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Creating Scalable and Addressable Biomimetic Membrane Arrays in Biomedicine

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1. Introduction

Biomimetic membrane arrays that mimic biological cell membranes, with the ability to support membrane protein or peptide reconstitutions, are increasingly being recognised as an important platform for biomedical applications. High-throughput screening (HTS) systems based on membrane arrays may become an important alternative to cell-based screening of potential drug candidates on membrane protein targets (Fang *et al.*, 2006). The advantages of such membrane arrays are the ability to address specific drug-on-target interactions and to identify potential unintended effects on cell membrane properties or interactions with secondary unwanted proteins. The transport properties of channel proteins or peptides may also be utilized in novel sensor based platforms such as stochastic sensors for detection of organic molecules in solutions for use in medicine or environmental monitoring (Ashkenasy *et al.*, 2005; Capone *et al.*, 2007; Gu *et al.*, 1999; Nikolelis & Siontorou, 1996).

Provided that the effective membrane area can be scaled sufficiently, protein channel-based membrane arrays may be applied in larger scale biomedical applications. An example is aquaporins, which are water selective proteins that function to filter water, for example in the mammalian kidney. Aquaporin-based large scale biomembranes may be envisaged as the new generation hemodialysis systems for kidney patients, or be applied in general water purification systems.

Biomimetic membrane peptide or protein based arrays are however not currently applied in commercial biomedical or biotechnological applications. While creation of a single lipid bilayer membrane across a Teflon aperture is a well-established technique, the creation of biomembrane arrays comprises a relatively new concept in the scientific field of biomimetics. The reasons are amongst others associated with the inherent difficulties of reproducible creating planar suspended membranes and a generally low stability of established biomembranes. Moreover, amongst the general challenges in biomimetic membrane design is scale up of membrane effective areas to create stable and addressable membrane arrays with long lifetimes (> days).

This chapter will give an overview of recent advances in the development of planar biomimetic membrane arrays, and will discuss strategies and general challenges for creating stable and scalable biomembranes for use in biomedical applications.

2. Biomimetic membrane design

Current planar membrane designs include vertically and horizontally positioned arrays in a chamber or device, which typically relies on membrane arrays being established either by manual, robotics or microfluidic techniques. The choice of design may depend on the nature of the membrane molecule to be incorporated (peptide or protein) and the biomedical application in question.

2.1 Membrane array scaffolds

The fabrication method as well as membrane array geometries are important parameters to consider when designing chambers and devices for sensor and separation applications based on biomimetic membrane arrays.

Membrane proteins function among others to facilitate passive-mediated or active transport of small molecules and substances across the membrane, or function as receptors mediating intracellular signal transduction pathways upon extracellular ligand binding to the receptor. To utilize membrane protein function in model membrane designs, suspended membranes may be created that allow for transport processes to take place across the artificially made membranes. A membrane scaffold supporting planar suspended membrane array formations is illustrated in Fig. 1A.

To create medical screening platforms or microarray assays, the multi aperture scaffold may further be embedded in a polymer-matrix to create individually well-defined wells as is known from microtiter plates or immobilized soluble protein dot-blot microarrays. The design illustrated in Fig. 1B shows a composite half-sandwich scaffold design with well-defined wells. The matrix may be designed to be porous to maintain ion and solute diffusion across the established membranes. This is necessary if electrophysiological measurements of receptor or protein channel properties are included in the design as a read-out parameter.



Fig. 1. Biomimetic membrane array designs using micro-structured ethylene tetrafluoroethylene (ETFE) as scaffold. A) ETFE membrane scaffold for freely suspended planar membrane arrays. B) Composite half-sandwich membrane scaffold consisting of an ETFE partition partly embedded in a porous support structure to create individually well-defined wells (grey). C) Complete composite scaffold sandwich structure. Shown in the illustrations are the ETFE membrane scaffold (green), surface modifications of the ETFE scaffold (yellow), biomimetic membranes (red), proteins (white), aqueous layers or hydrogel polymers (transparent) and porous supportive structures (grey).

To support applying a hydrostatic or an osmotic pressure across the membrane, separation applications based on protein channel properties require that the established biomimetic membrane arrays are stabilized by a complete sandwich composite structure (Fig. 1C). As illustrated in Fig. 1, the membrane array scaffold can be created as a modular design based on the actual aperture scaffold and from this design multi composite/encapsulated scaffolds may be created depending on the design criteria.

Single aperture partitions can be created by various mechanical methods such as micro drilling, needle puncturing (Ginsburg & Noble, 1974), heated wire (Benz *et al.*, 1975; Montal & Mueller, 1972; Wonderlin, Finkel & French, 1990) or electrical sparks (Minami *et al.*, 1991). However, common for these methods are that they are generally not suitable for fabricating scaffolds comprising an array of apertures. The reasons are that these methods cannot produce consistent aperture sizes and position the produced apertures closely and precisely, and moreover these techniques have tendencies to create groin and burr edges that do not support stable membrane formations.

Methods described suitable for the fabrication of membrane scaffold arrays include hot embossing of silicon wafers (Heyderman *et al.*, 2003), lithography techniques (Le Pioufle *et al.*, 2008; Mayer *et al.*, 2003; Suzuki, Le Pioufle & Takeuchi, 2009), UV excimer laser ablation (O'Shaughnessy *et al.*, 2007; Sandison & Morgan, 2005) and CO_2-laser ablation (Vogel *et al.*, 2009). The ability to produce consistently sized and closely positioned apertures are important parameters to enable successful formation of stable membranes in array. Of the three mentioned techniques for creating highly defined aperture arrays, the CO_2 laser ablation technique is likely the most versatile and cost efficient technique. It has the ability to ablate Teflon films with different thicknesses (micrometers to >1 mm), enable fast scaffold production times (milliseconds-seconds) and support easy scale up. Fig. 2 shows rectangular and hexagonal aperture scaffolds, respectively, micro-structured with the CO_2 laser ablation technique.

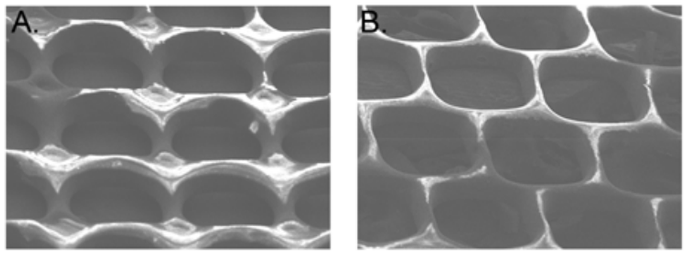


Fig. 2. Scanning electron microscopy images of CO_2 laser fabricated ETFE multi-aperture scaffolds. Images show middle sections of A) Rectangular 8×8 aperture array and B) Hexagonal 8×8 aperture array. EFTE micro structuring was performed as described by Vogel *et al.* (Vogel *et al.*, 2009).

2.2 Chamber designs for membrane array formation

There exist numerous chamber designs to encompass a membrane array scaffold, albeit there are common features relating to the strategy of membrane formation. Fig. 3 schematically illustrates some of the chamber design strategies recently developed in our laboratory, and we will discuss current trends and common features in chamber designs from these examples.

The vertical chamber design strategy (Fig. 3A, D) is a classical chamber design approach originally described for painting or folding a lipid bilayer across a Teflon partition aperture (Montal & Mueller, 1972; Mueller & Rudin, 1969). This design provides easy access to the chambers via wells from the top of the chamber and to each side of the established membranes. This allows for addition of solutes (e.g. creation of osmotic gradients), substances (e.g. ligands), transmembrane peptides, membrane proteins, liposomes or proteoliposomes close to established membranes. At the same time it allows for sample collecting via the accessible top chamber wells. In this manner, the horizontal chamber design has, among others, been applied to characterize vesicle fusion events with planar artificially made membranes (Kendall & MacDonald, 1982; Perin & MacDonald, 1989; Woodbury & Hall, 1988a; Woodbury & Hall, 1988b; Zimmerberg, Cohen & Finkelstein, 1980b). The hydrophilic dye calcein was used as a traceable marker that was encapsulated into lipid synaptic vesicles and added to one side of the membrane (Zimmerberg, Cohen & Finkelstein, 1980a). Membrane fusion events with the established planar membrane resulted in calcein release to the other side of the membrane, which could subsequently be sampled and the fluorescent calcein content quantified (Zimmerberg *et al.*, 1980a).

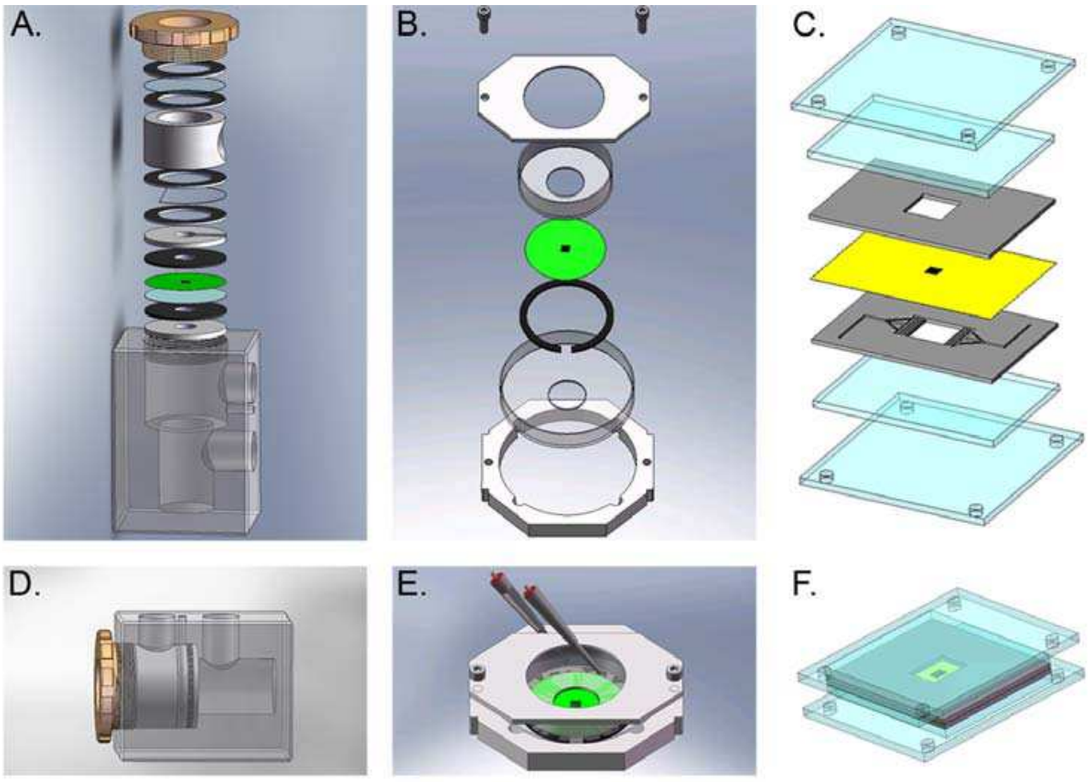


Fig. 3. Chamber designs for creating biomimetic membrane arrays A), D) Automation technique chamber design strategy for establishing vertically oriented membrane arrays (Hansen *et al.*, 2009b). B), E) Horizontal chamber design that supports combined optical–electrical measurements of established biomimetic membranes (Hansen *et al.*, 2009a). C), F) Automated microfluidic chamber design for microfluidic filling and establishment of biomembrane arrays (Kamila Pszon-Bartosz *et al.*, manuscript in submission).

We recently developed a vertical chamber based on the classical design (Fig 3A, D), where the membrane formation strategy was modified to comprise a novel membrane array formation technique; the so called automation technique for the establishment of vertically positioned membrane arrays (Hansen *et al.*, 2009b). Electrophysiological recordings across the membrane demonstrated that functionally membrane arrays were created in this design. Moreover, this technique supported membrane formations of 5×5, 8×8 and 30×21 arrays having average aperture diameters of 300 µm (Hansen *et al.*, 2009b).

In general, the vertical chamber design allows for electrophysiological recordings across the membrane, but the simultaneous visualization of established membranes by surface sensitive techniques such as fluorescence microscopy is not straightforward in this design. Therefore, the current trends in chamber design are directed towards the development of horizontal chambers that fit, or can be adapted, into modern array scanners (Le Pioufle *et al.*, 2008; Suzuki *et al.*, 2009) or fluorescent microscope stages (Hemmler *et al.*, 2005; Wilburn, Wright & Cliffel, 2006). Such designs are typically created to support more than one read-out parameter such as having voltage-clamp read-outs combined with optical imaging.

Membrane array formation in horizontal chambers is typically carried out manually by painting the membrane array across the scaffold or by applying microfluidic techniques to establish fully automated membrane formations. The rationale behind manually painting membranes onto scaffold arrays is that it may be adapted to robotic-based membrane deposition techniques, such as robotic array spotters or printers, or be re-designed to include microfluidic membrane formation techniques.

The chamber fabrication time and the material costs are important parameters to ensure that biomimetic membrane based arrays are made economically feasible for the pharmaceutical industry or creating commercially available medical point-of-care microdevices. Therefore, preferred biomimetic membrane designs comprise single-use chambers or microarray devices that are based on low-cost materials, easy to produce and which are easy and efficient to handle. Our suggestions of how to meet these design criteria are illustrated in Fig 3B-F. Fig 3B, 3E illustrate a single-use chamber design based on clamping membrane scaffold arrays between 35-mm and 50-mm culture dishes, whereas Fig. 3C, 3F show a fully automated and closed microfluidic device based on poly(methyl methacrylate) (PMMA), in which all materials are cut and micro structured by CO_2 laser ablation.

2.3 Considerations of membrane design criteria

Membrane design criteria should preferably be defined on the basis of the biotechnological application in question. A commonly accepted membrane quality criterion is that established membranes should exhibit >1 Giga-Ohm sealing resistance in order to achive low ion leakage. (Reimhult & Kumar, 2008). This is however a somewhat misleading membrane quality criterion. Ohmic sealing that may be obtained for a given membrane is inversely related to the effective membrane area, meaning that >1 Giga-Ohm seals cannot practically be achieved with large membrane arrays. Instead, for large biomimetic membrane arrays it therefore makes more sense to define membrane quality as membranes having a large effective area as evidenced by a large value for the electrical capacitance and low ionic permeability as evidenced by a low value for the electrical conductance compared to the effective membrane area.

Another important design criterion for biotechnological/pharmacological applications may be peptide or protein reconstitution yield, because this likely depend on the application.

Less peptides or proteins are likely needed to create a sensitive screening platform in drug discovery compared to creating a membrane based separation technology. Thus when setting up design criteria, strategies and goals for the peptide or protein reconstitution yield need to be taken into consideration.

Additional design criteria for HTS systems or mass transfer flow applications may include a high perforation level of the membrane scaffold material so that the artificial membrane platform is scalable to meet various requirements for individual technical applications (Hansen *et al.*, 2009b). For example functional membrane units can be arranged in arrays to facilitate rapid screening (e.g. by microplate readers).

Membrane stability is a key parameter to be considered for biomimetic membrane based devices. There is a general consensus that biomimetic membranes should have lifetimes for > 1 day (Reimhult & Kumar, 2008). This will also depend on the application in question and on whether a membrane-based assay relies on the end user to create the membrane arrays as a step in the assay protocol, or if the membranes will be fully assembled in ready-to-use devices before reaching the end user. In addition, the membranes or precursor membrane solutions should exhibit transportation robustness and be storable for defined time periods.

The methodology for membrane formation should be considered during the design of novel biomimetic sensor and separation platforms. This may also relate to cost efficiency and feasibility to enter a competitive market. Membrane formations by robotic spotting techniques is likely more expensive than microfluidic-based membrane formations, but robotic deposition techniques may be designed for an application where the total cost would still allow for a competitive product. Thus biomimetic membrane device fabrication processes and materials costs should be considered as a whole during product development.

3. Formation of functionally stable and scalable membrane arrays

Although, it is straightforward to set up specific design criteria for a given biomimetic based platform technology, there are several inherent challenges of biomimetic membrane formations that tend to make it difficult or challenging to meet defined design criteria in practice. Challenges with poor membrane stability, limited scalability and low membrane formation reproducibility must be solved in order to create a general commercially available biomimetic membrane based platform technology.

3.1 Biomimetic membrane stability

Poor membrane stability is a recognized challenge with artificially made membranes. This is an even more pronounced general challenge when working with arrays of biomimetic membranes. To understand why poor membrane stability is a general challenge, it is necessary to realize the properties and dimensions that apply for artificial biomimetic membranes.

The lipid bilayer is only a few nanometers thick and varies with the acyl chain length from 4-10 nm for natural occurring phospholipid species (Lewis & Engelman, 1983; White & Thompson, 1973). The partition scaffold is typically in the range of 20 to 50 micrometers in thickness, meaning that the aperture scaffold is thousand times thicker than the lipid bilayer. Lipid bilayers established across partition apertures are therefore surrounded by an annulus of thick parent lipid solution to compensate for the dimension differences between scaffold and bilayer thickness (White, 1972) (Fig. 4). Solvents such as alkanes (e.g. *n*-decane) are typically used to precondition the scaffold to membrane formations; so called partition

prepainting. It is believed that the solvent of the preconditioning step and/or the solvent present in the lipid bilayer slowly diffuses from the annulus, resulting in membrane destabilization and eventually membrane collapse (Malmstadt, Jeon & Schmidt, 2008).

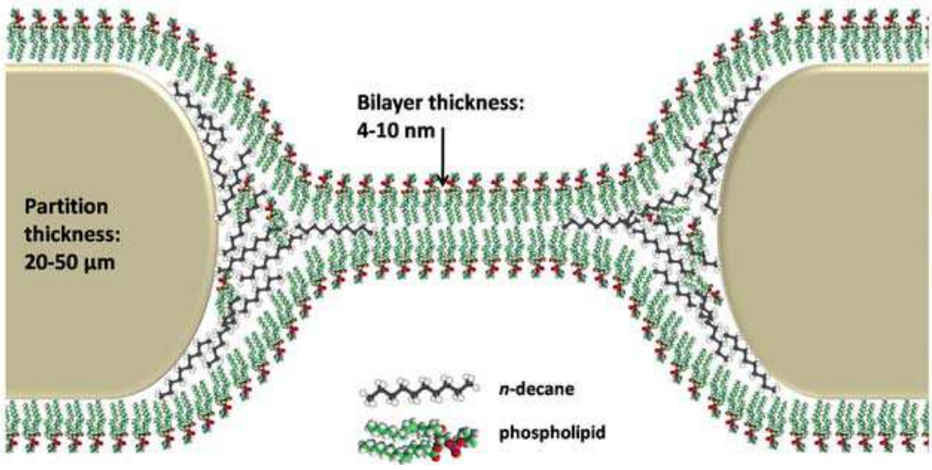


Fig. 4. Schematic illustration of typical dimensions of a lipid bilayer and partition aperture. Typical lipid bilayer thicknesses are 4-10 nanometers (nm), whereas partition thicknesses generally range from 20 to 50 micrometers (µm). The figure is not drawn to scale.

Malmstadt *et al.* showed that membrane stability may be significantly increased (> days) by stabilizing the membrane surroundings by hydrogel encapsulation, which was explained to result from a slowing down of the solvent diffusion out of the annulus, thereby prolonging the membrane lifespan (Malmstadt *et al.*, 2008). This approach is promising and may also be crucial for creating stable and portable devices.

We noticed that the typical partition preconditioning step resulted in inhomogeneous coverage of the preconditioning solution on the partition. Since the membrane stability is dependent on sufficient hydrophobic interactions between the bilayer forming solution and the partition scaffold we speculated that a more homogenous surface pretreatment coverage could result in increased membrane stability (Hansen *et al.*, 2009b). To investigate this, we developed an airbrush technique to homogenously cover the partition with preconditioning solution. This resulted in a markedly increased reproducibility in membrane formation, but did not increase the membrane lifetimes correspondingly (Hansen *et al.*, 2009b).

Ries *et al.* showed that the membrane electrical characteristics, dynamics of membrane formation and the membrane stability are strongly dependent on the partition substrate (Ries *et al.*, 2004). Inspired by this, we studied the effect of covalently modifying the partition substrate using surface plasma polymerization (Perry *et al.*, submitted). By this technique we were able to increase the membrane stability significantly. Using double-sided *n*-hexene partition surface modifications we were able to increase membrane lifetimes from an average of 100 min (Hansen *et al.*, 2009b) to average membrane lifetimes of approx. 70 hours, while 20% of established membranes lasted 140 hours (Perry *et al.*, submitted). These results underline that long term stability of established biomimetic membranes is critically dependent on a sufficient interaction with the hydrophobic surface of the partition and the bilayer forming solution.

Another approach to increase membrane stability has been based on biomimetic membranes consisting of semi-synthetic or synthetic biomimetic polymers. It was recently demonstrated

that 8×8 arrays of triblock copolymers could successfully be established by the automation technique for creating biomimetic membrane arrays (Gonzalez-Perez *et al.*, 2009). Membrane stability could be achieved with lifetimes up to 23 hours. Also cross-linkable lipids have been suggested to being able to increase membrane stability (Benz, Praß & Ringsdorf, 1982; Daly *et al.*, 2006; Shenoy *et al.*, 2005), but more work is needed to show if this strategy may sufficiently increase membrane lifetimes.

Besides the membrane annulus and the biomimetic membrane composition, the aperture diameter is also a crucial determining factor for membrane stability. The membrane stability generally increases with decreasing aperture diameters. This has motivated designs based on nano-sized biomimetic membrane arrays (Han *et al.*, 2007; Hemmler *et al.*, 2005; Studer & Tiefenauer, 2007). The nano-sized aperture diameters should in principle favour long lived membranes, but they may also increase the risk of creating non-functional membranes, because the nanoscale aperture diameter may preclude sufficient membrane thinning.

An impressive silicone nitride chip array comprising 960,000 nano-membranes has been developed (Han *et al.*, 2007). The membrane lifetimes achieved using this technique were up to 144 hours. These lifetimes are comparable to our best membrane array lifetimes using plasma polymerization as pretreatment. In comparison the total membrane effective area of 0.045 cm^2 for the previously described 8×8 arrays is about 150 times larger than the total membrane area in the silicon nano-membrane chip array. Practical use of small nano-sized chip arrays could be in microelectronic devices or novel nanotechnology applications.

While membrane stabilities of > 1 day can be achieved with recent advances in biomimetic membrane research, it is still difficult to create storable and transportable biomimetic membrane devices. However, recent developments in membrane encapsulation strategies suggest that robust portable biomimetic membranes may be created (Jeon, Malmstadt & Schmidt, 2006; Kang *et al.*, 2007; Malmstadt *et al.*, 2008; Oliver *et al.*, 2008; Uto *et al.*, 1994). Efforts are none the less still required to create a general stable and transportable biomimetic membrane design.

3.2 Membrane array scalability

A general biomimetic membrane platform supporting different biotechnological applications would preferably be scalable to meet various application requirements. Fig. 5 illustrates scalability in biomimetic design. Biomimetic membrane scalability is not straightforward, and also represents a new concept in formation of biomimetic membrane arrays. The tendency in biomimetic membrane work has actually been to scale down the designs. The reason is likely related to the inherent membrane instability, but recent advances in membrane stability (as discussed in section 3.1) have led to the acceptance that scaling up the arrays may indeed be practically feasible. In this relation, we recently demonstrated that scaling up from rectangular 8×8 arrays (64 membranes) to rectangular 24×24 (576 membranes) or hexagonal 24×27 (648 membranes) arrays may actually provide an overall higher membrane stability evidenced by significant longer lifetimes (Hansen *et al.*, 2009a).

An emerging concept, relying on a high degree of membrane scalability, is novel separation technologies based on reconstitution of functional membrane protein channels. A study of the solute transport characteristics and permeability of aquaporin water selective channels incorporated into polymer vesicles indicated that the water permeability and salt rejection of aquaporin based biomimetic membranes would potentially represent a novel separation

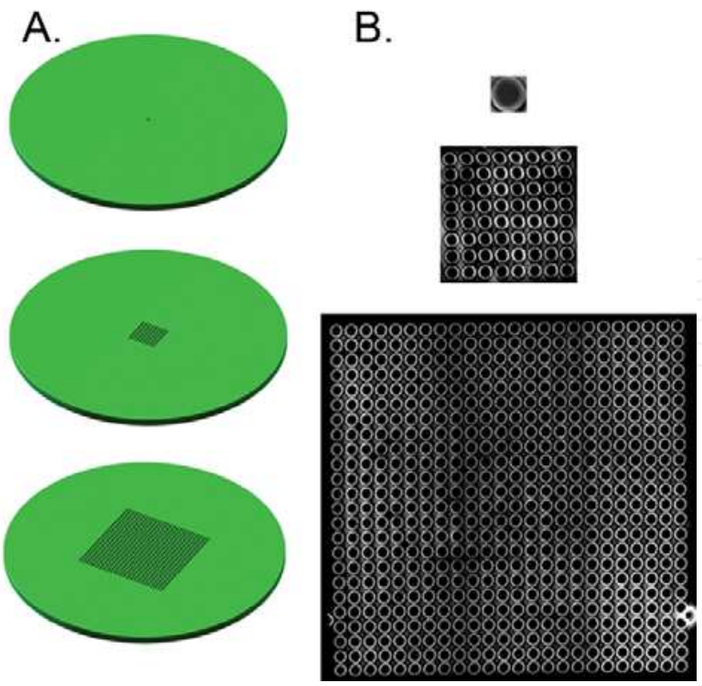


Fig. 5. Illustration of scalability of biomimetic membranes A) Schematic presentation of scalable aperture scaffolds with 300 μm diameter apertures supporting establishment of suspended biomembranes. B) Fluorescent images of established membranes in ETFE microstructured scaffolds corresponding to panel A). Established lipid membranes of 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine were doped with the fluorescent lipid analogue 1-oleoyl-2- [6-[(7-nitro-2-1, 3-benzoxadiazol-4-yl) amino]hexanoyl]-*sn*-glycero-3-phosphocholine (NBD-PC) for visualization. Membranes shown in panel B) were established in the chamber design depicted in Fig. 3B and 3E. The membranes of panel B) are not shown to scale.

technology that would be able to exceed current reverse osmosis and forward osmosis membranes in performance (flux and salt-rejection) (Kumar *et al.*, 2007). Novel membrane protein based separation technologies may be explored, provided that the biomimetic membrane effective area can be scaled sufficiently. Extended research activities in biomimetic membrane scalability is therefore required to produce first generation biomimetic membrane based separation technologies.

3.3 Membrane addressability in membrane arrays

Biomimetic membrane arrays offer a platform for generating large membrane protein arrays, where a lot of information can be achieved with extremely low sample volumes (Suzuki & Takeuchi, 2008). A unique feature of biomimetic membrane arrays is that it offers the opportunity of multiplexed measurements on several levels within the same technology platform.

The trend in biomimetic membrane design is to create chambers that support electrical recordings of membrane, protein or peptide electrical properties combined with fluorescence microscopy. The most straightforward designs include voltage-clamp measurements of an entire array to ensure that functional membranes are established

(Hansen *et al.*, 2009a; Hemmler *et al.*, 2005; Wilburn *et al.*, 2006). In principle the electrical voltage clamp recordings could be adapted to individually address the membrane electrical properties of a membrane array. The concept of multiplexed electrical recordings was explored in a proof-of-concept study by Suzuki *et al.*, demonstrating that each well of a 96-wells microplate format could be electrically addressed (Suzuki *et al.*, 2009). Each well of the 96-wells plate however comprised a membrane array of 3×3 membranes, and individually membrane addressability was not addressed in this study, but would in principle be achievable in the presented design.

A solution to enable individual addressability of membranes of a biomimetic membrane array could be achived by creating a microelectrode array that would be positioned beneath the membrane scaffold. A microelectrode array has been successfully created for electrochemical detection of soluble enzyme activities (Lin *et al.*, 2008). Although the microelectrode array was demonstrated using immobilized soluble proteins, there is no principal hindrance in adapting this concept to biomimetic membrane array designs. Fig. 6 schematically illustrates how such microelectrode arrays may be envisaged to be adapted in biomimetic membrane designs in order to create individually addressable membranes.

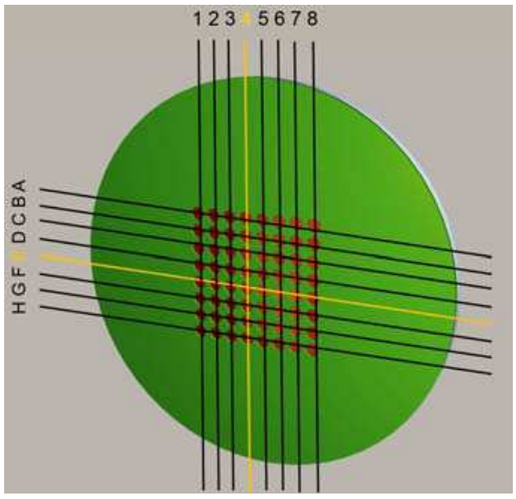


Fig. 6. Schematic illustration of how electrical multiplexing of biomimetic membrane arrays may be designed. Shown are the aperture partition (green), biomimetic membranes (red), electronic grid (black and yellow lines). The yellow lines show the individually addressed membrane of the array (where line E crosses line 4).

Electrical membrane array multiplexing using microelectrode arrays combined with in situ fluorescence assays would offer results read-out on two levels. The latter has been demonstrated on solid supported protein immobilized G-protein coupled receptor (GPCR) microarrays, where receptor-ligand interactions could be detected using fluorescently-labelled ligands (Fang, Frutos & Lahiri, 2002; Fang, Lahiri & Picard, 2003).

A third level of biomimetic membrane readouts could be created by detection of small solutes, ions or other substances transported across the membrane facilitated by proteins or peptides reconstituted into the biomimetic membrane arrays. Hemmler *et al.* demonstrated this principle in practice by visualizing transport of the aqueous calcein fluorescent dye across individual membranes reconstituted with α-hemolysin membrane protein pores (Hemmler *et al.*, 2005). Next generation of individually addressable membrane arrays could

be envisaged to comprise ligand-receptor assays, where extracellular binding of ligand to receptors would lead to a secondary signaling pathway on the other side that subsequently would lead to a fluorescent or colorimetric signal, which could be quantified. For microfluidic devices the chamber outlets could be connected in line with fluorescence or absorbance detection, or alternatively connected to HPLC instruments for quantifying solutes or molecules. Functional demonstration of such novel conceptual ideas are however still to be proven in practice.

To sum up, a lot of information may be achieved with extremely low sample volumes from a single biomimetic membrane array by designing platforms that enable multiple results read-out. Such design strategies may aim at combining microelectrode multiplexing, in situ fluorescence assays and quantification of transport processes across the membranes by sample collecting followed by solute determination or concentration measurements.

3.4 Incorporation of membrane peptides and proteins into membrane arrays

Functional incorporation of membrane molecules into biomimetic membranes is essential to create peptide or membrane protein based sensor or separation applications using biomimetic membrane arrays.

Small fusiogenic membrane spanning depsipeptides or peptides generally insert spontaneously into established membranes, and may in many instances be dissolved in aqueous buffers, or other solvents (e.g. alcohols), that can be added directly to established membranes (Zagnoni *et al.*, 2007). The ease of incorporation is one of the main reasons why they are often used to demonstrate that functional biomimetic membranes have been successfully established. In this sense incorporation of small fusiogenic peptides constitute a quality control parameter for functional biomimetic membrane formation. Moreover, some fusiogenic peptides may be relevant in biotechnological and biomedical applications such as for creation of ion-sensing electrodes or biosensors for detection of small solutes and analyte molecules (Borisenko, Zhang & Woolley, 2002; Capone *et al.*, 2007; Frant & Ross, 1970; Nikolelis & Siontorou, 1996; Schar-Zammaretti *et al.*, 2002).

In contrast, medium to large membrane proteins (35-500 kDa), especially α-helical membrane proteins, do generally not readily self-insert into pre-established membranes (Zagnoni *et al.*, 2007). Although, the light-driven α-helical proton-pump bacteriorhodopsin (BR) as well as several *E. coli* outer membrane (β-barrel) porins (e.g. OmpA, OmpF and FomA) may be reconstituted into planar membranes directly from a detergent solubilized state (Arora *et al.*, 2000; Pocanschi *et al.*, 2006; Schmitt, Vrouenraets & Steinem, 2006), this is general not applicable for most membrane proteins. Therefore, a general reconstitution methodology is required for incorporation of membrane proteins into biomimetic membranes preserving correct protein function (e.g. ensuring correct orientation in the membrane).

Another, largely unresolved challenge is how to reconstitute different proteins into individual membranes to create large membrane protein microarrays, as is known from commercially available DNA microarrays or immobilized protein dot-blot arrays.

4. Biomedical application of biomimetic membranes

The function of a biomimetic membrane array depends not only of successful reconstitution of membrane proteins in stable host lipid/polymer membranes. Also the sensitivity or

signal-to-noise ratio in the output-signal of the device must be high in order to allow for detection of analyte concentrations down to the single-molecule level. Thus sufficient amplification of the signal is vital for biomedical application of biomimetic membrane devices. This amplification generally arises as an intrinsic amplification in the biomimetic membrane material per se combined with external amplification.

Due to the relative ease by which pA currents can be resolved many biomimetic membrane based sensor platforms have built on incorporation of ion channels (Nielsen, 2009). The linear gramicidins present a versatile system that can be easily engineered. The preferred conformation of gramicidin in lipid bilayers is a $\beta^{6.3}$ helical fold of the pentadecapeptide. Upon dimerization of two gramicidin monomers from each opposing lipid monolayers an ion conducting dimer permeable to monovalent cations is formed. This system has revealed many basal features of ion channel function, for reviews see (Andersen & Koeppe, 2007; Koeppe & Andersen, 1996). In a freestanding bilayer gramicidin monomers diffuse randomly in each monolayer leaflet and dimerization is a random process. When a transmembrane potential is applied over a membrane separating two aqueous electrolyte compartments dimerization is evident as discrete amplitude changes in the recorded current-trace corresponding to appearance and disappearance of ion conducting dimers.

Sensors based on matrices with engineered gramicidin channels have been presented (Cornell *et al.*, 1997; Wiess-Wichert *et al.*, 1997). These sensors operate by changing the conformational equilibrium between gramicidin monomers and dimers. Concretely the sensors are built with a lower lipid monolayer tethered to an electrode and a mobile upper lipid monolayer where each monolayer leaflet contains engineered gramicidins. The gramicidins in the lower monolayer are covalently tethered to the electrode substrate whereas the upper monolayer gramicidins are covalently linked to specific antibodies moieties which can recognize specific analyte molecules. Another set of antibodies are covalently linked to the upper headgroup of bilayer spanning bolalipids with the lower bolalipid headgroup linked to the electrode substrate. This arrangement effectively anchors the bolalipid-linked antibodies relative to the gramicidin monomer-linked antibodies in the upper monolayer.

If no analyte molecules are present, the conformational equilibrium between monomers and dimers results in a randomly fluctuating current with a mean value effectively dependent on the total gramicidin concentration. When an analyte is present it may cross-link antibodies attached to the mobile outer layer channels with those attached to membrane spanning bolalipid-based tethers. The result is a decreased mean value of the fluctuating current as the outer monomers now are 'captured' by the analyte mediated crosslinking and therefore not available for dimerization with their immobilized lower monolayer channel partners. The increase in the effective transmembrane resistance (equivalent to a decrease of membrane admittance) with time provides a means to estimate the concentration of the analyte.

Gramicidin channels are low molecular weight peptides and unique in the sense that channel function (i.e. transfer of ions across the membrane) depends on dimerization of two (identical) monomers. In general membrane spanning ion channels are high molecular weight oligomeric structures with large hydrophilic moieties where the oligomeric interactions may depend only on weak interactions. However, recently the voltage-gated HERG potassium channel has been successfully reconstituted in biomimetic membranes tethered on mercury showing that large 'bulky' channel forming oligomeric proteins can be functional in a confined cushion geometry (Becucci *et al.*, 2008).

Ion channel gating (i.e. opening and closing of the ion conducting pathway) is a result of complex conformational changes in the protein. Although our structural understanding of ion channel gating is still limited, sensing devices based on detecting ion channel gating has been proposed. For example the ligand-gated nicotinic acetylcholine receptor (nAchR) ion channel (with acetylcholine as the 'natural' ligand) has been reconstituted in free-standing lipid bilayers (Boheim *et al.*, 1981; Eray *et al.*, 1995) as well as in lipopetide supported biomimetic membranes (Schmidt *et al.*, 1998). Using nAchR modified with two bispecific antibodies the channel remains open until both antibodies bind to the same antigen (Eray *et al.*, 1995).

Also the rectifying voltage gated Kv1.5 potassium channel has been reconstituted in free standing and solid supported membranes (Dhoke *et al.*, 2005; Matsuno *et al.*, 2004). The current/voltage relations display symmetric sigmoidal shapes. This highlights one of the major challenges in biomimetic membrane design based on reconstituted ion channels. The sigmoidal I/V relationship indicates that approximately half of the channels are inserted so they are rectifying the ionic current in one direction and the other half is rectifying currents in the opposite direction. For some application orientational randomness is problematic as membrane proteins generally have distinct intracellular and extracellular binding sites – thus for a sensor based on ligand detection directional control over protein insertion is imperative. However by careful optimization reconstitution procedures a more or less pronounced unidirectionality may be achieved.

The application of gated ion channels in biosensors exemplifies how signal amplification occurs both intrinsically (e.g. the binding of a single ligand gives rise to currents with 10^6 ions/second per channel) and externally though e.g. the I/V conversion and amplification of the current signal in voltage-clamp amplifiers. As we gain more insight into channel gating through more high-resolution structures of ion channel proteins in various conformational states the use of complex ion channel proteins in biomimetic membrane sensors may evolve from an 'all-or-nothing' type of response to more complex read-outs based on detecting ion channel sub-conductance states.

Ion channels represent one important class of biomedical 'targets'. Another important class is comprised of GPCRs. GPCRs generally detect molecules outside the cell and initiate downstream signalling in the form of a cascade of biochemical reactions leading to changes in cellular function. Since GPCRs are targets for more than 50% of all medicinal drugs there is a huge interest in understanding GPCR mediated signalling. Although the signalling process is generally well-described it is also very complex because it can involve several GPCRs simultaneously and the signalling may also occur by other pathways not requiring G-proteins. Also different ligands can result in different signals from the same GPCR depending on the cell type and *vice versa:* the same ligand can result in different signals in different cells. This complexity presents a major obstacle in our understanding of GPCRs but recently an elegant biosensing method has pointed to a way of overcoming some of the complexities. The sensor is based on coupling ion channels with GPCRs. Thus when the GPCR binds an agonist, its conformation changes, and this changes the structure of the coupled ion channel. (Moreau *et al.*, 2008). The conformational change in the GPCR is thus transduced into a change in ion channel current. In order to make this (and other ion channel based methods) technologically feasible electrical measurements must be integrated with membrane array designs allowing for parallel current recordings.

Another strategy is to take advantage of the electrical properties of bilayers and use them as insulating surfaces. Any defect in this surface is easily detectable as a change in impedance

and as the defect locations create strong non-specific binding sites the sensitivity of such a device is high (Steltze, 1993). Impedance analysis on supported lipid bilayers can also be used to dissect the action of channel forming peptides e.g. the bee venom melittin (Becucci *et al.*, 2006a), the potassium specific valinomycin (Becucci *et al.*, 2005), and channel forming proteins e.g. the bacterial outer membrane porin Omp F (Becucci, Moncelli & Guidelli, 2006b) on the bilayer. This approach has also been used in black polymer membranes (BPMs) where protein driven energy transduction was realized by incorporation of cytochrome c oxidase (COX) (Ho *et al.*, 2004).

Light driven transport across membranes constitutes a particular interesting biosensing mechanism behind the design of membranes for energy conversion and advanced photoresponsive/optical devices (LaVan & Cha, 2006). BR and halorhodopsin (HR) are examples of light-driven ion pumps for protons (Oesterhelt & Stoeckenius, 1973) and chloride (and other halide) ions respectively (Essen, 2002; Schobert & Lanyi, 1982). BR occurs natually in highly ordered two-dimensional arrays (purple membranes) in *Halobacterium salinarium* (Oesterhelt & Stoeckenius, 1973). BR may be reconstituted in proteoliposomes (Kayushin & Skulachev, 1974; Oesterhelt & Schuhmann, 1974) but direct adsorption of purple membrane fractions onto suitable substrates takes direct advantage of the natural two-dimensional layout of BR in the native membrane (Ganea *et al.*, 1998). This approach has been refined by forming lipid membranes on a porous alumina substrate and then adsorbing purple membrane patches onto the membrane (Horn & Steinem, 2005). Also HR (Essen, 2002; Varo, 2000) has been reconstituted in proteoliposomes (Duschl, McCloskey & Lanyi, 1988) and lipid bilayer membranes (Bamberg, Hegemann & Oesterhelt, 1984).

Biomimetic sensing with reconstituted rhodopsins rely on an optical input, but changes in optical properties of supported biomimetic membranes may also be used as output signal. For example the application of supported membranes in the design of biosensors mounted on electro-optical devices is attracting considerable interest. Using surface plasmon resonance (SPR) allows for real-time measurements of ligand binding to immobilized proteins (Löfås & Johnsson, 1990) and thus opens for the possibility to detect ligand binding to membrane spanning proteins. Immunosensing can be seen as a special case of ligand binding sensing and detection of *Staphylococcus* enterotoxin B (SEB) in milk has been demonstrated in a microfluidic system with supported bilayer membranes with biotinylated anti-SEB IgG (Dong, Scott Phillips & Chen, 2006).

Although most work on biomimetic membrane sensors is based on incorporating membranes proteins, some sensor designs may also be realized in protein free systems. A recent example is a membrane supported by nanoporous aluminium oxide providing a high surface area and a protective environment against dewetting (Largueze, Kirat & Morandat, 2010). The membrane contains polyethylene-glycol (PEG) conjugated lipids as hydrating, protective and tethering agents and ubiquinone which is a naturally occurring redox lipophilic mediator embedded within the acyl chains of the lipid bilayer. The sensing system is based on cyclic voltammetry and can detect alterations of lipid membranes that are induced by the addition of surfactants (exemplified by the commonly used non-ionic detergent: Triton X-100). Biomedical application of this system could for example be screening of pulmonary surfactant candidates, monitoring enzymatic degradation by lipases, and studying peptide bilayer insertion.

Another method relies in the use of infrared absorption spectroscopy (IRAS) measurements. A recent study combining IRAS and voltage-clamp has demonstrated changes in specific regions in spectra obtained with solvent containing biomimetic membranes formed with

D_2O in the electrolyte solutions providing insight into membrane formation (Hirano-Iwata *et al.*, 2009). An OD stretching peak (arising from D_2O) appeared immediately after lipid application (painting). The band intensity increased with time with a concomitant decrease in bandwidth which could reflect gradual changes in the ordering of interfacial water molecules. Also specific bands could be assigned to CH*x* stretching modes of acyl chains. The intensity of these bands was about ten times higher than that of the C=O modes of PC. Thus these CH*x* bands likely arose from *n*-decane rather than from phospholipid acyl chains. The band intensities decreased with time, suggesting that *n*-decane was slowly expelled. These results demonstrate that IRAS can detect self-thinning of the lipid solution to form biomimetic membranes, the resulting expulsion of the *n*-decane solvent, and reordering of interfacial D_2O. By extending this methodology combined IRAS-voltage-clamp may be used to detect spectroscopic signals due to specific conformational changes in lipid acyl chains induced by pharmaceutical compounds as well as a biomedical screening assay for investigating the properties of naturally occurring (antioxidant) membrane residing solvents e.g. ubiquinone.

5. Future research

By virtue of mimicking cellular membranes, model systems based on one or few lipid species and reconstituted proteins have attracted considerable interest in biomedical research since the first appearance of black lipid membranes in the 1960ties. With the recent advances in nanotechnology over the last two decades this interest has now manifested itself in laboratory model devices with multiparameter detection of membrane dynamics and protein function. Recent developments in membrane design have led to the concept of biomimetic membrane arrays that may provide powerful HTS assays in drug discovery and in creation of novel separation technologies based on membrane peptide and protein function.

A reason that biomimetic membrane array devices are not already commercially available is related to the difficulty of creating stable and transportable devices. Although, major efforts have resulted in increased biomimetic membrane stability and lifetimes (several days to weeks), further improvements are still required. Especially transportation robustness needs further attention. With further developments in multi composite materials, sandwich structuring and encapsulation strategies storable and transportable biomimetic membrane based biomedical devices appear feasible.

While general protocols for reconstitution small fusiogenic membrane-spanning depsipeptides and peptides are relatively well-established, general reconstitution strategies are urgently required for controlled and reliable incorporating medium to large membrane proteins (35-500 kDa) that do not reliable self-insert into established biomimetic membrane arrays. Moreover, designs that support reconstitution of different membrane proteins into individual membranes would enable fabrication of membrane peptide or protein microarrays similar to current DNA microarrays and protein dot-blot arrays.

With the recent advances in biomimetic membrane array design and with further developments of biomimetic designs to comprise laboratory-on-a-chip (LOC) and micro-total-analysis systems (μTAS) the ultimate goal of industrially fabricated devices for drug-discovery, toxicologial testing, and other biomedical/pharmaceutical applications based on biomembrane function seem within reach.

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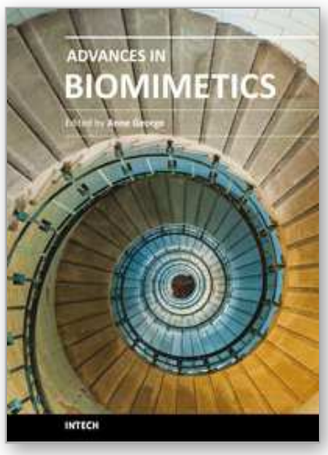
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