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REVIEW

Ion transport across biomembranes and model membranes

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Abstract The milestones formerly achieved in the comprehension of ion transport across biological membranes on the basis of electrochemical concepts and/or instrumentation are briefly summarized. The various types of model membranes presently employed for the investigation of ion transport across biomembranes are reviewed and their requirements for the incorporation and functional investigation of membrane proteins are examined. The potential of model membranes for the elucidation of many problems in molecular membrane biology and for the realization of microarray sensors individually addressable to membrane proteins by electrochemical means is assessed.

Introduction

Bioelectrochemistry can be defined as that area of science in which electrochemical foundations and techniques are exploited to investigate processes of biological relevance. By far, the most important electrified interfaces in living systems are biological membranes. They consist of a bimolecular layer of lipids (the bimolecular leaflet) incorporating proteins. Lipid molecules are "amphiphilic", i.e., consist of a hydrophobic section (the hydrocarbon tail) and a hydrophilic section (the polar head). In biological membranes, the two lipid monolayers are oriented with the hydrocarbon tails directed toward each other and the polar heads turned toward the aqueous solutions that bath the two sides of the membrane. The resulting lipid "bilayer" is a matrix that incorporates different proteins performing a variety of functions. Biomembranes form a highly selective barrier between the inside and the outside of living cells. They are highly insulating to inorganic ions, and large electrochemical potential differences can be maintained across them. The permeability and structural properties of biological membranes are sensitive to the chemical nature of the membrane components and to events that occur at the interface or within the bilayer. For example, biomembranes provide the environmental matrix for proteins that specifically transport certain ions and other molecules, for receptor proteins and for signal transduction molecules. The membrane proteins in charge of ion transport across membranes are ion channels and ion pumps. Ion channels are responsible for the flow of hydrophilic ions across biomembranes along their electrochemical potential gradient, namely from the membrane side where the electrochemical potential of the ion is higher to that where it is lower (passive transport). Ion pumps are responsible for "active transport" of ions across biological membranes, namely transport of ions from the membrane side where their electrochemical potential is lower to that where it is higher. Since such an ion transport is endergonic, it can proceed only if it is driven by an energy source, which may be solar energy or an esergonic chemical reaction. Several ion pumps are "ATP-ases", in that the esergonic chemical reaction

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that drives them is the release of a phosphate group by adonisine triphosphate (ATP), with formation of adenosine diphosphate. The various responses observed in biomembranes are concentration-dependent, usually very rapid and reversible, and frequently voltage-dependent.

The past

The first application of electrochemical foundations and instrumentation to biological membranes was made by the two electrophysiologists Alan Lloyd Hodgkin and Andrew Fielding Huxley, whose work was focused on membranes responsible for the transmission of nerve impulses [1]. These membranes envelope the nerve cells, called neurons and, in particular, the elongated portion of neurons, the axon. The experiments of Hodgkin and Huxley on the giant axon of the squid represent a milestone in the understanding of ion transport across membranes. The K^+ ion concentration inside cells is about two orders of magnitude higher than outside them, while the opposite is true for Na^+ ions. Hodgkin and Huxley identified the driving force for the flux of these ions across the cellular membrane with the difference between the potential applied across the cellular membrane (the transmembrane potential) and the potential expressed by the corresponding Nernst equations (the Nernst potentials). When they did their work, they did not know what the cellular membrane looked like on the nano scale. They did not know about the existence of ion channels and ion pumps in the membrane. Nonetheless, they were able to conclude that changes in axon permeability to Na^+ and K^+ ions were dependent on membrane potential, and not on membrane current. Originally, they supposed that sodium ions crossed the membrane via negatively charged lipid carrier molecules. What they observed, however, proved that this was not the case. Therefore, they concluded that sodium movement depends on a distribution of charged particles that do not act as carriers in the usual sense. We now know that these "charged particles" are the sodium channels. To determine the potential dependence of axon permeability to Na^+ and K^+ they used a potentiostatic system based on operational amplifiers, which they called "voltage clamp". The transmembrane potential was controlled by placing one Ag|AgCl electrode inside the axon and another one just outside it. Two further electrodes were placed on the opposite sides of the axon membrane to allow the flow of electric current across it. Whenever the transmembrane potential deviated from a preset value, the potentiostatic system forced the current to flow between the latter two electrodes to maintain the transmembrane potential at the preset value. At each preset potential value, Hodgkin and Huxley adjusted the Na^+ concentration outside the axon at the value satisfying the Nernst equation for the given preset value, so as to annihilate the driving force for Na^+ flux. Therefore, the resulting current was exclusively due to axon permeability to K^+ ions, which could be determined at different potential values. By subtracting the K^+ current so determined from the overall current flowing for non-Nernstian Na^+ concentrations outside the axon, the Na^+ current (and hence the axon permeability to Na^+ ions) could also be determined. Based on this series of experiments conducted in the 1950s, Hodgkin and Huxley developed a mathematical model for the transmission of nerve impulses. In 1963, they were awarded the Nobel Prize in Physiology or Medicine for their achievements.

A further milestone was achieved in the late 1950s by Rudin et al. [2], while they were investigating the ion specificity of Langmuir–Blodgett lipid mono- and multilayers. Newton's observation of the so-called black soap films, whose thickness had been measured by several investigators, combined with Gorter and Grendel's bimolecular leaflet model for the plasma membrane, prompted Rudin to form a black film in aqueous solution by extending the methods used in black soap film approach. To this end, a lipid solution in decane was spread beneath an aqueous phase across an aperture, several millimeters in diameter, drilled through a partition (septum) of Teflon. The spontaneous organization of the amphiphilic lipid molecules into a bilayer was driven by the strong self-association of water molecules, which inhibits the mixing of water and amphiphile. The layer of the lipid solution in the nonpolar solvent became gradually thinner, with rainbow interference colors appearing on it, followed by black spots; finally, the whole layer became completely black. The blackening marks the transition of the lipid layer from a multimolecular to a bimolecular film, called bilayer (or black) lipid membrane (BLM), which is a non-reflecting optically black film (see Fig. 1). This system has been studied extensively as a model for the lipid bilayer of cell membranes, by incorporating integral proteins, photoactive pigments and biomolecules involved in biophysical, biochemical and physiological studies. The possibility of incorporating single integral proteins or smaller lipophilic biomolecules into BLMs has permitted researchers to isolate and investigate their functions, separately from the other proteins that are normally embedded in a biomembrane. This serves to reduce complex membrane processes to well-defined interactions between selected proteins, lipids, and ligands. The thin bimolecular lipid film in Rudin's BLM contains organic solvent molecules dispersed within the bilayer and in the form of microlenses floating in the bilayer [3]. A method for forming bilayers from lipid monolayers that eliminates solvent was subsequently developed by Montal and Mueller [4]. A major drawback of BLMs is their fragility, high sensitivity toward vibrations and mechanical shocks and low resistance to

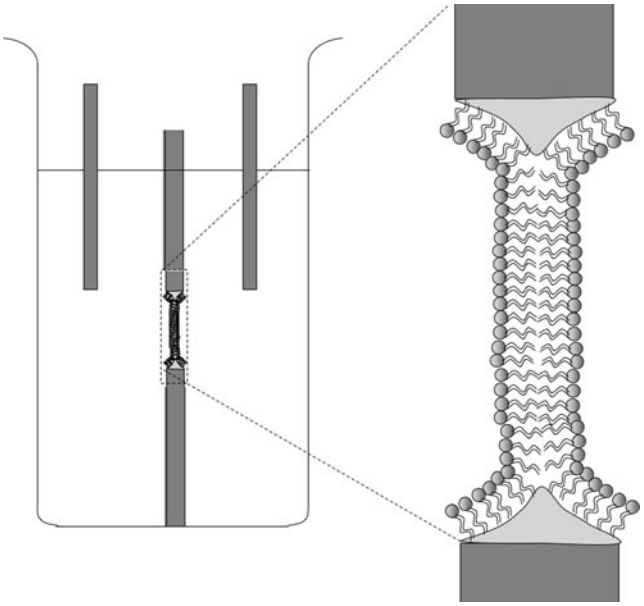

Fig. 1 Schematic picture of a lipid bilayer spanning a hole in a Teflon septum interposed between two aqueous solutions containing two Ag/AgCl reference electrodes

electric fields; thus, they hardly last more than 8 h and collapse for potential differences (transmembrane potentials) greater than 100÷150 mV between the solutions that bath the two sides of the BLM; moreover, they do not lend themselves to investigations with surface-sensitive techniques. Recently, attempts have been made to form more robust BLMs by spanning them over nanopores interposed between two aqueous and/or hydrogel phases [5]. The observed 30 times increase in stability by reducing the pore size by a factor of four demonstrates the benefit from using nanopores. Besides stabilizing lipid bilayers, small apertures increase the signal to noise ratio to an appreciable extent. Peptides and small channel-forming proteins insert spontaneously into these preformed bilayers. Bulky membrane proteins are incorporated either by fusion of proteoliposomes (i.e., unilamellar vesicles containing proteins) to preformed bilayers or by direct fusion of proteoliposomes to nanopores.

A third milestone was achieved by Erwin Neher and Bert Sakmann in the late 1970s and early 1980s with the development of the patch clamp technique, a remarkable refinement of the voltage clamp technique [6]. This electrophysiological technique allowed the recording of the currents of single ion channels for the first time, proving their involvement in fundamental cell processes such as action potential conduction. Neher and Sakmann received the Nobel Prize in Physiology or Medicine in 1991 for their achievements. The patch clamp technique can be applied to a wide variety of cells, but is especially useful in the study of excitable cells such as neurons and muscle fibers. As an electrode, it uses a glass micropipette with a tip inner diameter of about 1 μm, which contains an aqueous solution and an AgCl-coated silver wire. The composition of this solution can be changed, and drugs can also be added to study ion channels under different conditions. The micropipette is placed next to a cell, and a gentle suction is applied through it to draw a piece of the cell membrane (the "patch") into the micropipette tip, as shown in Fig. 2; the tip forms a high resistance "seal" with the cell membrane, which reduces the background electrical noise to one or few picoampères. The resulting configuration, with the intact cell clamped to the micropipette tip, is called "cell attached". If the micropipette is then pulled away from the cell, the patch may get detached from it, sealing the tip and giving rise to the "inside out configuration". The patch area is sufficiently small to contain only a few ion channels, thus permitting the recording of the opening and closing of single ion channels by application of a suitable potential difference between the Ag|AgCl electrode inside the micropipette and an identical electrode in the external solution. If, in addition to the protein under study, other proteins capable of interfering with the recording are present in the patch or in the clamped cell, specific inhibitors are used to block their function. The protein of interest is often "expressed" in the oocytes of the *Xenopus laevis* frog, which transcribe and translate the injected

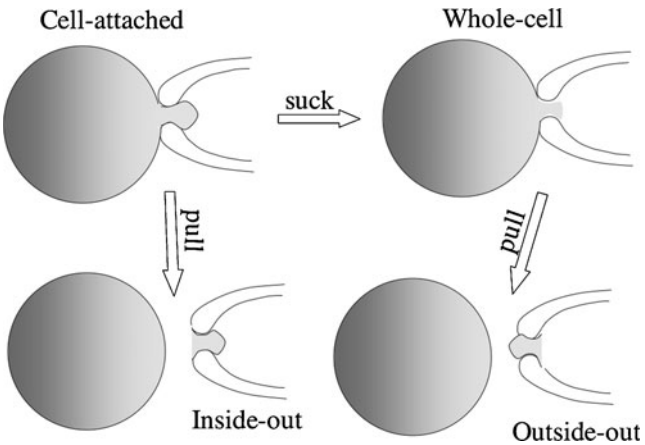

Fig. 2 Different configurations of the patch–clamp technique, exemplified by a micropipette tip and a single cell. Touching the cell with the micropipette tip while sucking mildly, the "cell-attached" configuration is obtained. By quickly withdrawing the micropipette from the cell, a membrane patch is ripped off the cell, sealing the tip and yielding the "inside-out" configuration, in which the intracellular surface of the membrane is exposed to the external solution. If, starting from the cell-attached configuration, more suction or a short voltage pulse is applied, the patch ruptures causing the liquid inside the cell to come in contact with that inside the micropipette ("whole-cell" configuration). Starting from the latter configuration, a quick withdrawal of the micropipette from the cell causes the neck of the membrane to remain attached to the micropipette and to seal the tip by forming a patch in which the extracellular surface of the membrane is exposed to the external solution ("outside-out" configuration)

genetic information. Due to their great availability, large size and lack of interfering proteins, these cells are particularly convenient for patch clamp investigations.

The present

Due to the inherent fragility of BLMs, research was subsequently directed toward the fabrication and characterization of lipid monolayers and bilayers self-assembled on metals, with formation of rugged functionalized electrodes capable of incorporating integral proteins in a functionally active state. Thanks to their particular robustness, these "biomimetic membranes" have potential not only for the investigation of the function of membrane proteins, but also for biosensor applications. With only a few exceptions, biomimetic membranes consist of a more or less complex architecture that includes a lipid bilayer. In order of increasing complexity, they can be classified into: solid-supported bilayer lipid membranes (sBLMs), tethered bilayer lipid membranes (tBLMs), polymer-cushioned bilayer lipid membranes, S layer stabilized bilayer lipid membranes (ssBLMs), and protein-tethered bilayer lipid membranes. Schematic pictures of some of these biomimetic membranes are shown in Fig. 3. Mercury-supported lipid monolayers and alkanethiol/lipid "hybrid bilayers" are particular biomimetic membranes that contain a single lipid monolayer.

To be able to incorporate integral proteins in a functionally active state, biomimetic membranes consisting of lipid bilayers should meet a number of requirements: (1) they should be robust enough for long-term stability, and be easily and reproducibly prepared; (2) they should have the lipid bilayer in the liquid crystalline state and with a good lateral mobility; (3) they should have water (or, at least, a highly hydrated hydrophilic region) on both sides of the lipid bilayer; (4) they should be sufficiently free from pinholes and other defects that might provide preferential pathways for electron and ion transport across the lipid bilayer [7]. Requirements 2 and 3 are necessary for the incorporation of integral proteins into the lipid bilayer in a functionally active state. In fact, integral proteins have a hydrophobic domain buried inside the biomimetic membrane, which must be sufficiently fluid to accommodate this domain. Often, they also have hydrophilic domains that may protrude by over 5 nm outside the lipid bilayer. To avoid denaturation of the integral proteins and to promote their function, their incorporation into biomimetic membranes must ensure that their extramembrane hydrophilic domains are accommodated in a hydrophilic medium on both sides of the lipid bilayer. Moreover, the transport of hydrophilic ions across a solid-supported lipid bilayer via ion channels or ion pumps is only possible if an aqueous or hydrophilic layer is interposed between the bilayer and the support. Requirement 4 is needed to make the biomembrane model sufficiently blocking as to characterize ion channel or ion pump activity by electrochemical means without the disturbing presence of stray currents due to defects.

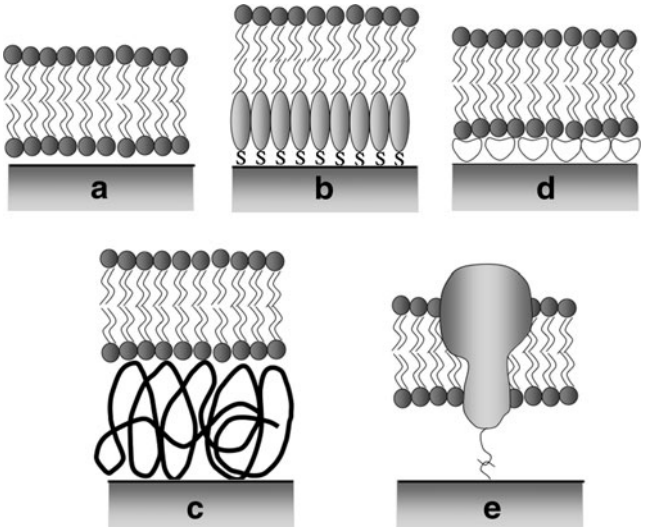

Fig. 3 Schematic picture of different biomimetic membranes: **a** a solid supported BLM (sBLM), **b** a tethered BLM (tBLM) consisting of a thiolipid monolayer with a lipid monolayer on top, **c** a polymer-cushioned BLM (pBLM), **d** a S layer stabilized BLM (ssBLM) consisting of a S layer with a lipid bilayer on top, **e** a protein-tethered BLM (ptBLM)

Apart from lipid molecules, the molecules that are most commonly employed for the fabrication of biomimetic membranes are "hydrophilic spacers" and "thiolipids". Hydrophilic spacers consist of a hydrophilic chain (e.g., a polyethyleneoxy or oligopeptide chain) terminated at one end with an anchor group for tethering to a support and, at the other end, with a hydrophilic functional group (e.g., a hydroxyl group). Sulfhydryl or disulfide groups are employed as anchor groups for tethering to metals such as gold, silver, or mercury (see an example in Fig. 4b); methyl-, methyloxy- or chloride-substituted silane groups are used for tethering to glass, quartz, silica, or mica. The latter supports are nonconducting and cannot be investigated by electrochemical techniques. Hydrophilic spacers serve to separate the lipid bilayer from a solid support, to compensate for surface roughness effects, to prevent any incorporated peptides or proteins from touching the support surface (thus avoiding loss of their functionality due to denaturation), and to provide an ionic reservoir underneath the lipid bilayer. Thiolipids differ from hydrophilic spacers in that the hydrophilic chain is covalently linked to one or, more frequently, two alkyl chains at the opposite end with respect to the anchor group, as shown in Fig. 4a. The alkyl chains simulate the hydrocarbon tails of a lipid molecule and provide one half of the lipid bilayer to the biomimetic membrane.

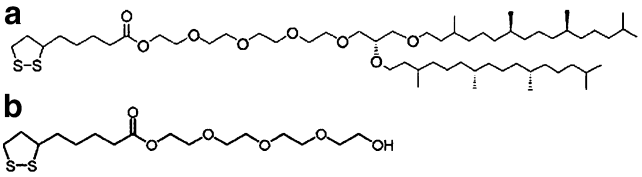

Fig. 4 **a** Structure of a widely adopted thiolipid, called DPTL. It consists of a tetraethyleneoxy chain terminated at one end with a lipoic acid residue and covalently linked at the other end to two phytanyl chains. **b** Structure of the corresponding hydrophilic spacer (TEGL), in which the two phytanyl chains are replaced by a hydroxyl group

Supported lipid monolayers

A simple and easily prepared biomembrane model obtained by noncovalent self-assembly consists of a phospholipid monolayer supported by a hanging mercury drop electrode. The lipid coating is obtained by spreading a solution of the lipid in pentane on the surface of an aqueous electrolyte, allowing the pentane to evaporate and immersing a hanging mercury drop electrode in the electrolyte [8]. This procedure gives rise to a lipid monolayer, with the hydrocarbon tails directed toward the hydrophobic mercury surface and the polar heads directed toward the solution. The defect-free support provided by liquid mercury to the lipid monolayer and the complete absence of pentane in the film impart high mechanical stability, resistance to electric fields and reproducibility to the monolayer. This self-assembly procedure exploits the fact that mercury is the most hydrophobic metal. Thanks to the liquid state of the mercury support, this simple biomembrane model is fluid and allows lateral mobility of the lipid molecules. However, it has no hydrophilic ionic reservoir on the metal side of the lipid film, and consists of a single-lipid monolayer. Consequently, it is not suitable for the study of the function of integral proteins. It can be used to investigate the behavior of the polar heads of the lipid monolayer with varying pH [9, 10], the behavior of small lipophilic biomolecules incorporated in the lipid film [11] and that of peripheral proteins adsorbed on the film surface [12].

Great interest has been focused on self-assembled films that are attached to a gold or mercury support by formation of a covalent linkage between the self-assembled molecules and the solid support, yielding structures of long-term, mechanical stability. Monolayers of alkanethiols on gold are probably the most widely used and best characterized of all self-assembled films to date. Self-assembly involves the anchoring of the thiol to the metal surface through the sulfhydryl group, often accompanied by its deprotonation and electron transfer from the sulfur atom to the metal. The hydrocarbon chains are, therefore, directed toward the aqueous solution. The self-assembly is normally carried out by keeping the metal electrode immersed in an ethanol solution of the thiol from 12 to 24 h for Au and from 5 to 20 min for mercury. The deposition of a phospholipid monolayer on top of an alkanethiol monolayer tethered to gold can be accomplished by several different preparation techniques, by exploiting the attractive hydrophobic interactions between the hydrocarbon tails of the alkanethiol and those of the phospholipid. The most common procedure for lipid deposition onto alkanethiol monolayers is vesicle fusion [13]. Solid-supported alkanethiol/phospholipid bilayers are unsuitable for incorporation of channel-forming peptides and membrane proteins, because they do not fulfill the aforelisted requirements. No hydrophilic layer is interposed between the hybrid bilayer and the electrode surface, thus excluding the space and water required for the proper folding of the extramembrane domains of integral proteins. Moreover, the flexibility and fluidity of the chemisorbed alkanethiol monolayer in direct contact with the electrode are much less than those of BLMs; this lack of flexibility and fluidity makes these mixed bilayers practically impermeable to lipophilic molecules of biological interest.

Solid-supported bilayer lipid membranes

The term “solid supported bilayer lipid membrane” or, simply, “solid supported membrane”, is commonly used to denote a biomimetic membrane consisting of a lipid bilayer in direct contact with a solid support, as shown schematically in Fig. 3a. These biomimatic membranes are typically formed on a hydrophilic solid support by immersing it in an aqueous dispersion of small unilamellar vesicles (SUVs), which slowly rupture and spread on the surface of the support. Alternatively, they can be formed by Langmuir–Blodgett and Langmuir–Schaefer transfers. When appropriately formed, these sBLMs are separated from the support surface through a water layer, estimated between 6 and 15 Å thick [14]. Several theoretical and experimental investigations of water near-polar hydrophilic surfaces suggest that it is more ordered than bulk water, with higher viscosity and lower dielectric constant. The lubrification effect of the water layer imparts a significant long-range lateral mobility to the lipid bilayer. However, significant frictional coupling between the bilayer and the underlying substrate slows down lateral diffusion and may be accompanied by a breakdown of the two-dimensional fluid nature of the membrane. The above biomembrane models are not suitable for studying the function of integral proteins that have extramembrane hydrophilic domains on both sides of the lipid bilayer. In fact, to avoid their denaturation and to promote their function, these proteins must be incorporated in a lipid bilayer having a hydrophilic medium on both sides. Not surprisingly, embedded membrane-spanning proteins usually show no lateral

diffusion, because of their interaction with the substrate, even though some of them maintain their function if their active site is far from the solid substrate. The majority of sBLMs are formed on nonconducting supports such as silica, glass, or mica. Consequently, they are not amenable to electrochemical measurements. A remarkable exception is represented by phospholipid bilayers self-assembled on Au(111) single crystal faces by Lipkowski et al. [15] either by vesicle fusion or by Langmuir–Blodgett and Langmuir–Schaefer transfers [16]. The latter procedure yields bilayers with a higher packing density and with smaller tilt angles of the alkyl chains with respect to the surface normal. These bilayers have been characterized by charge density measurements, photon polarization modulation infrared reflection absorption spectroscopy and neutron reflectivity.

Tethered bilayer lipid membranes

Tethered bilayer lipid membranes refer to architectures in which the lipid bilayer is separated from the support through a monomolecular layer tethered to the support via a sulfhydryl or disulfide group (for gold or mercury supports) or via a silane group (for silica and glass supports). The monolayer interposed between the support surface and the lipid bilayer should have a well-defined composition and geometrical arrangement, as distinct from polymer-cushioned bilayer lipid membranes. The most widely used tBLMs consist of a thiolipid monolayer tethered to an electrode surface, with a lipid monolayer on top of it, as shown schematically in Fig. 3b. A "thiolipid" molecule consists of a hydrophilic polyethyleneoxy or oligopeptide hydrophilic chain terminated at one end with a sulfhydryl or disulfide group, for anchoring to the support, and covalently linked at the other end to two alkyl chains simulating the hydrocarbon tails of a lipid, as shown in Fig. 4a. A tethered thiolipid monolayer exposes a hydrophobic surface to the bulk aqueous phase and provides one half of the lipid bilayer. The other half is obtained by forming a lipid monolayer on top of the thiolipid monolayer, usually by vesicle fusion. The cross-sectional area of a hydrophilic polyethyleneoxy chain is smaller than that of the two alkyl chains, if it is in its fully extended conformation, but not if it is coiled. In the former case, it is sufficiently hydrated to provide a satisfactory ionic reservoir; conversely, in the latter case it may accommodate only a limited amount of water molecules. Whether the conformation is extended or coiled depends both on the interfacial electric field and on the nature of the metal support. A particularly convenient thiolipid of this type, called DPTL, was synthesized in the Max Planck Institute of Polymer Science in Mainz [17]. It consists of a hydrophilic tetraethyleneoxy chain covalently linked to a lipoic acid residue for anchoring to the metal at one end, and bound via ether linkages to two phytanyl chains at the other end (see Fig. 4a). Its cross-sectional area is of about 55 $Å^2$. A structural and functional characterization of a DPTL monolayer tethered to gold has shown that the tetraethyleneoxy moiety is only partly hydrated at the more positive potentials [18, 19]. Its hydration increases at negative potentials, close to DPTL desorption. TBLMs fabricated with DPTL have a resistance of $5 \div 10$ MΩ cm^2 and a capacitance of about 1 μF cm^{-2}, which are comparable with those of BLMs.

"Thiolipopeptides" consist of an oligopeptide chain terminated at one end with a sulfhydryl group and covalently linked at the other end to the polar head of a phospholipid. They are considered to assume a helical structure. In this case, their cross-sectional area amounts to about 75 $Å^2$. They are usually obtained by tethering to a gold electrode a "thiopeptide" consisting of an oligopeptide chain terminated with a sulfhydryl group at one end and with a carboxyl group at the other end. This thiopeptide monolayer is then coupled in situ with dimyristoylphosphatidylethanolamine [20]. TBLMs fabricated with thiolipopeptides have been employed for the incorporation of a number of integral proteins; they exhibit high capacities (from 2 to 10 μF cm^{-2}) and low resistances, of the order of 10^4 Ω cm^2, which are about three orders of magnitude lower than those of conventional BLMs. The low resistance is ascribed to a not perfectly homogeneous coverage of the thiolipid monolayer by the distal lipid monolayer, generating an appreciable number of pinholes and other defects in the bilayer.

Thiolipid-based tBLMs anchored to gold do not meet the requirement of fluidity and lateral mobility. The thiolipid molecules are rigidly bound to the metal surface atoms. In principle, the lipid molecules on top of the thiolipid monolayer might be free to move laterally. In practice, however, their lateral mobility is hindered by the presence of adsorbed or hemifused vesicles and by the roughness of the metal support [21]. Moreover, the hydration of the polyethyleneoxy moiety of thiolipids anchored to gold is low [18, 19], while the incorporation of proteins with extramembrane domains requires a significant hydration of the spacer. Only small peptides, such as gramicidin [22], and ionophores, such as valinomycin [23], can be accommodated in the lipid bilayer moiety of polyethyleneoxy-based tBLMs, via incorporation from their aqueous solutions. Nonetheless, Au-supported oligopeptide-based tBLMs have been reported to incorporate a few bulky proton pumps, such as F_0F_1 ATPase [24, 25] and cytochrome *c* oxidase (COX) [26]. In all these cases, the lipid monolayer on top of the thiolipid monolayer was formed from a suspension of vesicles, via vesicle splitting and spreading on the thiolipid monolayer; the proton pump was then incorporated in the tBLM from its aqueous

solution in detergent. The current following activation of the proton pump was assumed to be due to proton flux to or from the hydrophilic spacer through the membrane protein spanning the lipid bilayer. It should be noted that vesicles have a low propensity to fuse on the hydrophobic surface exposed to the aqueous solution by a gold-supported thiolipid monolayer, especially if they incorporate an integral protein; rather, they are adsorbed or partially fused [27]. In this case, incorporation of the above proton pumps in a gold-supported tBLM from their solutions in detergent may easily take place in the membrane of adsorbed or partially fused vesicles, since the vesicular membrane is clearly interposed between two aqueous phases. In this respect, the functional activity of the above proton pumps may be successfully verified even with vesicles or proteoliposomes adsorbed and/or partially fused on a gold-supported thiolipid monolayer, without the need for a spacious ionic reservoir beneath the lipid bilayer. In fact, their activation may cause an increase in the proton concentration on top of the thiolipid monolayer (in the case of F_0F_1 ATPase activated by ATP) or its decrease (in the case of COX activated by ferrocytocrome *c*). In view of the relative permeability of the leaky thiolipopeptide monolayers to protons, this may determine an increase or a decrease in the proton electroreduction current on gold, as actually observed.

Gold-supported DPTL|DPhyPC tBLMs on microchips (where DPhyPC stands for diphytanoylphosphatidylcholine) have been recently employed to record single-channel currents of peptides and proteins. To this end, a microelectrode array device consisting of many (100×100 μm^2) "sensor" pads was employed [28–30]. A DPTL monolayer was tethered to the gold-coated sensor pads from a DPTL solution in ethanol; a lipid monolayer was then formed on top of it by vesicle fusion. In view of the very small surface area of the pad, the resistance of the resulting tBLM ranged from 1.5 to 17 GΩ. This resistance was high enough to reduce the background electrical noise to the low level required for the use of the patch–clamp technique. This devise has allowed the recording of single-channel currents of gramicidin A [28], the high-conducting Ca^{2+}-activated K^+ (BK or Maxi-K) channel, the synthetic M2δ ion channel [29] and the mechanosensitive channel of large conductance from *Escherichia coli* [30]. All these peptides and proteins were incorporated into lipid vesicles, before fusing them onto the DPTL-coated sensor pad. With the exclusion of the gramicidin channel, the unitary conductance of these ion channels was found to be from one third to one tenth of that obtained with conventional BLMs. In this connection, one cannot exclude the possibility that, even in this case, the channels responsible for single-channel currents are located in the membrane of adsorbed or partially fused vesicles. In fact, if this is the case, then the capacitive coupling between the vesicular membrane and the tBLM is expected to decrease the unitary conductance of the channels. The channel lifetimes for both open and closed states were normally found in good agreement with those obtained in BLMs under similar conditions.

As distinct from Au-supported thiolipid-based tBLMs, Hg-supported thiolipid-based tBLMs may incorporate bulky proteins, such as OmpF porin from *E. coli* [31] and the HERG potassium channel [32] in a functionally active state, thanks to the fluidity imparted to the thiolipid monolayer by the liquid mercury surface. Incidentally, no hazard is involved in the manipulation of the small amount of mercury used for the preparation of tBLMs. Upon incorporating gramicidin [33] or valinomicin [34], the tetraethyleneoxy (TEO) moiety of DPTL in aqueous KCl solution undergoes a conformational change ascribable to its elongation, as the applied potential is stepped from a fixed initial value of −0.200 V/SCE to a final value of −0.500 V/SCE [34]. As the final value of this potential step becomes progressively more negative, the charge of K^+ ions accommodated in the TEO spacer increases rapidly, attaining a maximum limiting value of about 45 $\mu C\ cm^{-2}$ at –0.8 V/SCE [35]. This corresponds to three potassium ions per DPTL molecule, denoting an appreciable hydration of the spacer. A drawback in the use of mercury-supported tBLMs is represented by the notable difficulty in using surface-sensitive techniques for their structural characterization. With respect to solid metal supports, mercury has the advantage of providing a defect free, fluid and readily renewable surface to the self-assembling thiolipid/lipid bilayer. Moreover, it imparts lateral mobility to the whole mixed bilayer. In addition, the self-assembly of a lipid monolayer on top of a thiolipid monolayer is readily carried out by simply immersing a thiolipid-coated mercury drop in an aqueous electrolyte on whose surface a lipid film has been previously spread [34]. Thanks to the hydrophobic interactions between the alkyl chains of the thiolipid and those of the lipid, this simple procedure gives rise to a lipid bilayer anchored to the mercury surface via the hydrophilic spacer moiety of the thiolipid. By avoiding the use of vesicles, this procedure excludes any artifacts due to partially fused vesicles. These advantageous features make the incorporation of membrane proteins in mercury-supported thiolipid-based tBLMs easier and safer than in solid-supported tBLMs.

Polymer-cushioned bilayer lipid membranes

The spaciousness of the ionic reservoir of tBLMs may not be sufficient to accommodate bulky extramembrane domains of membrane proteins. The problem is particularly serious with cell adhesion receptors, whose functional extracellular domains can extend to several tens of nano-

meters. This problem can be circumvented by separating the lipid bilayer from the solid substrate using soft polymeric materials of typically less than 100 nm thickness, which rest on the substrate and support the bilayer, as shown schematically in Fig. 3c. These stratified films are often referred to as polymer-cushioned or polymer-supported bilayer lipid membranes (for a review, see Ref. [36]). This approach reduces the non-specific binding of proteins to the solid support and the frictional coupling between proteins and the support, preventing the risk of protein denaturation due to direct contact between protein subunits and the bare support surface. In some cases, the cushion may assist self-healing of local defects in lipid bilayers deposited on macroscopically large supports. To increase the stability of polymer-cushioned membranes, the polymer was also tethered both to the substrate and to the membrane [37]. Usually, polymer cushions are anchored to supports such as glass, silica, and mica, by using polymers derivatized with alkyl silanes or triethoxysilane for covalent linkage to silanols at the surface of the silicate substrates. More rarely, they are anchored to gold [38] or to semiconductors such as indium tin oxide [39] via thiolated polymers.

S layer stabilized bilayer lipid membranes

Monomolecular crystalline arrays of protein subunits, called S layers, are common surface structures of archea and bacteria [40, 41]. They constitute the outermost component of the cell envelope of these procaryotic organisms. S layer subunits can be aligned in lattices with oblique, square, or hexagonal symmetry. Since S layers are monomolecular assemblies of identical protein subunits, they exhibit pores of identical size and morphology. S layer subunits of most bacteria interact with each other through non-covalent forces and can be set free with high concentrations of agents that break hydrogen bonds, such as guanidine hydrochloride, or urea. Once the S layer lattice of a bacterial cell is completely disintegrated and the disintegrating agent is removed by dialysis, the S layer subunits have the unique capability to reassemble spontaneously in suspension, at the liquid/air interface, on solid surfaces, on spread lipid monolayers, and on liposomes. Recrystallization starts at several distant nucleation points on the surface and proceeds until neighboring crystalline areas meet. The natural tendency of S layers to interact with membranes has been exploited to insert them as an intermediate layer between a lipid bilayer and a substrate, giving rise to the so-called “ssBLMs” (see Fig. 3d). In the case of bacterial S layer proteins, it has been demonstrated that protein domains or functional groups of the S layer lattice interact via electrostatic forces with some head groups of lipid molecules. A well-characterized S layer protein, SbqA from *Bacillus sphaericus* CCM 2177, was used as an ultrathin crystalline, water-containing hydrophilic layer between a gold electrode and a lipid bilayer [41]. The SbqA protein recrystallizes in monomolecular square lattices. A morphological unit, about 170 nm in diameter, consists of four protein monomers. The pores are of identical size and morphology, with a diameter of about 3.5 nm.

Protein-tethered bilayer lipid membranes

In all the biomimetic membranes previously described and allowing the incorporation of proteins, the protein orientation in the membrane is purely casual. At most, if one of the two extremembrane domains of the protein is much bulkier than the other, incorporation in a tBLM occurs preferentially with the bulkier domain turned toward the aqueous phase, in view of the limited spaciousness of the hydrophilic moiety of the tBLM. Moreover, the packing density of the reconstituted proteins in the lipid bilayer is not well controlled. The need for a well-defined protein orientation with respect to the electrode surface is particularly felt with redox membrane proteins, in which the electrons involved in a chain of redox centers are conveyed across the membrane in a well-defined direction. To overcome this problem, Knoll et al. [42] have developed a novel methodology based on tethering proteins, rather than lipids, to the electrode surface; the lipids are then allowed to self-assemble around the tethered proteins. To this end, a recombinant membrane protein is engineered to bear a stretch of six consecutive histidine residues. A gold surface is then functionalized by attaching a molecule terminated with a nitrilotriacetic (NTA) moiety at one end and with a sulfhydryl group for anchoring to gold at the other end. Complexation of Ni^{2+} ions to both the NTA functionality and the histidines of the stretch causes the protein to be anchored to the gold surface from its solution in detergent, as shown schematically in Fig. 3e. To retain full functional integrity, the membrane protein is incorporated into a lipid bilayer. For this purpose, the protein layer tethered to gold is mixed with detergent-destabilized lipid vesicles. By removing the detergent with microporous biobeads, the tethered proteins are surrounded by lipid molecules that form a lipid bilayer around them, as verified by surface plasmon resonance and electrochemical impedance spectroscopy; a water layer remains interposed between the lipid bilayer and the NTA moiety, acting as an ionic reservoir. The capacity and resistance of the bilayer amount to about 6 $\mu F\ cm^{-2}$ and 800 $k\Omega\ cm^2$ [43]. This high capacity and low resistance denote a loosely packed lipid bilayer, partly ascribable to the presence of a high protein content.

This approach has been adopted to investigate the function of COX from the proteobacterium *Rhodobacter sphaeroides* [42], the last enzyme in the respiratory electron

transport chain of bacteria. In this protein-tethered bilayer lipid membrane, the orientation of the protein with respect to the membrane normal depends on the location of the histidine stretch (his tag) within the protein. Two opposite orientations of the protein were investigated, either with the cytochrome *c* binding side pointing away from the electrode surface or directed toward the electrode, simply by engineering the his tag on the C terminus of subunit SU I or SU II, respectively. The functional activity of COX was verified by cyclic voltammetry with both protein orientations. The cyclic voltammogram of COX oriented with the cytochrome *c* binding side directed toward the electrode was used to determine the functional activity of the enzyme as a function of its surface density [44]. This density was varied by diluting the thiol functionalized with the NTA moiety with a non-functionalized thiol that did not bind to the enzyme. At low COX surface densities, the bilayer does not effectively form, and protein aggregates are observed; on the other hand, at very high surface densities, very little lipid is able to intrude between the closely packed protein molecules. In both cases, redox activity is low. Redox activity is preserved in the biomimetic membrane only at moderate surface coverages, in which a continuous lipid bilayer is present and the protein molecules are not forced to aggregate.

The future

Several efforts are presently made to realize biomembrane models consisting of a lipid bilayer anchored to a solid electrode through a hydrophilic spacer and satisfying those requirements of ruggedness, fluidity, and high electrical resistance that are necessary for the incorporation of integral proteins in a functionally active state. The capacitive currents resulting from the activation of ion pumps, transporters, channel proteins, and channel-forming peptides incorporated in these biomembrane models can be analyzed over a broad potential range by electrochemical techniques, which are by far less expensive than other techniques presently adopted. The realization of these biomembrane models allows fundamental studies on the function of integral proteins. Biomimetic membranes are ideally suited to elucidate many problems in molecular membrane biology, by permitting a reliable and rapid functional screening of a large number of mutant receptor proteins. This will open the way to the elucidation of structure–function relationships in ligand–receptor and protein–protein interactions.

Moreover, the development of biomimetic systems that incorporate therapeutically or diagnostically important natural proteins will open the door to the realization of sensors targeting biological analytes. Many practical applications are foreseen for these sensors, such as the detection of drug candidates modulating the function of ion channels and pumps or targeting membrane receptors. In this respect, there is strong need to develop novel, rapid, and highly sensitive methods for drug screening, capable of selecting and analyzing a huge number of compounds. At present, screening of pharmacologically active compounds follows traditional procedures that apply time-consuming ligand-binding studies and receptor–function tests separately. Thus, the direct, predominantly electrochemical determination of the function of ion channels and pumps in biomembrane models reconstituted from purified components addresses a strongly felt need for the development of new drug candidates or diagnostic test systems.

Microarray technology allows the simultaneous analysis of hundreds of parameters within a single experiment. DNA microarrays, which have revolutionized many areas of biology, have pointed out the generic advantages of microarray technology. With these microarrays, microspots of “capture” molecules are immobilized in rows and columns onto a solid support and exposed to samples containing the corresponding binding molecules. Even though the overall amount of capture molecules immobilized in the “microspots” of the array is low, their density in the microspots is high, giving rise to a high local signal. Moreover, the number of binding molecules captured by the microspots is low, due to their small area. As a result, the capture process does not change significantly the concentration of the target molecules in the sample. The notable importance of quantifying all proteins of a cell and of determining how their translational modifications depend on cell state and environmental influences has raised an enormous interest in transferring microarray technology from DNA to protein molecules. At present, efforts in this direction have been made almost exclusively with water soluble proteins. To the best of our knowledge, no microarrays specifically addressed to membrane proteins by electrochemical means have been reported in the literature so far, even though a large part of proteins is incorporated in biomembranes; moreover, integral membrane proteins represent about 50% of the targets to which drugs are currently addressed. Thus, e.g., about one third of yeast proteins are membrane proteins. The major difficulty encountered in fabricating microarrays of membrane proteins is that they are normally functionally active only in their natural lipid environment; it is, therefore, necessary to reconstitute them in this environment after purifying them. In order to retain the desired supramolecular structure, a supported lipid bilayer must remain hydrated at all times. This creates a significant challenge for fabricating arrays of supported lipid bilayers. Groves developed the first procedure for patterning surfaces with solid supported lipid bilayers [45]. A typical procedure involves the patterning of photoresist on fused quartz

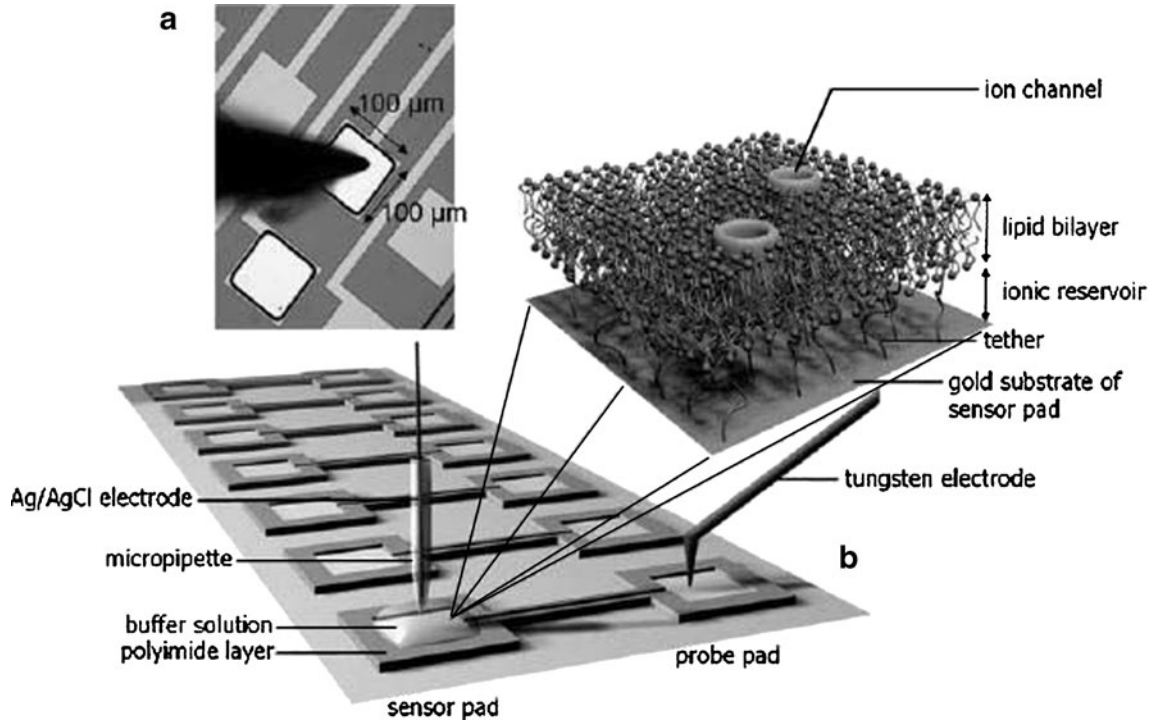

Fig. 5 The tethered bilayer membrane array. **a** An optical microscope image of the probe pad and the tungsten electrode tip. **b** Graphical representation of the tethered bilayer membrane array. The lower left corner shows the gold sensor pad covered with a tBLM that incorporates ion channels. The inset shows a tBLM formed at the gold surface of the sensor pad. (Keizer et al. [29] ChemBioChem 8:1246. Copyright Wiley-VCH Verlag GmbH & Co. KgaA. Reproduced with permission)

wafers by means of standard photolithographic techniques. Several materials that do not readily support continuous lipid bilayers can function as barriers to lateral fluidity when patterned onto a membrane compatible substrate. SUVs are fused onto the substrate between barriers, creating a lithographically patterned array of essentially identical planar-supported lipid bilayers. Although lipids are frequently present on the surface of barrier materials in the form of adsorbed, unfused vesicles, lipid long-range lateral diffusion is arrested [46]. Grids of barriers partition a lipid bilayer into an array of isolated "corrals", where the lipid bilayer is continuous and fluid. In addition to simple membrane partitioning, "spatially addressed" arrays of solid-supported lipid bilayers have been fabricated. Spatial addressing allows complete control over the chemical composition of each address in a supported bilayer array. A method for addressing lipid bilayers is the direct pipetting of SUV solutions into photolithographically pattern arrays. In this way, each bilayer can contain any desired composition of lipids or proteins, independent of the chemical composition of its neighbors [47]. These microarrays use nonconducting supports and, hence, can be "individually addressable", say, by fluorescence microscopy, upon using fluorophore-labeled lipids. Microarrays individually addressable by electrochemical means are still in their infancy. If micropatterns of supported membranes

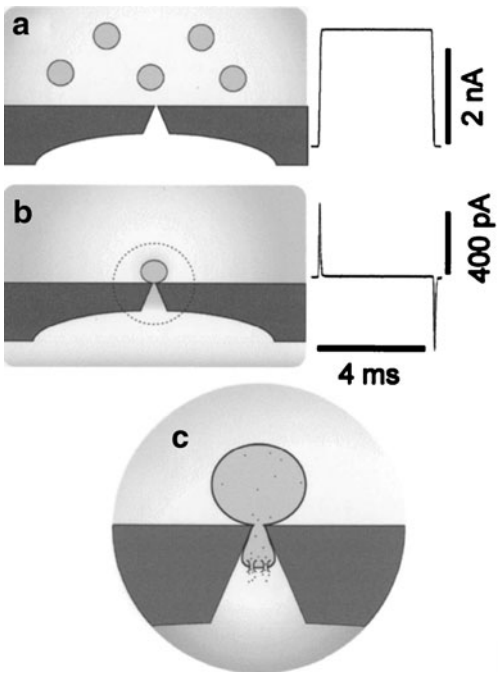

Fig. 6 Measured current response to a voltage pulse before **a** and after **b** a cell is sealed onto the aperture by suction. **c** Close-up view of the mechanically and electrically tight contact of the cell membrane and the chip in cell-attached mode. (Fertig et al. [51] Appl Phys Lett 81:4865. Copyright American Institute of Physics. Reproduced with permission)

with different ion channels are deposited on arrays of field-effect transistors with a comparable sensor area, such composite material would allow parallel monitoring of channel activity from individual membrane patches [48]. Combined with microfluidic devices that enable controlled delivery of analytes to each compartment, this would provide a powerful tool for high-throughput screening. A first attempt to realize a microarray for membrane proteins individually addressable by electrochemical means was recently made by Duran et al. [28–30]. The microarray consists of a number of gold (100×100 μm) sensor pads coated with a DPTL|DPhyPC mixed bilayer, as shown in Fig. 5. The distal DPhyPC monolayer is obtained by fusing vesicles incorporating the protein or peptide under study. Each sensor pad is electrically connected to a probe pad. A drop of buffer solution is deposited on the sensor pad, and a conventional patch micropipette containing an Ag|AgCl electrode and filled with the buffer solution is brought into contact with the drop. A tungsten tip is positioned onto the probe pad connected to the sensor pad where single-channel current measurements are to be carried out, with the tungsten tip and the Ag|AgCl electrode connected to a patch–clamp amplifier. Even though this devise cannot be easily automated for parallel monitoring of channel activity, it may represent a first step in this direction. The ultimate goal is the realization of a membrane-protein microarray platform allowing very small quantities of individual channel proteins to be effectively screened against a large set of drugs or diagnostic targets, without the need of labeling the protein or its target.

Attempts to fabricate microarrays of cell membranes by the patch–clamp technique are presently made by the "planar patch clamping" technique. This technique makes use of glass-based planar patch–clamp chips with a micrometer-sized aperture. In conventional patch clamping, the micropipette tip is placed onto the surface of the membrane of a cell under optical control via a microscope. Then, a tight seal is established by gentle suction, and an "omega"-shaped protrusion of membrane is drawn into the patch pipette (see the cell-attached configuration in Fig. 2). In the case of a planar patch–clamp chip with a single aperture, the procedure is very similar, but still somewhat different. Here, a suspension of cells is placed on top of the chip. Then a single cell is positioned onto the aperture in the chip by application of suction. In contrast to the classic patch–clamp technique, it is the cell that is moved to the aperture and not the pipette that is moved to the cell [49]. As in conventional patch clamping, a seal is obtained by application of suction and the membrane can then be ruptured for whole cell access either with suction or voltage pulses (see Fig. 6). The result is an electrical connection to the inside of the cell allowing for current recording. Nowadays, microfluidic cartridges containing a glass substrate with several patch–clamp apertures, each of which individually addressable by microfluidic channels on both sides of the substrate, are employed [50]. This design allows perfusion of cells and compounds by robotic pipetting means, making the whole approach very suitable for automation. In the case of multiple apertures on a single chip, individual, feedback-controlled suction lines are required for positioning and sealing of the cells. In addition to scaling up the number of recording channels, the throughput capability is increased by automated application of drugs by a pipetting robot.

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