Review

Membrane biosensor platforms using nano- and microporous supports

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Lipid membranes are versatile and convenient models for the study of properties of natural cell membranes. In particular, surface-supported membranes have attracted considerable attention because the whole range of surface-sensitive techniques, including interface-sensitive electrochemical measurements, can be used with them. Here we describe recent advances and current directions in the development of nano- and microporous substrates for electrochemical characterization of membrane protein-containing lipid bilayers. Improved techniques for lipid membrane self-assembly and membrane protein incorporation on these substrates have led to great improvements in measurement sensitivity, membrane stability and packaging. These advances suggest that nanopore-spanning membranes are leading contenders for a breakthrough in membrane protein screening and biosensing applications.

Introduction

Cell membranes are effective diffusion barriers, upholding concentration gradients of ions and molecules across the membrane. Through their function as scaffolds for membrane proteins, membranes also control the selective transport of molecules and ions across the lipid bilayer to create and modify ion gradients. These signal transduction events guide many important cellular processes, consequently, >50% of all current drugs target membrane proteins [1]. It is thus essential to be able to characterize membrane barrier properties and the changes that occur when these proteins are incorporated into a lipid membrane, activated or blocked.

Creation of robust *in vitro* membrane systems enabling direct incorporation and integrated electrochemical or voltage clamp measurements of membrane protein function (i.e. ion, charge and liquid transport) could potentially revolutionize current technologies in drug screening. Methods currently used for screening and profiling in the pharmaceutical industry are limited to the monitoring of binding events; but even extraction of binding affinity requires multiple experiments at a range of concentrations. Direct measurement of function in response to (drug) stimuli will enable quicker and better selection of leads and toxic hits. The same kind of platforms could be used for advanced biomimetic sensing. Possible applications would be electronic tongues and noses or light harvesting for energy and biochemical purposes. Important classes of membrane proteins, such as G-protein-coupled receptors (GPCRs) and their analogues, can be exploited for this purpose. From a fundamental scientific point of view, a convenient way of studying membrane constituents down to the single molecule level would help bridge the gap in information and understanding between holistic biological studies of function in whole cells and the expanding knowledge about membrane protein structure and *in silico* simulation of their function.

These tasks are difficult to carry out directly on native cell membranes with techniques such as the patch clamp. Hence, a host of attempts to find generic ways of achieving integration of biosensors with membranes have been attempted. The main obstacle to taking research on advanced *in vitro* membrane function from the academic laboratory to commercial applications has been the difficulty in incorporating membrane proteins into biosensor platforms in a manner that allows measurement of the function of the membrane proteins.

Membrane platforms for studying membrane protein function

A selection of proposed membrane sensor platforms of relevance for this review are schematically depicted in Figure 1, and Figure 2 provides brief descriptions of the most common ways to form a lipid membrane on a sensor substrate. For an in-depth review of the many suggested platforms we refer the reader to a recent review by Janshoff and Steinem [2]. Among the *in vitro* systems, the first developed and most widespread method for studying ion-channel function has been lipid membranes spanning apertures between two aqueous compartments, the so-called black lipid membranes (e.g. [3]). Although black lipid membranes have been a successful approach in the laboratory, this platform has suffered two major drawbacks for general application: (i) instability manifested in the collapse of the membrane within a few hours of preparation and (ii) preparation requiring solvents that stay in the membrane, making it incompatible with many proteins. Interesting recent developments in this area have included working with interconnected membrane bubbles in organic solvents without a substrate aperture [4,5], which has resulted in higher stability and throughput. However, with this approach mechanical robustness and the presence of solvent is still a concern. Having a solvent-free membrane is of particular importance in drug screening applications because the conformation, and thus the activity, of many sensitive ion channels and transporters is affected by the presence of organic solvents.

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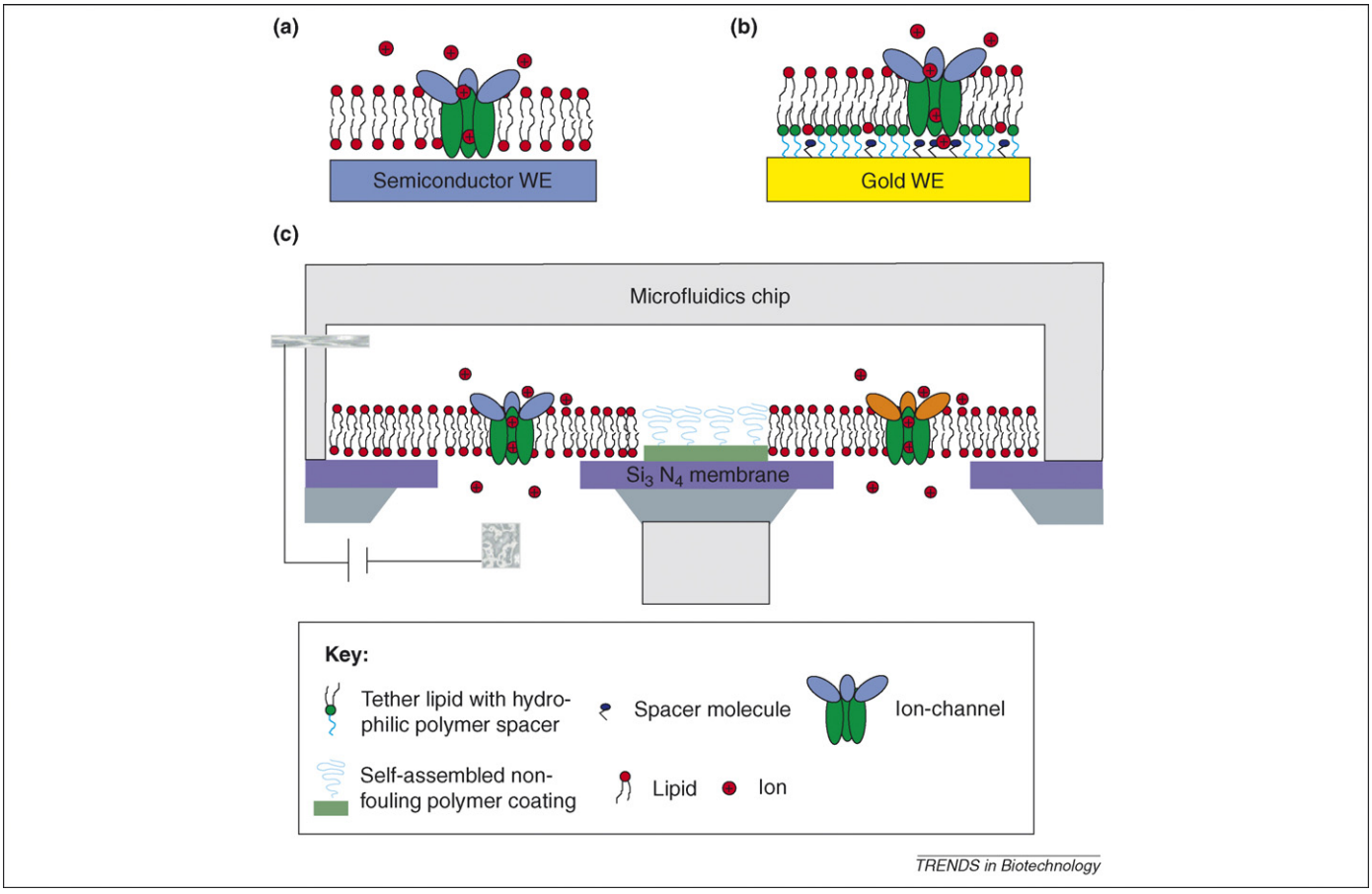

Figure 1. A selection of membrane sensor platforms **(a)** Supported lipid bilayer on hydrophilic support [52]. Typically a hydrophilic semiconductor or oxide substrate has to be used for direct assembly of a supported lipid bilayer (SLB). The support will act a as a working electrode (WE) for electrochemical measurements. **(b)** Tethered supported lipid bilayer. Covalently attached hydrophobic molecules with a hydrophilic linker – often derived from lipids – are used to tether and support the lipid membrane to a gold WE [53,54]. **(c)** Free-spanning membrane or black lipid membrane as it could look in a self-assembled membrane sensor array [16,17,23,27]. Lipid membranes containing ion-channels separated into functional spots, for example by non-fouling polymer barriers, span apertures in a solid support. It has free liquid access on both sides to accommodate large proteins and to easily conduct electrochemical measurements. The schematics show how such a platform combined with microfluidics could be used for parallel voltage clamp measurements on different single transmembrane proteins.

As a result, there has been an increasing emphasis on surface-based membrane platforms in recent years. These offer inherent stability because of the underlying supporting surface, as long as they are kept hydrated. In their original form – the solid-supported lipid bilayers (SLB, e.g. [6], Figure 1a) – have a severe drawback for studying membrane protein function in that they offer little space between the lipid membrane and the solid support to accommodate hydrophilic domains of the integrated protein [2]. The limited aqueous compartment also restricts electrochemical measurements because only a limited number of ions can be transported to the proximal side of the membrane before the osmotic gradient becomes too high [2,7]. However, advances have been made in this area enabling both creation of membranes on suitable electrode substrates [6,8] and demonstration of electrochemical measurements on inserted membrane proteins [9,10]. The most significant improvements in terms of properties, preparation and functionality in the area of solid-supported membranes have come from strategies aiming to decouple the membrane from the underlying electrode substrate. This decoupling has been achieved primarily by forming a supported lipid bilayer on a short hydrophilic polymer spacer, typically oligo(ethylene glycol) with a lipid anchor [11], so-called tethered lipid bilayers [2,11,12] (tSLB, see Figure 1b). The increased space of a few nanometers allows integration of membrane proteins without undesired surface interaction, but the reservoir is still too restricted to monitor continuous ion transport and the preparation can be difficult for solvent-free membranes [11]. Membranes can also be formed on top of hydrogels and other thicker (~10 nm) polymer cushions [13,14] creating a greater reservoir, but there are few successful demonstrations of electrochemical measurements [14,15].

Factors driving the current development of supported lipid membrane sensors over other techniques for membrane sensing, such as cell patch clamp, whole-cell sensing and black lipid membranes, are the ease of formation of membranes, control of complexity, stability and the applicability of a large range of highly sensitive surface analytical techniques. To date, no single approach has successfully combined solutions to all of the main design criteria for membrane biosensors summarized in Table 1. This article highlights recent advances aiming to combine the advantages of the original black lipid membranes, referred to here as free-spanning membranes, and supported lipid bilayers. Through combination of nano- and microfabrication with self-assembly of free-spanning membranes it has been demonstrated in recent years that a platform could be designed that fulfils the criteria for drug

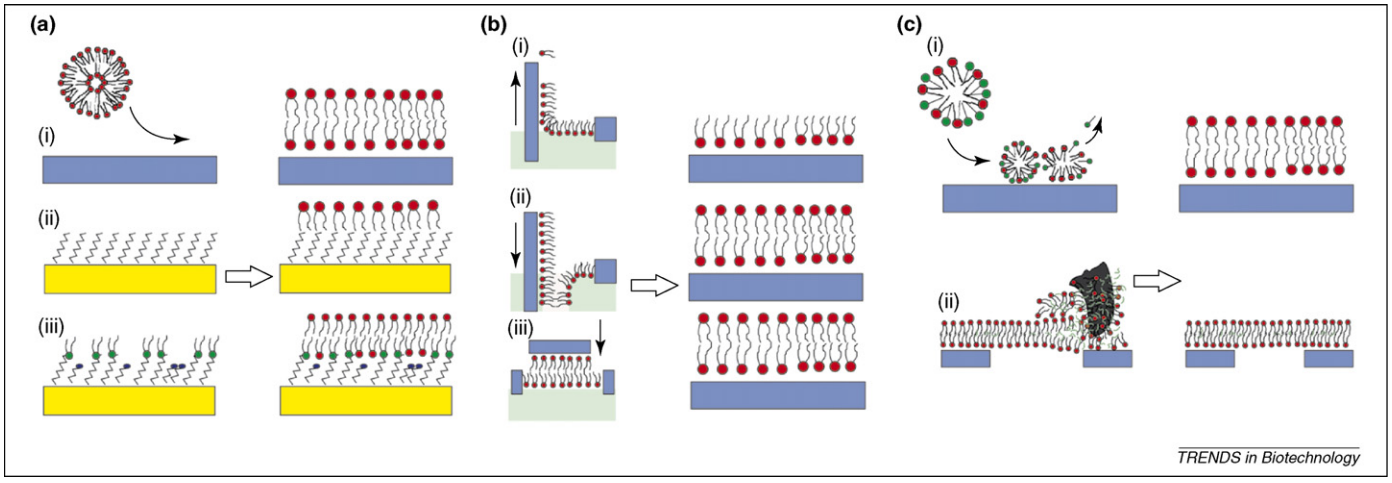

Figure 2. Methods used to assemble lipid membranes on sensor substrates. **(a)** Vesicle fusion (i) Self-assembly through vesicle fusion is a simple technique in which unilamellar lipid vesicles rupture upon contact with a surface and form a supported lipid membrane covering the interface [55–57]. Vesicle fusion occurs spontaneously on some hydrophilic substrates (e.g. silicon oxide and silicon nitride) to form planar bilayers [55,58]. (ii) On hydrophobic surfaces (e.g. pre-formed thiol-alkyl monolayers) lipid monolayers are formed instead of bilayers from lipid vesicles [9]. (iii) More complex tethers providing additional aqueous space under the self-assembled lipid layer such as hydrophilic spacers with covalently bound lipids (see Figure 1b) can be used to drive liposome fusion similar to (ii) [11]. **(b)** Langmuir-Blodgett techniques: (i) A motorised stage is used to move the substrate between an aqueous (green background) and a gas phase (white background). A lipid monolayer is held at a defined tension at the interface, which controls the packing density. Using a hydrophilic substrate and starting in solution, a lipid monolayer can be deposited by removal of the substrate [16]. (ii) Vertical reinsertion of the lipid monolayer formed in (i) through the interface deposits a (second) monolayer on top, which results in a lipid bilayer. (iii) If the substrate is instead taken through the interface horizontally the same result is achieved, but it is referred to as Langmuir-Schäfer deposition or sometimes as 'tip-dip' when capillaries are used [20]. **(c)** Detergent dialysis and painting. (i) Detergent dialysis: by forming micelles of lipids mixed with detergents, lipid material can be deposited from aqueous solution at a solid interface. The detergent is continuously removed from the micelles by dialysis leading to decomposition of the micelles and the formation of a planar lipid bilayer at the interface [12]. (ii) Painting and solvent extraction: a drop of organic solvent (solvent molecules depicted as green shapes in the membrane in the figure) containing dissolved lipids is added to a surface in an aqueous phase. The amphiphilic lipids will align at the solvent interface. When the solvent is extracted the lipids at the interface fuse to form a bilayer at the substrate–aqueous solution interface [17].

screening, biomimetic sensing and transmembrane protein research. Major steps have been taken with this approach in terms of sensitivity, versatility, ease of membrane formation and stability. Although a general means for functionalizing free-spanning membranes with transmembrane proteins still needs to be demonstrated, we discuss some novel approaches to overcoming this problem and for stabilizing and packaging free-spanning membranes for commercial biosensor applications. Together, these advances have the potential to enable long-term stable membrane functionalized sensor platforms with which continuous recording of charge transport even through single ion-channels and transporters can be achieved.

Demonstration of increased stability for free-spanning membranes

Reducing aperture dimensions for free-spanning membranes on a biosensor chip down to the nanometer range – as depicted in Figure 1c and a typical substrate shown in Figure 3b – has been envisaged as the most straightforward way to increase membrane stability compared with traditional black lipid membranes, which could span apertures of hundreds of μm to several mm. Increase in mechanical stability with reduced aperture size has recently been demonstrated by Simon *et al.* who compared lipid bilayers formed by the Langmuir-Blodgett technique (Figure 2b) across 1000 nm, 650 nm and 300 nm apertures

Table 1. Design criteria and current developments for free-spanning membrane biosensor platforms for commercial applications

Design criterion	Requirement	Current developments
Sensitivity	'Gigaohm seal' enabling measurements of single ion-channels and transporters	Single ion-channel sensitivity achieved (e.g. [26,45])
Stability	Membrane stable >1 day for screening applications	Achieved in several formats (e.g. [17,23,44])
Ease of formation of solvent-free membrane	Absolute reproducibility in formation by a parallel process. Requires self-assembly	Not fully solved but examples demonstrated (e.g. [18,21,23,35])
Ease of membrane protein insertion	Insertion of wide classes of membrane proteins without need for full purification and reconstitution of each protein. Should also include large proteins sensitive to unfolding	No general method demonstrated, but specific examples demonstrated (e.g. [33,35,37])
Robustness	Can be packaged and stored for months (or regenerated). Can withstand mild drying conditions, for example brief air exposure during sample mounting	Mechanical stabilization but not drying demonstrated. Membrane properties need further verification (e.g. [42,44])
Arrays	It should be possible to miniaturize the platform onto a chip with multiple sensing spots for parallel measurements both for multiplexed sensing and for parallel screening applications	Not demonstrated for small apertures but recent examples for black lipid membranes in microfluidic devices [51]
Cost	Needs to be economically competitive compared with less information-rich methods such as fluorescence assays on cell fragments for drug screening. Implies parallel measurements and that expensive reconstitution and handling of membrane components should be avoided	Not investigated

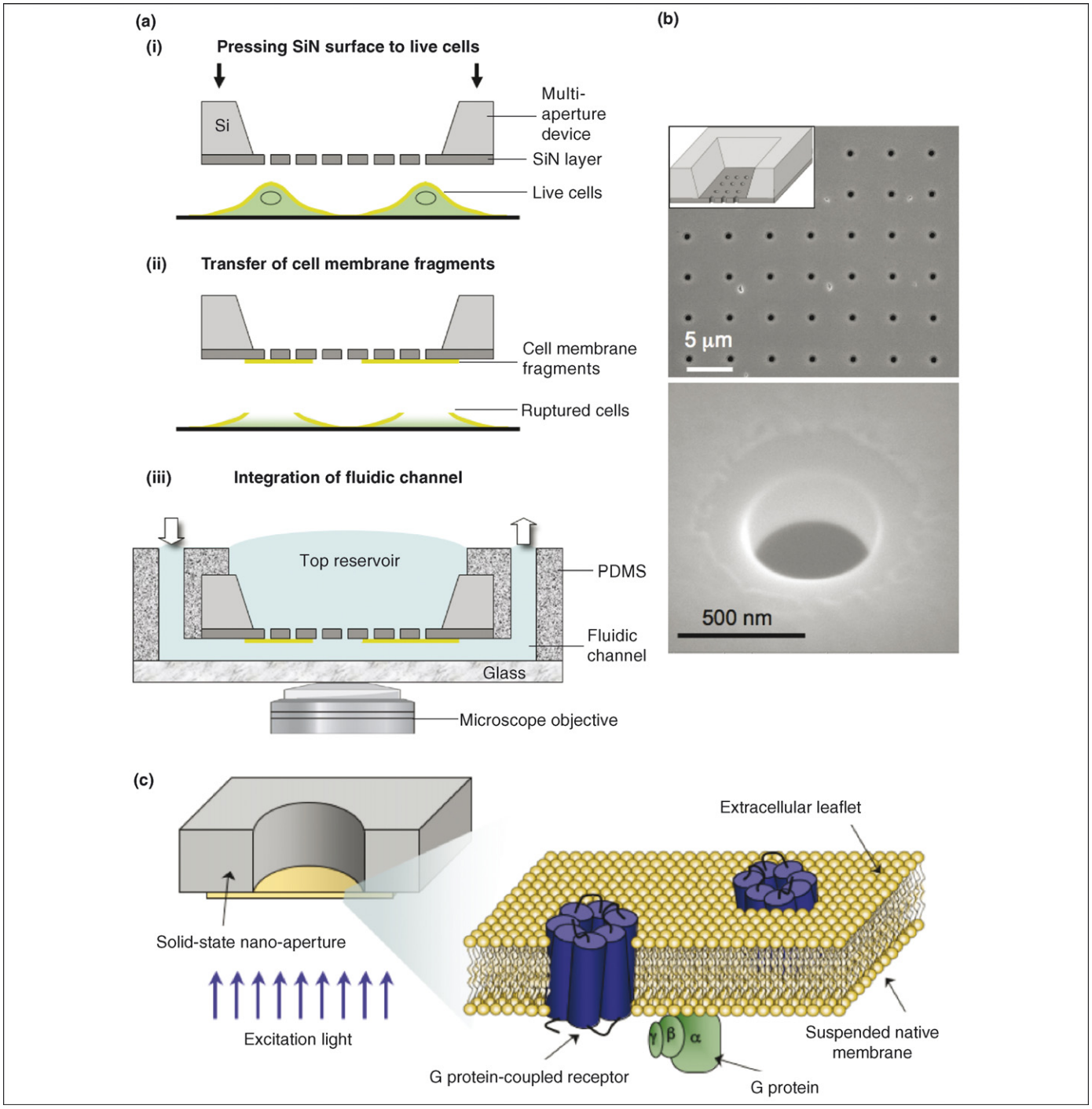

Figure 3. Platform developed by Vogel *et al.* for spanning cell membrane sheets on nanoporous sensor substrates integrated in a polydimethylsiloxane (PDMS) microfluidic device [35]. **(a)** Native membrane fragments are transferred onto the nanopore arrays by steps (i)–(iii). **(b)** Scanning electron micrographs of 5 μm lattice array (top) of 500 nm holes fabricated in a 500 nm thick silicon nitride film (bottom). The inset shows a scheme of the 3D structure of the silicon chip. Similar chip structures have been use in several other studies using apertures in the 100–1000 nm range. **(c)** Schematic view of a planar native membrane covering a nanoaperture. In this case, GPCRs already present in the native membrane were studied by fluorescence microscopy. More commonly, the free-spanning membrane is first formed from a synthetic lipid composition and ion channels inserted into the membrane, which allows for some measure of control of the density and position of the membrane protein. Reprinted, with permission, from [18,35]. Copyright 2006 American Chemical Society. Copyright 2006 Swiss Chemical Society.

in silicon [16]. The bilayers were tested under different mechanical stresses by increasing the force applied by an atomic force microscope cantilever. As predicted, bilayers spanned over 300 nm apertures were the most mechanically stable and had the longest lifetimes of ~24 h. Tiefenauer *et al.* [17] also recently tested free-spanning lipid bilayers over apertures in the same size range produced by solvent spreading (Figure 2c). Membrane resistance sufficient for single ion-channel sensing was achieved for 3–4 days for 200 nm free-spanning membranes and 800 nm free-spanning membranes showed the same resistance for 1–3 days. Although the membranes were robust and had high resistance, this method does not yield solvent-free bilayers. These studies demonstrate that membrane stability is improved for a given membrane–substrate system by reducing the diameter of the aperture and that

fabrication of nanoporous substrates for free-spanning membranes will yield high enough long-term stability (several days) for screening applications.

Slightly larger pores have been used by Vogel *et al.* who attempted to electrophoretically position and subsequently rupture giant lipid vesicles in microfluidic channels. For this system they demonstrated sufficient resistance for single ion-channel sensing using giant vesicles and planar bilayer membranes spanning a 2 μm aperture [18]. This work was a continuation of previous work demonstrating the same principles for membrane formation – including incorporation of alamethicin channels – over 0.6–7 μm apertures in silicon nitride membranes. In the earlier studies the electrochemical seal of the membrane was improved by covering the substrate with positively charged poly-L-lysine and fusing negatively charged liposomes [19]. Fusion by poly-L-lysine funtionalized surfaces has the drawback of not being a general approach for all membranes and is likely to induce significant distortion in the adsorbed membranes.

Sub-100 nm apertures enable self-assembly of durable solvent-free membranes

Solvent-free solid-supported lipid bilayers that will also be more compatible with sensitive transmembrane proteins can be formed by self-assembly from small and large unilamellar vesicles (Figure 2a) or by the more cumbersome Langmuir-Blodgett deposition techniques (Figure 2b). Homogeneous vesicle fusion is greatly facilitated by the use of unilamellar vesicles with a diameter of 200 nm or smaller. For the creation of free-spanning membranes by vesicle fusion, aperture diameters in the sub-100 nm range would be required, which would also further increase stability compared with the membranes described in the previous section for micron-scale apertures.

Cremer *et al.* recently demonstrated a method for decreasing the size of apertures down to the sub-100 nm range, investigating conical apertures molded into glass capillaries [20]. Bilayers were subsequently spanned using a 'tip-dip' method, which is comparable to the Langmuir-Schäfer method applied to an aperture (Figure 2biii). It was shown that the tip and the aperture were predominantly covered by a lipid bilayer. However, the most interesting observation was that decreasing the total membrane area surrounding a free-spanning membrane on a hydrophilic support carries a penalty. The 1–2 nm water film between the SLB and the substrate can carry a high ionic current. With the membrane formed only at the tip of a capillary this current dominated the electrochemical response and made this format unsuited for single ion-channel measurements. The seal was recently made sufficient by creating a hydrophobic sealing area on the glass capillary [21], but the platform will be difficult to miniaturize and integrate into a chip format.

Instead of single nanopores, Steinem *et al.* have developed a set of platforms based on nanoporous array substrates in the sub-100 nm range [22–28]. Leakage currents are avoided by spanning large areas consisting of densely packed pores. Large areas also enable working with lower protein concentrations or alternatively with proteins that have a low charge translocation rate, such as ion-transporters. Lipid membranes spanning the apertures have been formed by vesicle fusion (Figure 2a) and painting (Figure 2cii). To form membranes by solvent-free vesicle fusion, the substrate had to be rendered hydrophobic by a self-assembled monolayer (SAM). The hydrophobic surface provides enough driving force for formation of a lipid monolayer on top of the alumina scaffold and a lipid bilayer spanning the pores.

Membrane formation by painting also achieved good results on hydrophobized substrates and yielded higher surface coverage and membrane resistance than for vesicle fusion without notable loss of membrane stability. However, painted membranes still contain solvents used during their formation. Large arrays of free-spanning membranes were shown to be stable for tens of hours, with sequential rupture of the free-spanning membranes over the nanopores leading to only a slow reduction of membrane resistance. Various channel peptides and proteins have been studied with single-channel recordings on these platforms [25–27]. Favero *et al.* achieved comparable results on similarly hydrophobized nanoporous substrates with non-solvent-free membranes [29]. Ion-channel and receptor protein functionality was measured for up to 10 h and the blocking capacity of the membrane was observed for up to 6 days.

Incorporation of membrane proteins into free-spanning membranes

Insertion of transmembrane proteins into a newly formed free-spanning lipid membrane is one of the major challenges for membrane sensing platforms. Usage of apertures in the micron and sub-micron range necessitates control over the insertion and localization of single proteins to correctly analyze the results of single protein activity measurements. The most common method is the insertion of small ion channels directly from solution [2,4,27,30], but this method cannot be applied to the vast majority of membrane proteins.

Transmembrane proteins can be reconstituted after purification into liposomes. This process, although difficult, has been demonstrated for supported membranes [9,12]. The fusion of proteoliposomes either forming the supported membrane [31,32] or fusing to an already formed supported or free-spanning membrane [33,34] is regarded as the most promising approach for membrane protein functionalization of surface-based membrane platforms. Although the first approach has not been combined with electrochemical sensing, the latter approach has been demonstrated on free-spanning membranes by Morgan *et al.* using a salt gradient across the membrane and an antiobiotic-sterol pair to induce fusion. The use of proteoliposomes faces many technological bottlenecks, including the purification and reconstitution of the desired membrane proteins. Methods for direct insertion of membrane proteins into membrane platforms without the purification and reconstitution steps are highly desirable.

Vogel *et al.* worked on a novel approach in which cell membrane fragments were directly transferred onto a silicon nitride membrane patterned with arrays of nanopores [18,35] (Figure 3). The silicon nitride substrate was coated with positively charged poly-L-lysine. This substrate was

pressed against the negatively charged membrane of a cell, which resulted in the ripping off and attachment of native membrane fragments. Although it allows cell membrane proteins to be studied in a completely native membrane environment, this method at present lacks the specificity that would be necessary for a functional microarray of single proteins. Complete coverage of the pores, which is crucial for measuring membrane seal and membrane function, was not achieved, although it is possible in principle by way of further miniaturization of the chip.

Bayley *et al.* have developed methods for the insertion of channel proteins into pre-formed free-spanning lipid bilayers. This also avoids reconstitution but is compatible with any membrane-sensing platform. Purified single proteins are inserted into the free-spanning membrane by means of using an agarose dome at the end of a manually operated tip, which is brought into contact with the lipid bilayer [36]. The method was later refined to incorporate the use of a sharp (~5–50 μm in diameter) glass tip instead, with which channel proteins expressed in the outer membrane of *E. coli* could be harvested and transferred directly into the sensor membrane [37]. Although the lifetime of the sensor was not determined, rapid screening of 100 colonies of proteins was shown using this system [38], which demonstrates that rapid transfer of ion channels from cell membranes to sensing platforms is possible without reconstitution. However, this method is probably not suitable for larger and less robust transmembrane proteins.

Stabilization of free-spanning membranes

Supported membranes are disrupted by drying. In addition to this problem, free-spanning membranes suffer low temporal stability and typically rupture within hours even when kept hydrated To stabilize aperture-spanning membranes Sleytr *et al.* have developed a novel approach based on the so-called S-layer proteins found on the exterior of the *Bacillus sphaericus* bacterium. They formed continuous lipid bilayers by Langmuir-Blodgett deposition over micro-filtration membranes with pores of an average size of 400 nm, which had been pre-coated with an S-layer [39–41]. Characterized by impedance spectroscopy, the lipid bilayers showed considerably prolonged lifetimes ranging from 22 to 72 h. The dense protein layer thus provides a stabilizing scaffold that extends the life time of the suspended lipid membrane across the larger aperture, in the same way as the S-layer protein crystal stabilizes the *sphaericus* bacterium membrane. Several ion-channels were also successfully inserted into the bilayer. Lipid membranes have also been stabilized on top of filtration membranes by Nikolelis *et al.* by inclusion of monomers that can be polymerized to loosely encapsulate and stabilize the membrane after formation [42,43].

A different approach to stabilizing lipid membranes spanning large apertures by hydrogels has recently been demonstrated by two groups. After formation of a lipid membrane from a gelling solution containing agarose across an aperture 100 μm in diameter the membrane is sandwiched between two agarose gel films [44,45]. Self-inserting ion-channels could be added to the solution after membrane formation but before gelling [45]. Voltage clamp measurements on a single α-hemolysin pore have also been demonstrated [44]. Pore recordings could still be obtained 3 weeks after the membrane was formed and the stabilized membranes could be cut out and transferred to other sensing setups.

These advances are important steps towards membrane sensor platforms with the sensitivity and the necessary longevity for various commercial applications, such as in artificial tongues and in drug screening. In addition, the approaches using hygroscopic polymers to stabilize the membranes might also provide sufficient protection against drying in normal atmospheric conditions as has been recently demonstrated for supported lipid membranes [46,47]. This would greatly facilitate packaging of membrane sensor elements and increase shelf-life, both of which are important obstacles to commercialization.

Concluding remarks

The past few years have seen significant developments in nanoporous membrane sensor platforms, up to a point where these platforms seem to be the leading candidate for creating a viable chip format for functional transmembrane protein measurements. This is demonstrated by the current degree of fulfilment of the design criteria summarized in Table 1. The initial key advances have been the reduction of aperture size by nanofabrication to increase stability and the ease of formation of the lipid membrane. The optimal aperture size seems to be in the ~20–100 nm range, with the lower limit indicated by the scant success in using substrates with dense pores of only a few nm for electrochemical measurements on membranes [48–50]. Significant steps have also been made in the formation of stabilizing matrices around free-spanning membranes and the exploration of novel ways to incorporate membrane proteins into the sensor structure. These advances promise both robust and packagable commercial membrane sensors and sensors with a greater variety of membrane receptors and ion channels and/or transporters as functional components. Smaller but important steps have also been made in terms of surface functionalization protocols to promote lipid bilayer formation and analysis of single protein signaling (not discussed in detail here). Several of these developments have occurred in parallel, but remain to be integrated into a single platform.

In the near future we foresee further advances in inexpensive manufacturing of nanoporous sensor substrates driven by a need to find suitable alternatives to cumbersome and expensive techniques such as electron beam lithography. These will need to fulfil requirements for miniaturization and packaging, controlling factors such as chip integration, low leakage current and parasitic capacitance. An integration of solvent-free membrane preparation and membrane protein reconstitution techniques with nanopore-spanning membranes is also probable. In particular, the use of vesicle fusion protocols utilizing native cell membrane fragments is attractive in this respect. Direct use of native cell membrane material does not require protocols that expose the proteins to solvents and detergents and would thus be more reliable and cost effective for commercial applications. Finally, a move towards array-based sensing in which different

self-assembled membrane proteins are simultaneously screened in parallel measurements can be expected. The same development is needed for biosensors such as artificial tongues, in which many receptors have to be exposed and read out simultaneously to create the response. For research environments this might entail single-protein arrays, wherease for applications in drug screening the cost reduction upon densification of arrays will drive this development, as it has in the field of genomics.

In the longer term it is also possible that the sensing platforms would have to progressively evolve to include more of the signaling apparatus associated with *in vivo* membrane systems, because research focus is increasingly shifting from understanding single protein function to understanding signaling networks where protein–protein and protein–lipid interactions perform important regulatory functions. As more complex biological questions are addressed with increasingly sophisticated membrane structures it will become ever more important to use complementary characterization techniques for membrane processes. For example, electrochemical-signaling coding for function needs to be correlated with simultaneous interaction measurements that will use fluorescence microscopy methods or surface-sensitive optical techniques like waveguide spectroscopy. Several of the porous substrates discussed here are optically transparent and can be integrated in any chip-based design, which makes them compatible with most currently used assembly protocols and characterization and biosensor techniques. Thus, nanoporous substrates as sensor scaffolds are well poised to take on these extended challenges.

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