Nano for bio: Nanopore arrays for stable and functional lipid bilayer membranes (Mini Review)

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The usefulness of nanotechnology for biotechnological applications is frequently emphasized. The recent development for using nanostructured materials as supports for free-standing lipid bilayers is briefly reviewed. The authors then demonstrate that the stability of fragile free-standing lipid bilayers in nanopores is enhanced up to days depending on the surface chemistry, the lipid composition, and the diameter of the pores. The insertion of a pore forming protein into bilayers can be monitored over time as a stepwise decrease of membrane resistance. Since membrane proteins are major drug targets, such stable and functional proteo-bilayers integrated in microfluidics are the key components of *in vitro* devices for drug screening. This conference paper reviews the recent literature and provides preliminary results from own research. © *2008 American Vacuum Society.*
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I. REVIEWING RESEARCH ON SUPPORTED LIPID BILAYERS

A. Motivation for research

From a chemical point of view the idea of life is the separation of microscopic aqueous chambers by lipid bilayers to enable complex concerted (bio)chemical reactions. The main internal reaction chamber of a cell, the cytoplasm, is separated from the outside world by such a membrane, which consists of a typical lipid composition. Furthermore, bilayers form compartments within the cell, which carry out various functions, i.e., energy production in mitochondria, formation of new cellular structures in the endoplasmic reticulum, and controlled degradation of metabolic and structural biomolecules in lysosomes. Lipids spontaneously self-assemble in an aqueous environment due to their amphiphilic nature. However, biological membranes are much more than simple lipid bilayers.[1] Integrated proteins can act as gates and selectively transport ions across these barriers; others regulate metabolic reactions or communicate with the outside world. The complex interaction and dynamic regulation of membrane proteins is presently poorly understood and is a major topic of systems biology.[2]

Genome analysis dramatically extended our knowledge about membrane proteins. From the human genome sequence analysis the number of membrane proteins can be assessed to about 8000, which is approximately 30% of all human genes. The majority of membrane proteins have several hydrophobic helical domains, which span lipid bilayers. This structural motive has been deduced from x-ray diffraction patterns of protein crystals. The atomic 3D structure allows us to understand protein functions. However, the crystallization of membrane proteins is very difficult. In addition, post-translational modifications occur in mammalian cells resulting in glycosylated proteins, which are even more difficult to crystallize. Thus, from the 176 presently known unique membrane protein structures (Aug. 2007), less than 10 from *homo sapiens* are of a sufficiently high resolution to understand their function.[3] These represent only about 1% of the current 1160 membrane protein families, which can be categorized according to their function in receptors, transporters, ion channels, pumps, signal transducers, lipid metabolic enzymes (transferases), mitochondrial (oxidase), and structure proteins (nuclear porins).[4] This illustrates the importance of membrane proteins in sustaining life functions.

Modern biology and molecular medicine aimed at understanding the dynamic processes of life at a molecular level. Therefore, it is of pivotal importance to understand quantitatively the binding processes of natural effectors, i.e., of hormones and metabolic species to membrane proteins. Such data provide the basic knowledge that enables us to modulate their function. For an effective selection and design of artificial effector compounds, both structural information and quantitative analytical methods are required. Thus, functional assays for membrane proteins are needed to screen libraries of effector compounds that contain potentially useful drug candidates. Such versatile assay systems have a high commercial potential, since membrane proteins are main drug targets. In summary, for research as well as for drug development robust, simple and cheap assay systems are required for measuring the function of membrane proteins.

B. State-of-the art of supported bilayer systems

Membrane proteins are macromolecules consisting of a linear polyamide backbone chain of 100 up to 1000 condensed amino acids and their side-chains. The backbone forms to a great extent secondary structures such as alpha-helixes and beta-sheets, which are further combined to domains.[5] Such functional domains are rather rigid when assembled in bundles of alpha-helices or in sandwiches. The interaction between structural elements of the domains is the basic principle for the specific function of membrane pro-

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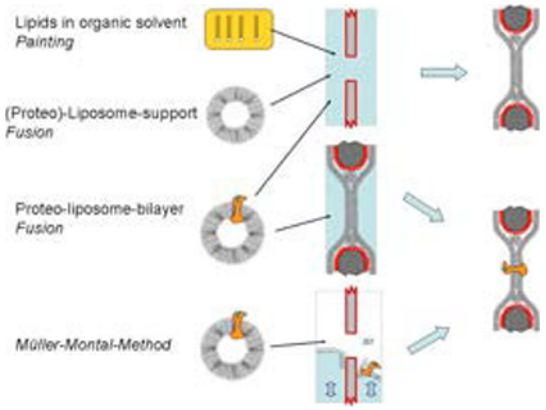

FIG. 1. The formation of planar lipid bilayers can be achieved by four different *methods* as sketched. Functional membrane proteins will be integrated in planar bilayers either by fusion of proteo-liposomes to preformed bilayers or by direct fusion of proteo-liposomes to nanopores. Using these methods the surface density of membrane proteins can easily be controlled. The Müller-Montal method is used to prepare and investigate the stability of planar proteo-bilayers as presented in Fig. 5.

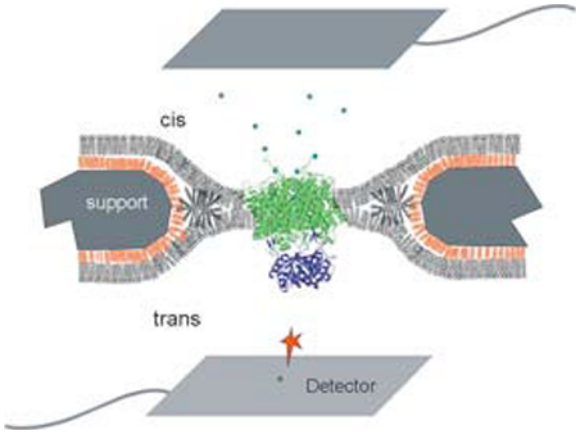

FIG. 2. Free-standing planar lipid bilayers in one pore of a nanopore array support for functional assays of membrane proteins. As a typical membrane protein the ammonium transporter AmtB (Ref. 63) (green) and the inhibitor GlnK (Ref. 64) (blue) are shown, which regulate the passage of ammonium/ammonia molecules (small circles) from cis- to trans-side of the membrane. Note that the three loops of GlnK closely interact with AmtB and a free access at the trans-side is required.

teins. For instance, the conformational changes of amino acid residues in the selectivity filter of a K^+ channel[6] result in a dislocation of pore helices and an opening of the vestibule followed by a specific flow of the K^+ ions across the embedded macromolecule. Thus, a sufficiently high flexibility is often a prerequisite for the function of an integrated membrane protein.[7] The snuggling of lipids to the proteins embedded in fluid bilayer membranes is poorly understood, but is often of pivotal importance for their function.[8] Therefore artificial bilayers must retain the high flexibility of the integrated proteins.

In the fundamental work of Müller[9,10] and Montal[11] planar lipid bilayers were formed on supports with a small aperture (0.1–1 mm), which separate two compartments. Addition of liposomes to the buffer in the compartments results in spread lipid monolayers at the air-water interface. When the buffer levels are raised alternatingly above the aperture, a lipid bilayer is formed (Fig. 1). Such so-called black lipid membranes (BLMs) were very helpful to investigate the effect of peptides integrating into bilayers.[12,13] Since BLMs are notoriously fragile,[14] a race began in the late 1980s to make them more stable. Several groups started to immobilize lipid bilayers on solid surfaces,[15–17] aimed at achieving systems suitable for analytical applications.[18] In developing stable and functional supported bilayers, one main topic of the TETHMEM conference meetings, researchers encountered the difficulties of squaring the circle. A stabilized bilayer is rigid and consequently not suitable to keep membrane proteins in a functional state, whereas bilayers of natural lipid mobility are usually not stable enough. The lateral mobility of lipids in the bilayer is calculated from the recovery time of fluorescent dye-labeled lipids required to refill a small photobleached spot in the bilayer.[19] In order to achieve stable *and* fluid lipid membranes, chemists have synthesized new organic molecules to bind lipid bilayers on surfaces by molecular tethers.[20–23] Alternatively, thin hydrophilic polymer films have been spread on the surface to generate a small aqueous gap between the bilayer and the surface.[24,25] It could be shown that the mobility of lipids in such molecular constructions is comparable to that of biological membranes and that even membrane proteins can be integrated in a functional state.[17,26–29] However, a major drawback remains for analytical applications: The very small space between the bilayer and the support (about 2 to 10 nm) is rapidly saturated by the transported species.[30] This makes quantitative dynamic measurements difficult.

The great potential of emerging micro- and nanofabrication methods for topologically structuring surfaces was recognized about 10 years ago[31] and the expectations for bioanalytical applications are promising.[32] When free-standing planar lipid bilayers are spanned over nanopores, their stability will presumably be enhanced and both sides are accessible (Fig. 2). Since such nano-BLMs strongly resemble BLMs in apertures of micrometer dimensions in Teflon sheets, the biophysical properties, especially the fluidity of the membrane will be similar.

C. Recent research activities using nanostructured supports

In the last 5 years different nanostructured surfaces were used as supports for lipid bilayers.[33] Painting lipids on single microfabricated pores with diameters of 50 to 200 μm resulted in free-standing lipid bilayers to which, in a second step, proteoliposomes have been fused by using a glass rod. From such micro-BLMs the activity of integrated ion channel proteins could be recorded.[34] The authors found an increase of the specific capacitance with smaller pore sizes (up to a factor of 2) and explained this by a higher fraction of bilayer compared to other systems where a larger solvent torus, the so-called annulus,[35] is observed. On hydrophobic

surfaces the organic solvent is presumably excluded to a higher degree. It remains unclear if the observed increased capacitance is attributed to the surface chemistry or to the pore size. In a systematic study comparing planar free-standing bilayers on Teflon chips with pore diameters ranging from 25 to 250 μm diameter,[36] the specific capacitance of the bilayers was almost constant at about 0.6 μF cm^{-2}, a value reported for BLMs[9] and tethered bilayers.[37] Such bilayers are stable for many hours even at applied voltages up to 400 mV. The ion channel activity of alamethicin and alpha-hemolysin (α-HL) could be recorded at a high signal to noise (S/N) ratio. The advantage of a high S/N ratio was also observed for bilayers generated by painting lipids on single micropores of 5 to 500 μm diameter in Teflon sheets.[38] However, the stability of such bilayers was limited to about 1 h under the applied potential of 150 mV. The authors[38] concluded that recording from multiple planar lipid bilayers in parallel would be an attractive approach for high throughput screening. A remarkable increase of stability of DPhPC-bilayers in micropores using an agarose gel has recently been reported.[39]

Polycarbonate membranes as used for ultrafiltration are a commercial source for nanoporous supports. The pores in these membranes are of uniform size of about 1 μm and they are irregularly arranged.[40] Such polycarbonate membranes have been covered with a gold layer to which thiolated alkane molecules were immobilized. After immersion into a phosphatidylcholine (PC) solution, free-standing bilayers were spontaneously formed in the pores and the effect of ion carrier insertion as well as the activity of the ionotropic glutamate receptor were recorded.[41] However, the stability of the PC-bilayers was only about half an hour and could be enhanced to about 10 h with 25% cholesterol. Although the feasibility of functional assays for membrane proteins has been demonstrated,[40,41] ultrafiltration membranes have some severe limitations: (1) The achieved sealing of about 10 MΩ cm^2 is too low for sensitive measurements;[33] (2) the pore density is rather low; (3) the pore distribution is random, resulting in merged pores in which bilayer stability may be affected; (4) the membrane is about 10 μm thick, leading to a high aspect ratio (pore size to membrane thickness) and a hindered diffusion across the support as it can be suggested from the slow response time; and (5) the stability of coated gold on the polymer carbonate may be too low for commercial applications.

Nanoporous alumina membranes were frequently used by Steinem's group to support free-standing lipid bilayers.[42–46] The formation of bilayers was confirmed by electrochemical impedance spectroscopy (EIS) analysis. An adequate equivalent circuit model is a prerequisite for fitting the measured data points aimed at providing resistance and capacitance values of the membrane. These two main parameters allow us to compare the quality of lipid bilayer preparations. For complex preparations consisting of thin layers it is difficult to find the adequate model, since the capacitance value is indirectly proportional to the thickness of the layer, i.e., molecular lipid monolayers contribute much more to the capacitance value than the thicker support. Furthermore, it has to be considered that the capacitance contributions of free-standing bilayers in nanopores and of the parallel bilayers on support between the pores are additive. Therefore, the exact pore area has to be known for a precise calculation of the specific bilayer capacitance. In their earlier work, the expected capacitance value of 1 ± 0.2 μF cm^{-2} was obtained for bilayers formed from giant vesicles that fused to nanopores of 20 and 50 nm diameter.[42] In a further development the nanoporous alumina supports have been coated with gold, allowing the immobilization of thiolated lipids as anchors for lipid bilayers consisting of the plant lipid diphytanoyl-sn-glycero-3-phosphatidylcholine (DPhPC). The stability of the so-called nano-BLMs was monitored by determining the membrane resistance value over time. The initial membrane resistance of 7 GΩ drops to 2 MΩ during 120 h. This high membrane resistance and the sufficiently long stability permit measurements of single channels.[43] Upon insertion of single bacterial outer membrane protein F (OmpF), trimers are formed in preformed nano-BLM on 60 nm pores and different opening states of OmpF channels could be discriminated by current measurements. At a holding potential of −100 mV, steps of 170 pA currents have been determined, and the corresponding conductance value of 1.7 nS obtained by statistical analysis has been interpreted as single ion channels.[45] Smaller current steps were detected during the so-called subconductance state and in the presence of the antibiotic ampicillin. These experiments show that single transmembrane proteins can be monitored using BLMs supported on nanoporous surfaces.

II. PRELIMINARY RESULTS USING NANOPORE ARRAYS TO SUPPORT LIPID BILAYERS

Some years ago a high throughput fabrication technique was reported for chips with a thin silicon nitride membrane in which nanopores are arranged in regular arrays.[47] Such nanopore arrays in a silicon nitride membrane were hydrophobically functionalized and bilayers are formed by the painting method (Fig. 1). It was found that painted lipid bilayers were exceptionally stable.[48] In a comparative study it has been demonstrated that the stability of free standing bilayers on 800 nm pores depends on the nature of the lipid. Bilayers formed from the cylindrically shaped phosphatidylethanolamine (PE) lipid exhibited a membrane resistance above the high threshold of 1 GΩ for more than 1 week. Bilayers of the plant lipid DPhPC and the common lipid 1-palmitoyl-2-oleoyl PC (POPC) were above the threshold for 2 days whereas bilayers of the naturally occurring soy PC mixtures were stable for only about 2 h. By reducing the pore size to 200 nm, the stability of POPC-bilayers was increased by about 50%, whereas soy PC-bilayers were now stable for 3 days, which is an increase by a factor of 30. A high stability of natural lipid mixtures in nanopores is very important, since most membrane proteins may require defined mixtures of lipids to remain functional. A frequently used and relatively simple quality test for bilayers is the insertion of ionophores. The functionality of DPhPC-bilayers

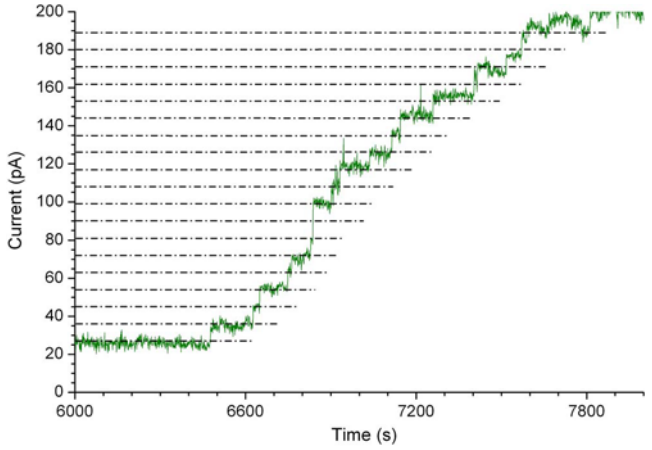

FIG. 3. Monitoring the formation of individual heptameric α-hemolysin pores in preformed painted planar POPC-bilayers present in 960 000 pores of 200 nm diameter arranged in arrays.[48] The current increases in steps of 9 pA after a long lag time. Experimental condition: 200 nM α-hemolysin in 150 mM KCl at an applied voltage of 50 mV.

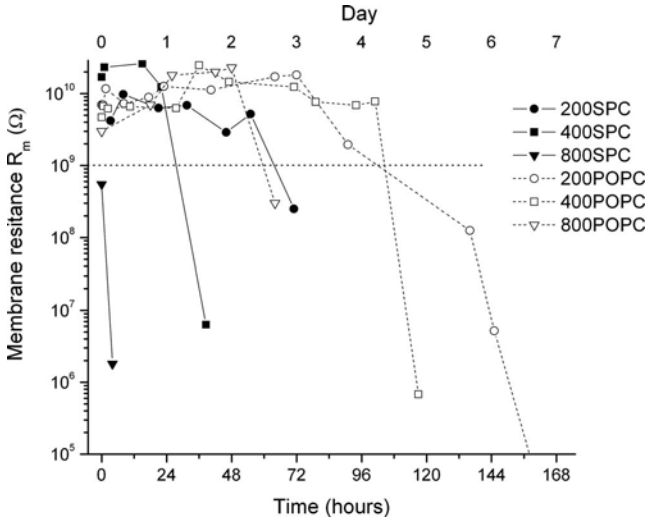

FIG. 4. Stability of bilayers of soy PC (SPC, ●, ■, ▼) and POPC (○, □, ▽) on nanopore arrays of different pore diameters (200 nm ●, ○, 400 ■, □, and 800 nm ▽, ▼). The dotted line indicates the very high threshold of 1 GΩ above which bilayers are present in all pores of the support. If only one pore opens, the membrane resistance drops to 200 MΩ (data not shown). Bilayers consisting of POPC are more stable than those of SPC, which need a pore diameter of 400 nm (■) or smaller to maintain a sealing above 1 GΩ for 1 day.

on 800 nm pore arrays has been demonstrated by adding valinomycin and subsequently increasing concentrations of KCl, resulting in a gradual decrease of the membrane resistance.[48] Furthermore, formation of protein pores in preformed POPC-bilayers can be monitored over time (Fig. 3). After a long lag time, which depends on the pore size and the α-HL concentration, the conductance of the lipid bilayer increased by steps of 9 pA under the indicated conditions. This value is in agreement with the reported single α-HL pore current of 7 pA determined at 30 mV and 250 mM KCl,[49] a recent study using variable conditions. The benefit of using nanopore arrays for monitoring single molecule signals may rely on at least two factors: the stabilization of lipid bilayers and the high S/N ratio as reported for small apertures[38] as confirmed by the low observed noise (see Fig. 3). For practical applications in drug screening the measurements of multiple events resulting in higher signals may be required.

Nanopores as support for free-standing bilayers are probably useful for commercial functional assay systems of membrane proteins. The fluidity of the free-standing bilayers within the pores is probably comparable to that of biological membranes and as a consequence transmembrane proteins will be mobile. The observed 30 times increase in stability by reducing the pore size by a factor of 4 demonstrates the benefit from using nanostructures. From an extended systematic study (Fig. 4) it can be concluded that a pore size $<$400 nm will probably be necessary to form stable lipid bilayers of natural lipid mixtures. Thus, reliable and simple methods for the fabrication of nanopore arrays support are required.

In addition to a small pore diameter, a suitable surface chemistry is required to stabilize free-standing bilayers. Thiol-compounds strongly bind to the gold surface, resulting in the observed high bilayer stability. However, the use of the gold-thiol combination also has two major disadvantages: (1) Coating of gold and preferentially of an adhesion layer of Cr are two additional steps. (2) The gold-thiol bond is prone to degradation by oxidizing agents. For practical applications a fast, simple, and reliable procedure is needed to form stable planar proteo-bilayers. Peptides and pore forming membrane proteins spontaneously insert into preformed bilayers. However, reconstitution of the majority of other membrane proteins is demanding and their integration in bilayers is a major challenge. The mentioned Müller-Montal method allows us to form bilayers from lipid[39] monolayer with integrated proteins without using organic solvent to dissolve the lipids. We found that bilayers formed from prokaryotic lipid mixtures in this way were less stable than painted bilayers (Fig. 5). Fur-

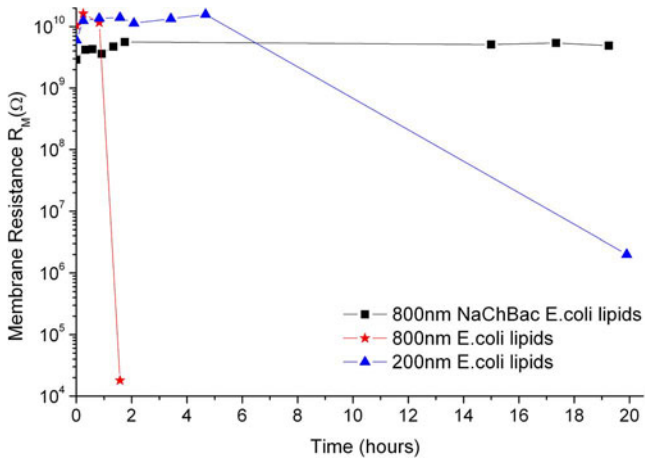

FIG. 5. Stability of free-standing (proteo)-lipid bilayers generated from proteoliposomes according to Müller and Montal.[9] A sodium channel (NaChBac) from *B. halodurans* was reconstituted in a lipid mixture of *E. coli* using beta-octyl glucoside and Biobeads for detergent depletion. The best of three preparations for each condition are shown. Note the effect of the membrane protein on the stability of the planar lipid bilayers.

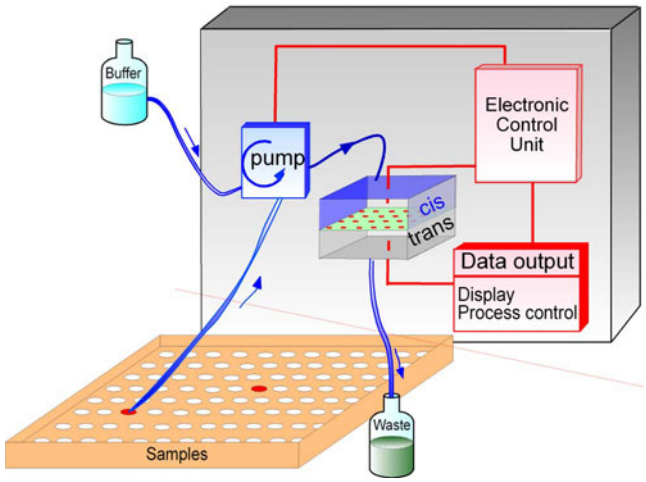

FIG. 6. Scheme of a microfluidic system for monitoring the function of membrane proteins in drug screening.

thermore, the stability of these bilayers is also higher in smaller pores and when membrane proteins are integrated therein (see ∀ in Fig. 5). However, the preparation of functional bilayers for screening applications must be much simpler. The formation of stable bilayers upon addition of proteo-liposomes to nanopore arrays should be achieved within minutes. Although many groups reported about fusion processes of (proteo)-liposomes to planar unstructured supports,[50–57] little is known about the influence of nanopores on the fusion of proteoliposomes of a similar size (see Fig. 1). Again, the influence of liposome composition and surface chemistry has to be investigated in a systematic way to find a suitable procedure for membrane protein immobilization.

III. OUTLOOK

For practical applications, planar proteo-bilayers need to be integrated in a system that allows (1) automated addition of reagents, (2) continuous control of the bilayer membrane quality, and (3) detection of the membrane protein activity (Fig. 6). Such automatic microfluidic systems have been developed for biosensors in general and recently also to generate lipid bilayers.[58–62] The formation of bilayers can be achieved by a controlled pumping of a low concentration of the lipid dissolved in decane to the cis-side whereas the trans-side is filled with electrolyte. Upon alamethicin and α-HL insertion into bilayers,[60] signals with a high S/N ratio have been recorded at a clamped voltage of 60 and 40 mV, respectively.[61] This experiment demonstrates that the mentioned three tasks can be performed by a relatively simple microfluidic system buildup of the commonly used material polymethylmethacrylate (PMMA).

The free access to both sides as shown in Fig. 1 opens new possibilities also for optical detection of molecules and ions transported across lipid bilayers by a membrane protein of interest. Since diffusion may not be impeded by nanopores with a low aspect ratio, the transport of chemical species can be visualized by measuring the fluorescence of dyed molecules in the trans-compartment. The simultaneous measurement of electrochemical and optical signals will significantly contribute to the understanding of transmembrane processes as well as to a simple and reliable continuous quality control of bilayers as required for screening applications.

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