies favoring stable, low leakage BLMs [15].

A number of planar aperture systems have been reported that enable BLM investigations not possible using other aperture formats, such as glass micropipettes. For example, a planar aperture system has been utilized to position BLMs within the focal plane of a microscope objective [16], allowing optical interrogation of membranes and membrane-bound processes. Many of the existing reports of planar aperture systems involve apertures formed in Teflon films [17–20]. Teflon meets dielectric and surface energy requirements, but its relative stability leaves few avenues for chemical micromachining. Thus, most Teflon apertures are fabricated by low precision physical machining processes that yield aperture diameters of several hundred microns. Furthermore, the lack of effective microfabrication processes makes the integration of Teflon apertures into precision fabricated microdevices particularly challenging.

Recently, trends have emerged in the precision microfabrication of planar apertures that will drive forward the integration of BLMs with automated microfluidic devices. Here, we describe two promising microfabrication trends, and the outlook for their applications in BLM-integrated microfluidic systems for sensing, sequencing and other applications.

Microapertures in silicon thin films

High precision silicon fabrication processes are well-established, and silicon wafers are common substrates in microfluidic devices. Thus, silicon appears a natural choice of aperture substrate material for integrating BLMs with microfluidic devices. Unfortunately, silicon substrates meet neither the dielectric nor surface chemistry criteria for effective BLM apertures. Although there are reports of microaperture fabrication in silicon [21–23], silicon aperture fabrication often involves additional, complex fabrication procedures to improve dielectric performance and manipulate surface chemistry. An alternative to forming microapertures in bulk silicon wafers is to employ insulator deposition processes common in silicon fabrication, such as vapor deposition or thermal oxide growth, to yield a thin film insulator substrate on a silicon wafer handle. The use of thin-film substrates offers the advantage of decreased substrate thickness, which facilitates BLM formation by allowing the leaflets of the bilayer to span a smaller distance before meeting to form a stable membrane. The presence of the silicon wafer handle allows common wafer bonding practices to be employed for integrating microfluidic networks.

Liu *et al.* described the fabrication of nanoapertures in a silicon oxide/boride thin film by electron beam lithography [24]. In this procedure, boron diffusion is used to form a thin boride layer at the surface of a silicon wafer. The boron diffusion layer acted as an etch stop in the backside etching of a well in the silicon wafer, leaving only the 2 μm -thick boride layer to serve as the aperture substrate. Thermal oxide growth on both faces of the wafer insulated the semiconductor wafer, improving dielectric performance. PMMA etch resist was patterned by electron beam lithography, and a nano aperture was formed in the boron and oxide film by deep reactive ion etching (RIE). The oxidized substrate was treated with hexamethyldisilazane to give a low surface energy that promotes membrane formation. By this method, apertures were fabricated with diameters as low as 660 nm that met the dielectric and surface energy requirements outlined above. The primary limitation of this approach, however, is the low throughput for fabrication by electron beam lithography.

Halza *et al.* described an alternate approach for aperture fabrication in silicon films that does not require electron beam lithography (Figure 2) [25]. This approach avoided many of the thin layer deposition and thermal oxidation steps described in the aforementioned work by Liu *et al.* by starting with a silicon on insulator (SOI) substrate, in which a 5 μm silicon film is isolated from a 300 μm silicon handle layer by a 500 nm buried oxide (BOX) layer (Figure 2A). The aperture was produced by conventional UV photolithography and Bosch dry etching of the silicon thin film, with the BOX layer acting as an etch stop (Figure 2 B and C). The same lithography and etching process was used to form a large well in the silicon handle layer, aligned with the aperture (Figure 2 D and E). The BOX layer was removed by RIE (Figure 2 F), and the silicon thin film was insulated by thermal oxide growth (Figure 2 G). Apertures were formed with diameters from 3 to 30 μm , and the parallel fabrication of 36 aperture devices on a single 4 inch wafer was possible. By relying on UV rather than e-beam lithography, this process trades sub-micron precision for parallel, high throughput fabrication.

Unquestionably, silicon thin film fabrication methods achieve high performance apertures with exceptional precision. Vapor deposition or thermal oxide growth processes yield conformal dielectric insulation of the silicon wafer, which addresses the challenges associated with silicon conductance. Still, the fabrication procedures are complex, and silicon substrates, especially those incorporating insulator thin films, are significantly more expensive than other common microfabrication materials. Although no study has addressed directly the long term use of insulator thin film apertures in sensor devices, the fragility of thin film substrates can reasonably be expected to pose a challenge in the development of robust and deployable sensor systems.

Microapertures in SU-8

Polymer materials offer improved dielectric performance, lower cost, and lower surface energies than silicon. The use of durable, photodefinable polymer substrate materials, such as SU-8, have enabled the photolithographic fabrication of robust, high aspect ratio microstructures with high fidelity, and in some cases with well-defined three-dimensional geometries [26, 27]. SU-8 is biocompatible, inexpensive, and commonly employed in the fabrication of microfluidic systems. These qualities combined with the amenable surface chemistry and dielectric properties, support the utility of SU-8 for the development of microfluidic-integrated BLM apertures. In the examples that follow, the ability to form three-dimensional structures in SU-8 allows the formation of microapertures with sloped walls, a geometry that offers a similar advantage to thin film substrates, by reducing the effective substrate thickness in the region of membrane formation [24, 28].

Liu *et al.* described a method for producing SU-8 microapertures by overexposure UV lithography [24]. In this method, a sacrificial positive photoresist layer was spin-coated on a silicon wafer (Figure 3, a), followed by a 10 μm layer of SU-8 (Figure 3, b). Since SU-8 acts as a negative photoresist, exposure to UV radiation (Figure 3, c) caused the sacrificial layer to solubilize in regions where the SU-8 was polymerized to form a solid aperture substrate. Whereas conventional SU-8 photolithography requires only seconds of UV exposure at typical UV power densities, Liu reported 3 to 15 minutes of UV exposure. During extended

exposure times, diffraction at the edge of photomask features allowed SU-8 in regions beneath the feature to be polymerized. The volume of exposed SU-8 beneath the photomask feature increased as the distance from the photomask increased, resulting in apertures with sloped walls (Figure 3, d). The polymerized SU-8 substrate was removed from the wafer by adhesive bonding to a supportive plastic layer (Figure 3, e), followed by dissolution of the sacrificial layer (Figure 3, f). While this approach yielded apertures as small as 10 μm in diameter, it was prone to production failures due to prolonged exposure times and irreproducible polymerization that resulted in sealed apertures [24].

We recently described an SU-8 aperture fabrication strategy based on incline and rotate photolithography [28]. In this method, a 75 μm layer of SU-8 was spin-coated directly onto the surface of a glass photomask treated with an adhesion inhibitor. The photomask both defines the aperture feature and serves as a supportive handle layer throughout the fabrication process. Collimated UV radiation was made incident on the photomask/SU-8 assembly at a controlled angle below the normal axis (Figure 4 A). During UV irradiation, the assembly was rotated in the plane of UV exposure, resulting in a well-defined three-dimensional volume of SU-8 left unexposed to radiation. Polymerized SU-8 substrates readily lifted from the photomask due to the adhesion inhibitor treatment. Apertures produced by this method, an example of which is shown in cross section in Figure 4B, had sloped walls similar to those described by Liu et al; however, incline and rotate photolithography allowed precision control over aperture wall angles across the range of 8° to 36° (measured from the normal axis) and produced symmetrical, high precision apertures with diameters as low as 7 μm . BLMs formed rapidly on the SU-8 apertures, showed characteristic conductance, and supported ion channel measurements. While this fabrication method was inexpensive, precise, and relatively straightforward, the smallest apertures observed with 100% fabrication success were 22 μm in diameter.

Precision aperture fabrication in SU-8 is highlighted by three factors: 1) SU-8 offers performance advantages in terms of dielectric properties, surface chemistry, and precision fabrication of three-dimensional geometries; 2) Microfabrication methods for these materials are more straightforward and less expensive than the processes involved in silicon fabrication; and 3) SU-8 has found widespread adoption in the fabrication of microfluidic devices, facilitating the integration of BLM technologies into lab-on-a-chip systems. Still, SU-8 microfabrication yields lower precision than that of silicon, and the lower limit of aperture diameters produced in silicon cannot yet be achieved in SU-8.

Outlook

The availability of precision fabricated microapertures in planar substrates will accelerate the development of technologies that incorporate BLMs. Here, we have placed emphasis on planar substrate materials that are readily integrated with existing microfabrication techniques because we believe that the integration of BLMs into microfluidic platforms holds key advantages for the development of ion channel-functionalized biosensors. Principle among these advantages is that the high degree of process integration and automation afforded by modern microfluidic technologies may allow on-demand BLM formation and characterization, addressing a key limitation discussed above. Specifically,

common microfluidic segmented flow strategies present an interesting avenue towards automated BLM formation, since phospholipids organize at aqueous/organic phase boundaries. Droplet microfluidics may present the most promising approach to automated BLM formation, since repeatedly exposing a planar aperture to lipid-containing aqueous/organic phase boundaries approximates the common manual practice of “painting” bilayers, but with improved reproducibility, throughput, and automation. In fact, automated droplet interface bilayer formation has been reported using a multiphase microfluidic approach [29, 30]. More work is needed to demonstrate the feasibility, repeatability, automation and integration of BLM formation in droplet microfluidic devices.

Next generation sequencing technologies that involve electrophoretically driving single DNA molecules through biological nanopores employ BLMs as key constituents [8–10]. A significant advantage offered by most of the aperture fabrication methods described here is the ability of highly parallel fabrication methods to yield high density aperture arrays. The high throughput requirements of accurate genetic sequencing stand to benefit significantly from high density array technologies, as the immense popularity of DNA microarray technologies have demonstrated [31, 32]. We believe that harnessing aperture fabrication strategies like those described here to achieve high density BLM arrays will play a key role in advancing next generation sequencing technologies, and the eventual application of these technologies to important genomic challenges.

Additionally, BLMs provide an important model that is utilized to understanding protein and lipid biophysics that occur at or within the cell membrane [33, 34]. Most commonly, such studies have centered on phenomena that alter ion flux across the membrane, since this is readily measured by high sensitivity current amplifiers. Optically interrogating BLMs is less common [16, 17], since orienting a suspended lipid membrane in the focal plane of a microscope objective presents a significant challenge. The fabrication methods discussed here offer reduced aperture dimensions, and thus improved membrane stability, over planar aperture systems employed in previous optical studies of membrane-bound phenomena. We believe that the potential to perform optical interrogation of BLMs with improved stability will facilitate studies that advance our understanding of membrane and transmembrane protein biophysics by correlating measurements in the optical and electrical domains.

In summary, recent developments in the precision microfabrication of planar apertures will play a key enabling role in the further development of technologies that incorporate BLMs and are thus poised to make significant contributions to a wide range of challenging analytical measurements..

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Fig. 1. Cross-sectional schematic of BLM formation on planar apertures

a BLMs form when lipid monolayers meet from opposite faces of a planar aperture, which connects otherwise fluidically and electronically isolated compartments. A solvent annulus forms in the region between the bilayer and the aperture wall. **b** Reduced thickness of the aperture substrate facilitates a smaller solvent annulus, which increases bilayer area relative to the aperture. Concurrent reduction of substrate thickness is required to achieve small diameter apertures.

Fig. 2.

Fabrication of microapertures in silicon-on-insulator wafers. A The SOI wafer consists of a 5 μm silicon film electrically isolated from a 300 μm silicon handle by a 0.5 μm oxide layer. B and C A microaperture is formed in the silicon film by conventional photolithography methods. D and E An access well is formed in the silicon handle by conventional photolithography methods. F the oxide layer is removed by reactive ion etching. G. Thermal oxidation is used to insulate the semiconductor device. Reprinted with permission from reference 25.

Fig. 3. Fabrication of microapertures by overexposure lithography of SU-8

a – d The microaperture is defined in SU-8 by overexposure lithography, yielding sloped aperture walls. **e** A supportive plastic chip is glued to the SU-8 substrate, and **f** the aperture is lifted from the Si wafer handle by developing the sacrificial positive photoresist layer. Reprinted with permission from reference 24.

Fig. 4.

Fabrication of microapertures by incline-and-rotate lithography. a A thin layer of SU-8 is spin coated directly onto a glass photomask bearing circular features. Collimated UV radiation is made incident on the photomask/SU-8 assembly at a fixed angle (θ_{UV}), and the assembly is rotated through greater than 360° during UV exposure. Following exposure, the photomask/SU-8 assembly is baked to complete SU-8 polymerization, unexposed SU-8 is removed with SU-8 developer solution, and the polymerized SU-8 is lifted from the photomask to give a well-defined microaperture with three dimensional geometry in a planar SU-8 substrate. b Scanning electron microscopy of a cross sectioned aperture reveals the resulting sloped-wall geometry. Adapted and reprinted with permission from reference 28.