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REVIEW

Natural and artificial ion channels for biosensing platforms

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Abstract The single-molecule selectivity and specificity of the binding process together with the expected intrinsic gain factor obtained when utilizing flow through a channel have attracted the attention of analytical chemists for two decades. Sensitive and selective ion channel biosensors for high-throughput screening are having an increasing impact on modern medical care, drug screening, environmental monitoring, food safety, and biowarefare control. Even virus antigens can be detected by ion channel biosensors. The study of ion channels and other transmembrane proteins is expected to lead to the development of new medications and therapies for a wide range of illnesses. From the first attempts to use membrane proteins as the receptive part of a sensor, ion channels have been engineered as chemical sensors. Several other types of peptidic or nonpeptidic channels have been investigated. Various gating mechanisms have been implemented in their pores. Three technical problems had to be solved to achieve practical biosensors based on ion channels: the fabrication of stable lipid bilayer membranes, the incorporation of a receptor into such a structure, and the marriage of the modified membrane to a transducer. The current status of these three areas of research, together with typical applications of ion-channel biosensors, are discussed in this review.

Keywords Biosensor · Ion channel · Lipid membrane · Planar patch clamp · High throughput · Drug sensing

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Introduction

Wilhelm Ostwald proposed in 1890 that the electrical signals measured in living tissue could be caused by ions moving in and out through cell membranes. This electrochemical idea rapidly achieved acceptance [1]. The notion of the existence of some type of narrow ion channel arose in the 1920s. Alan Hodgkin and Andrew Huxley made a major breakthrough at the beginning of the 1950s [2], but the existence of biological ion channels was still doubted as recently as the 1980s [3].

The binding of a particular analyte molecule to a biological receptor can initiate the flow of between several thousands and millions of ions or molecules across the cell membrane. The single-molecule selectivity and specificity of the binding process, together with the expected intrinsic gain factor obtained by utilizing the flow through a channel, first attracted the attention of analytical chemists in the 1990s [4]. At around the same time, Mark Keating and colleagues showed that defective potassium and sodium channels may be a cause of sudden cardiac death [5, 6]. Various diseases in which ion channels play an important role are now termed “channelopathies” [7].

More than 400 genes that code for ion channels have been found in the human genome. The selectivity of the receptor of a distinct ion-channel type reduces drug side effects in other types of tissue [8]. This makes ion channels

important targets for drugs [9]. The study of ion channels and other transmembrane proteins is expected to lead to the development of new medications and therapies for a wide range of illnesses [10]. Proteins are key players in the development of most diseases [11]. 60–70% of all drug targets are proteins, in particular those that reside in the cell membrane [12]. Sensitive and selective ion-channel biosensors for high-throughput screening are having an increasing impact on modern medical care, drug screening, environmental monitoring, food safety, and biowarefare control [13–15]. Even virus antigens can be detected by ion channel biosensors [16]. Single-molecule modulation of a gated event is considered a cornerstone of biomolecular electronics [17].

The need to preserve their natural lipid environment to avoid loss of function enforces strong prerequisites on a successful protein biochip [18]. In contrast to DNA microarrays, which have already proven beneficial in clinics [19], the establishment of robust protein arrays remains a challenge [20]. This is especially true of membrane proteins, which require the lipid membrane to retain their structural and functional integrity. Most of the biologically active compounds used in contemporary biosensor technologies, like nucleic acids, enzymes, and antibodies, work in aqueous solution, whereas the immobilization of transmembrane proteins—particularly receptors, ion channels, and pumps—on transducer surfaces includes a drastic change in their natural environment, resulting in the loss of activity [21].

Three technical problems had to be solved to achieve practical biosensors based on ion channels: the fabrication of stable lipid bilayer membranes, the incorporation of a receptor into such a structure, and the marriage of the modified membrane to a transducer. The utilization of artificial lipid membranes for the analysis of membrane proteins has several advantages over intact cell-based assays: proteins located in intracellular organelles can be accessed, the compositions of the lipid membrane and buffer can be precisely defined, and the population of membrane proteins can be controlled [22]. The first platforms utilized suspended bilayer lipid membranes (also called black lipid membranes, BLMs) [23], but these relatively large membranes are nonuniform and relatively fragile. The mechanical stability of BLMs can be improved by utilizing chemical additives like cholesterol, but additives often deteriorate the function of the embedded transmembrane proteins. Micromachined apertures stabilized the BLM, the formation of the latter was further improved by microfluidic technologies, and parallel recording in multiple independent wells in a single chip was realized [24]. Improved stability can be achieved by using supported lipid membranes (SLM), which are formed by incubating a liposome suspension on a substrate surface, often silicon or a polymer. SLMs can easily be patterned, but they are of limited use for electrophysiological measurements of ion channels owing to their high electrical permeability [22]. BLMs have been formed directly on a variety of materials [25], or tethered to a metal surface through a thiolated hydrophilic spacer [26]. BLMs spanning the holes of a microporous support are known as nano-BLMs, some of which have been reported to support ion channel function [27]. Microfabricated structures of silicon also allow the use of whole cells, which may be arranged in arrays of hundreds or even thousands of individual cell spots, providing powerful biosensory screening tools [28]. Although relatively low seal resistance was reported compared with the patch-clamp's gigaohm seal, whole-cell channel currents were observed with a good signal-to-noise ratio [29]. A whole cell may have several channel types in its membrane. The change in membrane potential due to the activation or blockage of one of these channel types can have a very small effect on the membrane potential due to the parallel conductance of other channel types [8]. The advantages and disadvantages of biosensors that exploit whole cells as detectors, and those which make use of one or several specific molecules—the most widely utilized being membrane proteins, such as G-protein coupled receptors (GPCRs) and ion channels—were discussed recently [30].

An alternative approach to the planar membrane is provided by liposomes (or lipid vesicles). These spherical lipid membranes are used as model cell membrane systems and as cell-sized containers. It is difficult to control their properties, such as their size, lamellarity, and entrapment efficiency [22]. On the other hand, their confined aqueous 3D structure can provide an environment that permits protein flexibility and movement while avoiding direct exposure of the protein to the surface [12]. Liposomes may also facilitate the development of biochips for membrane proteins.

From the first attempts to use membrane proteins as the receptive part of a sensor, ion channels have been engineered as chemical recognition elements [4, 31, 32]. These approaches mimic biological sensory functions and can be used with various types of receptors. Several engineered ion channels rely on gramicidin, a linear pentadecapeptide, which assembles within the hydrophobic interior of the lipid bilayer to form a half-membrane-spanning β-helix. After dimerization, it allows inorganic monovalent cations to travel without restriction through the bilayer. Upon tagging to gramicidin, high-affinity ligands cause the gramicidin-based sensor to act as a real-time monitoring system for protein–ligand interactions [33]. The ion current is switched off when mobile channels diffusing within the outer half of the membrane become crosslinked to antibodies immobilized at the membrane surface [4]. Several other types of peptidic or nonpeptidic channels have been investigated. Various gating mechanisms have been implemented in their pores [34]. Gated natural ion

channels have successfully been reconstituted into microfabricated orifices on optical [35] and electric transducers [25].

From an engineering perspective, the primary structure of a protein channel and pore is determined by its genetic sequence; the folded structure shows identical structures for virtually every copy of a protein. Exploiting a bacterial expression system makes it relatively easy and inexpensive to mass produce nanometer-sized channels [31]. Even chimeric proteins, which combine whole peptides and therefore contain all functional domains of the original proteins, have been produced in mammalian cells. Chimeric proteins allow the ion channel to adopt the appropriate conformational configuration for insertion into appropriate membranes while preserving the activity of the ion channel [36, 37].

Types and properties of ion channels

Biological molecular receptors are categorized into four different classes: ion channel receptors, G-protein-linked receptors, receptors with a single transmembrane domain, and enzyme-linked receptors [38]. Ion channels form water-filled pores through the cell membrane and regulate the ion transport across this membrane. The binding of specific chemicals (ligands) to the extracellular domain results in a conformational change in the channel protein [39]. This conformational change causes the channel pore to open (selectivity filter). This process is called gating. Ion channels can be ligand-gated, voltage-gated (different chemical potentials on either side of the lipid membrane), volume-regulated (mechanosensitive ion channels [40]) or light-gated. Several biological processes—the heartbeat and every perception—critically rely on the functions of gated ion channels. Many toxins and an estimated 90% of all warfare agents act on ion channels.

One class of natural ion channels is cation selective, while the other is anion selective (namely Cl^-). Many kinds of cation channels are specific in that they will primarily pass a single ion, e.g., Na^+, K^+ or Ca^{2+}. These ion channels are named according to their primary permeant ion, e.g., a K^+ channel or a Cl^- channel. Many subtypes exist. They are denoted by subscripts such as Kv (voltage-gated potassium channel) or Kcv (potassium channel from the chlorella virus). The rate of flow of ions through most ion channels exceeds 10^6 ions/s; in some channels it may even approach diffusion-limited rates [41].

The prototypic natural ligand-gated ion channel is the nicotinic acetylcholine receptor (nAChR, Fig. 1). It consists of a pentamer of protein subunits, with two binding sites for the neurotransmitter acetylcholine, which, when bound, alter the receptor's configuration and cause an internal pore with a diameter of about 0.65 nm to open. Nicotinic

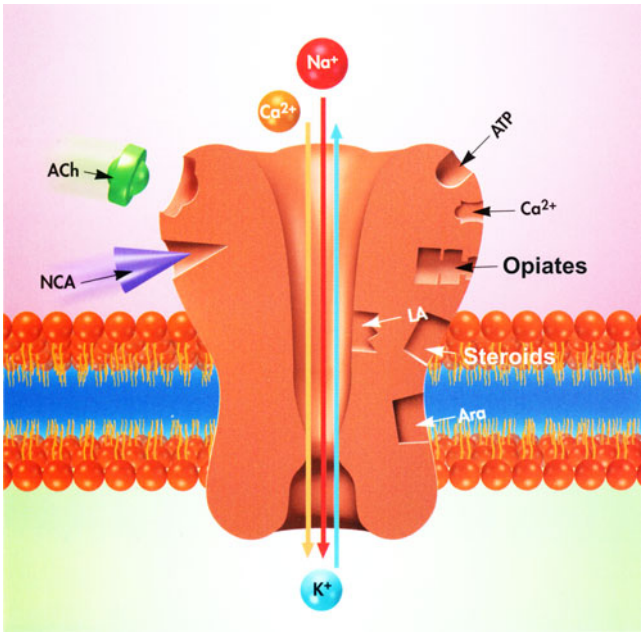

Fig. 1 Schematic diagram of the nicotinic acetylcholine receptor, a ligand-gated natural ion channel. Noncompetitive agonists (NCA) enhance the effect of the transmitter acetylcholine (Ach), while local anesthetics (LA), steroids, arachidonic acid (Ara), and opiates retard it (from [43] with permission)

receptors can also be opened by nicotine (hence the name "nicotinic"). This pore allows Na^+ ions to flow down their electrochemical gradient into the cell and K^+ ions to exit. The net flow of positively charged ions is inward. nAChR function can be modulated by toxins or drugs (agonists/antagonists) [42]. Nicotinic acetylcholine receptors are present in many tissues in the body and are the best studied of the ionotropic receptors [43, 44].

Besides acetylcholine, there are many different types of neurotransmitters (ligands), most of which are small hydrophilic molecules such as biogenic amines such as dopamine or serotonine (also known as 5-hydroxytryptophan or 5-HT) or aminoacids, such as γ-aminobutyric acid (GABA), as well as many others. Receptors are named after their ligands; subtypes are indicated by subscripts. The 5-HT_3 receptor is a ligand-gated ion channel; all other serotonin receptors are G protein-coupled receptors. Neurotransmitters are unable to cross or permeate passively through the hydrophobic membrane. Instead, they mediate their effects by interacting with a receptor embedded in the membrane.

The availability of gated natural ion channels has significantly improved in recent years. An important factor that limits the commercialization of biosensors based on gated ion channels is the stability of the protein. The delicate conformational changes that induce the gating are easily affected by changes in their vicinity [17, 45]. The function of a gated natural ion channel can be deteriorated by reduced lipid membrane fluidity. Reduced fluidity can

be caused by interaction with the support, by excessively strong tethering, or by adding more cholesterol to the membrane in order to improve its stability. Progress in developing the architecture of artificial membranes, as described in the next section, is necessary for further progress in this respect.

On the other hand, nongated ion channels like α-hemolysin, alamethicin or gramicidin are less delicate in handling, less complex structurally, and more readily available. Their properties are compatible with high-throughput platforms for drug screening and discovery [46]. α-Hemolysin (α-HL) forms a homoheptameric β-barrel in biological membranes. A β-barrel is a large β-sheet that twists and coils to form a closed structure in which the first strand is hydrogen bonded to the last. Barrel structures are commonly found in porins and other proteins that span cell membranes. Assembled α-HL is stable over a wide range of pH values and temperatures, its transmembrane pore stays open under normal conditions, α-HL can bind to various biological or synthetic lipid bilayers, the binding proceeds spontaneously, and it does not require specific ionic conditions. Using genetically engineered α-HLs, for which assembly and conductance can be triggered or switched on or off by external biochemical or physical stimuli (including light), a lipid bilayer can be made permeable at will for small solutes. Suspended in a lipid bilayer, an α-HL channel becomes a stochastic sensor when a molecular adapter is placed inside its genetically re-engineered stem, influencing the transmembrane ionic current induced by an applied voltage bias [47]. The reversible binding of analytes to the molecular adapter transiently reduces the ionic current. The magnitude of the current reduction indicates the type of analyte, while the frequency of the current reduction intervals reflects the analyte concentration [48].

Alamethicin is one of the simplest channel-forming peptide antibiotics, with 20 amino acid units. In cell membranes, it forms voltage-dependent ion channels through the aggregation of 4–6 molecules. Alamethicin is permeable to both cations and anions [49]. Gramicidin A is a linear 15-residue hydrophobic peptide. The chain assembles within the hydrophobic interior of the cellular lipid bilayer to form a β-helix. The helix itself is not long enough to span the membrane, but it dimerizes to form the elongated channel needed to span the whole membrane [50]. Dimerized gramicidin allows inorganic monovalent cations to travel through without restriction. The dimerization/dissociation rate depends, for example, upon membrane fluidity and the mobilities of the gramicidin monomers within their respective compartments of the lipid bilayer. If the gramicidin molecule in the outer compartment is combined with a recognition element, the mobility of this gramicidin molecule may become severely restricted after the recognition element binds to an analyte. This mimicked biological sensory function provided the basis for the successful development of a biosensor platform [4, 32, 51]. Unfortunately, such platforms are quite complicated to use as simple detection systems, in spite of their biological relevance [52]

Great effort went into the development of synthetic receptors as a means of mimicking natural receptors. One of the most promising application areas of synthetic receptors is differential sensing. Differential sensing can be explained as follows. Many synthetic receptors show exquisite selectivity, but they also suffer from cross-reactivity. Cross-reactivity is an attribute that is crucial in differential sensing schemes. An array of recognition elements must be created and the composite signal evaluated and interpreted by pattern recognition protocols. Therefore, both selective and nonselective synthetic receptors are finding use in analytical applications [53]. Synthetic ligand-gated ion channels (SLIC) combining a synthetic receptor and a synthetic ion channel have demonstrated their potential as biosensors for point-of-care diagnostics of infectious diseases or for evaluating vaccine development [54]. Biosensors of this kind can be produced using presently available technologies and components, such as automated high-throughput instruments (see the section on measurement techniques below).

So-called stochastic sensing is achieved by engineering the pore of a nongated ion channel like α-HL [55]. α-HL is especially open to engineering because its transmembrane domain is formed from β-barrels. The engineered pore contains the binding site for an analyte (inorganic ion, small organic molecule, or protein). Each time the analyte binds to the site, the ion flow through the pore is modulated. Individual binding events are monitored. The frequency of occurrence reveals the concentration of the analyte, while the current signature reveals its identity. An α-HL channel with a covalently attached adapter molecule inside the pore can continuously identify unlabeled nucleoside 5′-monophosphate molecules with accuracies averaging 99.8%. This highly accurate tool is suitable for integration into a system for sequencing nucleic acids and for analyzing epigenetic modifications [56].

An alternative approach to modifying the structures of natural ion channels like α-HL, alamethicin, and gramicidin A is to design peptides or nonpeptidic molecules. Any molecular architecture with novel characteristics that cannot be attained by natural channels could be designed [34]. The first successful proof of single-channel properties for synthetic molecules was reported in 1992 [57]. The initial basic concepts for the molecular design of artificial ion channels are summarized in [58]. Recent principles of nanopore devices obtained by genetic engineering are reviewed in [59, 60]. Very narrow nanopores (<10 nm) can even be used for studies on protein folding in confinement or other kinetic phenomena [61].

Calixarenes have received considerable attention over the past decade in host–guest chemistry [62]. Calixarenes offer an ideal platform due to the easy modification of their upper and lower rims. The cavities of calixarenes have mostly been studied for their encapsulation abilities towards alkali and alkaline earth metals, but calixarenes have successfully been used as starting structures for artificial ion channels too [34]. After integrating photochromic moieties into these types of calix[4]resorcinarenes, a family of gated nonpeptidic ion channels was obtained that can be reversibly switched between the open and closed positions using light irradiation [63].

Inspired by the functioning and responsiveness of biological ion channels, researchers are attempting to develop biosensor systems based on polymer and solid-state nanochannels. Such nanopore devices do not need lipid membranes, so they should be much more compatible with the requirements of applications. Attaching recognition sites to the channel walls leads to the preparation of sensors targeted at a specific molecule. There are many nanochannel platforms for the detection of DNA and proteins, but only a few are capable of detecting small molecules [64].

Types of bilayer membranes

The cell membrane, which forms a continuous barrier around almost all living organisms, is made of lipid bilayers. The lipid bilayer provides a protective container for cells and subcellular structures, and also hosts the machinery for cellular communication and transport across the cell membrane. Bilayer model systems are currently mainly used to investigate biological processes that occur at the cellular level, and thus provide information on ion channel properties [65], ligand–receptor interactions [66, 67], lipid dynamics [68], lipid domain (raft) formation [69], elastic properties of membranes [70], and lipid–DNA interactions [71].

Since the discovery of the possible solubilization of membrane proteins and their isolation from other membrane constituents (purification), different methods have been developed to reconstitute ion channel proteins into artificial lipid bilayers [72, 73]. These membrane proteins were then fully functional when correctly oriented and inserted into a lipid bilayer. The first reconstitution into a lipid bilayer for electrophysiology measurements was done in 1969 by Bean et al. [74]. The reconstitution of vectorial functions of membrane proteins was initiated by Racker [75–77]. Reconstitution plays a central role in identifying and characterizing the mechanisms of action of membrane proteins. The activity of the membrane proteins is studied using electrophysiology through different methods, such as black lipid membrane [78], or with the tip-dip bilayer technique [79]. Therefore, the structure–function relationship can be investigated to better understand the biophysical properties of membrane proteins in vivo. In the classical planar bilayer, which is formed either from bulk solution [80] or from a monolayer [78], protein insertion is initiated by adding a small diluted volume of protein solubilized in detergent to either side of the chamber [80]. An alternative method is to fuse small vesicles containing proteins to the bilayers using calcium, divalent or trivalent ions, and osmotic gradients [23, 82, 83]. The vesicles containing the proteins are usually obtained through dialysis of the proteins solubilized in detergent solution and excess lipids (forming micelles in detergents). Improvements in vesicle fusion were achieved in order to realize the best conditions for recording single-channel activity in the bilayer. This is required in order to study channel gating kinetics, such as controlling vesicle fusion by chelating divalent ions or re-establishing an equal osmotic gradient between each chamber. The conventional planar lipid bilayer and the mode of reconstitution of membrane proteins are well detailed in the literature [84–91].

There are a variety of lipid bilayer systems that can be used. Here we describe methods for the formation of artificial lipid bilayers.

Bilayers on patch pipettes

Also called the tip-dip technique, this method uses conventional patch clamp to form lipid bilayers on the pipette tip. This technique was introduced in the early 1980s by Wilmsen and Hanke [92–94]. The normal patch pipette is usually used to patch either native membrane proteins or purified proteins [95–97]. Basically, the pipette tip is immersed (Fig. 2a) in a monolayer lipid film formed at the air–water interface (Fig. 2b) in order to produce a lipid bilayer (Fig. 2c). When compared to the conventional planar lipid bilayer (BLM), the main advantage is a reduction in the capacitance due to the pipette tips, which usually have diameters in the range of 0.5 to 5 μm. Like the conventional patch clamp, handling is very difficult, and reconstitution can sometimes be difficult to achieve, as the proteins should initially be present in a monolayer to allow them to be incorporated into the bilayer.

Black lipid membranes (BLM)

The lipids are usually dissolved in an organic solvent (decane, hexadecane, pentane, etc.) or a mixture of different solvents (ethanol, methanol, chloroform, decane, hexadecane, pentane, etc). The lipid bilayers are formed over an aperture [80] in a movable septum separating two chambers. The septum contains at its center an aperture covered by a thin teflon film, or some other substrate, with a small aperture of around

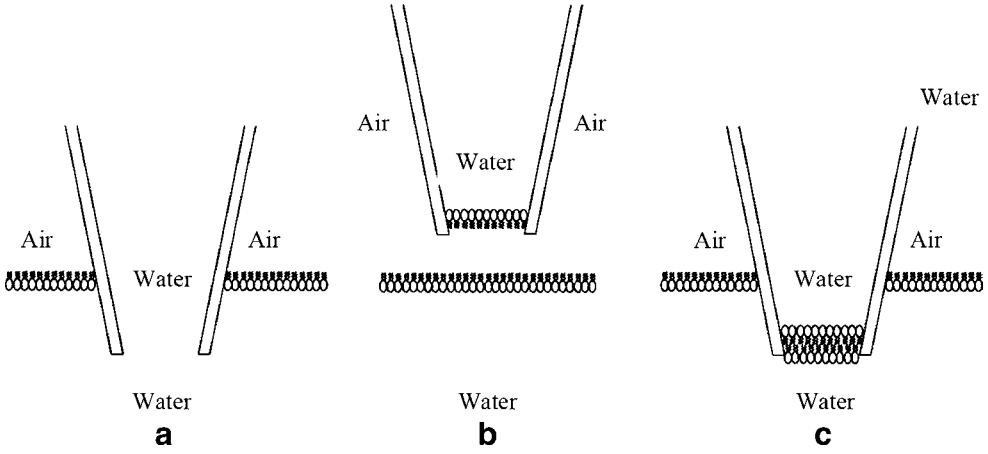

Fig. 2 Schematic formation of a bilayer from a phospholipid monolayer at the tip of a patch pipette. The lipid bilayer is formed by immersing the pipette tip in a phospholipid monolayer formed at the air–water interface. Channel proteins may be incorporated into the bilayer during formation if they are present in the original monolayer (from [145] with permission)

60–200 μm. Each chamber is filled with a buffered ionic solution and contains electrodes that are used to measure currents due to the flow of ions across the bilayers. It is important to have a hydrophobic film containing the aperture. The membrane bilayers can be made in a variety of ways, such as "painting" the aperture with lipids dissolved in an organic solvent and subsequently evaporating the solvent (painting bilayer technique) [80, 98]. This can be achieved using a paint brush or by blowing air bubbles onto the hole with a glass pipette with a rubber bulb until there a capacitance current forms in the voltage clamp. Alternatively, the bilayer can be formed by slowly raising the buffer level of each half chamber after a lipid monolayer has formed at the water/air interface (folded bilayer technique, Fig. 3). The monolayers themselves can be made from either an organic solvent based mixture of phospholipids [78], or from solvent-free lipid vesicles through self-assembly [81, 99, 100]. These techniques require extra equipment that is necessary for recording and to reduce the signal noise: a Faraday cage, an anti-vibration table, and an acoustic chamber. The basic principle of planar lipid bilayer recording is that it has a large capacitance. An estimate can be obtained from the area of a planar plate condenser:

$$C = \varepsilon_0 \varepsilon_m A/d \quad [\mu F/cm^2].$$

Here, d is the thickness of the membrane, ε_0 is the dielectric constant of the medium, ε_m is the relative dielectric constant of the membrane, and A is the area of the membrane. Thus, for a given lipid bilayer, the final value of the membrane capacity depends on the area of the aperture. Reducing the area of the lipid bilayer will decrease the capacitance, and this in turn increases the time resolution by decreasing the noise. The size of the aperture on the teflon film can be rather large (around 20 μm), depending on the techniques used to make the hole. Smaller apertures on the teflon film led to increasing difficulty in forming bilayers from lipid-containing organic solvents.

One of the most important aspects of BLM is the possibility of reconstituting membrane proteins into the

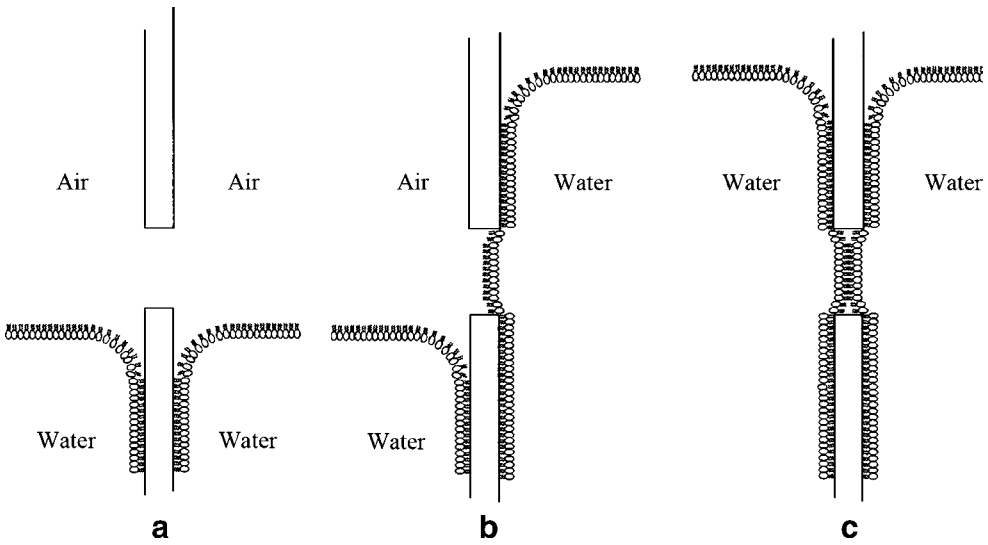

Fig. 3 Schematic formation of a bilayer on a teflon septum from preformed phospholipid monolayers. Monolayers are formed at the interface of an aqueous solution (water) and the air. The bilayer is formed by the successive raising of the level of the solution in the chambers on either side of the septum. Channel proteins may be incorporated into the bilayer if they are present in either of the monolayers (from [145] with permission)

lipid bilayer, and this has been shown in many studies with proteins [101, 102], peptides [103], and pore-forming toxins [104]. BLM has many advantages, but is also limited to the lifetime of the bilayer due to the poor stability of the membrane.

Tethered bilayer lipid membranes (t-BLM)

The tethered lipid bilayer membrane (t-BLM) increases the stability of the supported membranes by chemically anchoring the lipids to a solid substrate such as gold or some other inert surface. One of the main problems with t-BLMs is getting their electrical properties to be competitive with those of BLMs (capacitance of 0.5 mF/cm^2, resistance >1 MΩ cm^2) [105–108]. tBLMs usually involve bifunctional molecules that provide a lipophilic domain and a hydrophilic spacer. The hydrophilic (such as a thiol or disulfide group) spacer attaches the tethering molecule to the support and determines the hydrophilic environment as well as the volume of the submembranous space, which allows minimum interaction to be maintained with the substrate. Recently, applications of polyethylene oxide [108, 109] and peptide spacer groups were reported for the generation of highly insulating tBLM [106], which is necessary for investigating membrane proteins under a defined electric field. The lipophilic part inserts into one or both leaflets of the lipid membrane, and can consist of phospholipids [106, 110, 111], cholesterols [112], alkyl chains [4, 113], or phytanyl groups [105, 108, 109, 114].

Supported lipid membranes (SLM)

Supported lipid membranes or so-called SLBs (supported lipid bilayers; see Fig. 4) have been widely studied as model systems for elucidating the properties of membrane proteins or lipid membranes. Compared to BLMs, SLMs are more robust and stable in the presence of flow rates and vibrations, allowing solution exchange by perfusion, but only the upper face of the bilayer is exposed to solution. Often the main problem is that undesirable interactions occur between the lipid membrane and the surface that could result in the denaturation of the membrane proteins. Supported bilayers generally do not directly touch the substrate surface, but, depending on the substrate, they are separated by only a very thin water gap (1–2 nm thick layers) [115, 116]. There are different methods of fusing lipids to the solid surface: the transfer of a lower leaflet of lipids from the air–water interface by the Langmuir–Blodgett technique [117, 118], spreading lipid vesicles from an aqueous suspension on a substrate surface [119, 120], or even a combination of both methods. The advantage of SLMs is the possibility of using a direct physical interaction such as that in atomic force microscopy (AFM) [121]. Each different deposition has its advantages and its disadvantages. The Langmuir–Blodgett technique is useful for forming asymmetric bilayers [122, 123], but it is difficult to incorporate functional membrane proteins [119]. Fusing small vesicles to the substrate makes the reconstitution of membrane proteins easier. The proteins are reconstituted prior to the fusion [124, 125]. The formation of the lipid bilayer on a substrate depends on the properties of the individual lipids [126].

In this configuration of the lipid bilayer on a solid substrate, a membrane protein conducting ions cannot show electrical activity, and to obtain the proper biophysical properties of the protein, a system that is able to measure the ion movement is needed.

Droplet interface bilayers (DIBs)

Droplet interface bilayers (DIBs) are lipid bilayers formed by adding two droplets of aqueous solution to oil-containing lipids. Each aqueous droplet in the oil–lipid mixture instantaneously forms a self-assembled monolayer liposome. When the two droplets are brought together, the two monolayers form a lipid bilayer—the droplet interface bilayer—by displacing the oil between the monolayers [127–129]. The membrane proteins can be reconstituted into these lipid bilayers, as has been shown for different kinds of proteins [130]. One of the remarkable properties of the DIBs is the possibility of forming asymmetric lipid composition bilayers [130].

Free-standing membranes

By using a microstructured planar chip device for patch clamp recording [8, 131–137] instead of the common glass pipette, the fusion of the vesicles on the surface becomes an attractive method for electrophysiology and the reconstitution of membrane proteins into the lipid bilayer without denaturation.

Stable lipid bilayers are then formed by bursting a GUV (giant unilamellar vesicle) on the glass surface [138–141], forming a free-standing portion above the hole (Fig. 5).

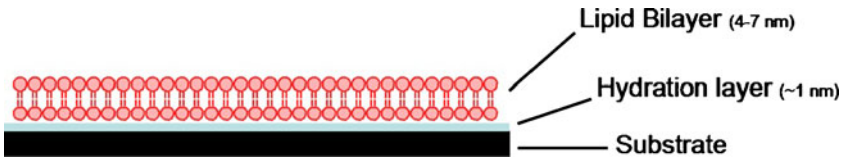

Fig. 4 Schematic diagram of an SLM. The lipid membrane can be obtained through either vesicle fusion or the Langmuir–Blodgett technique

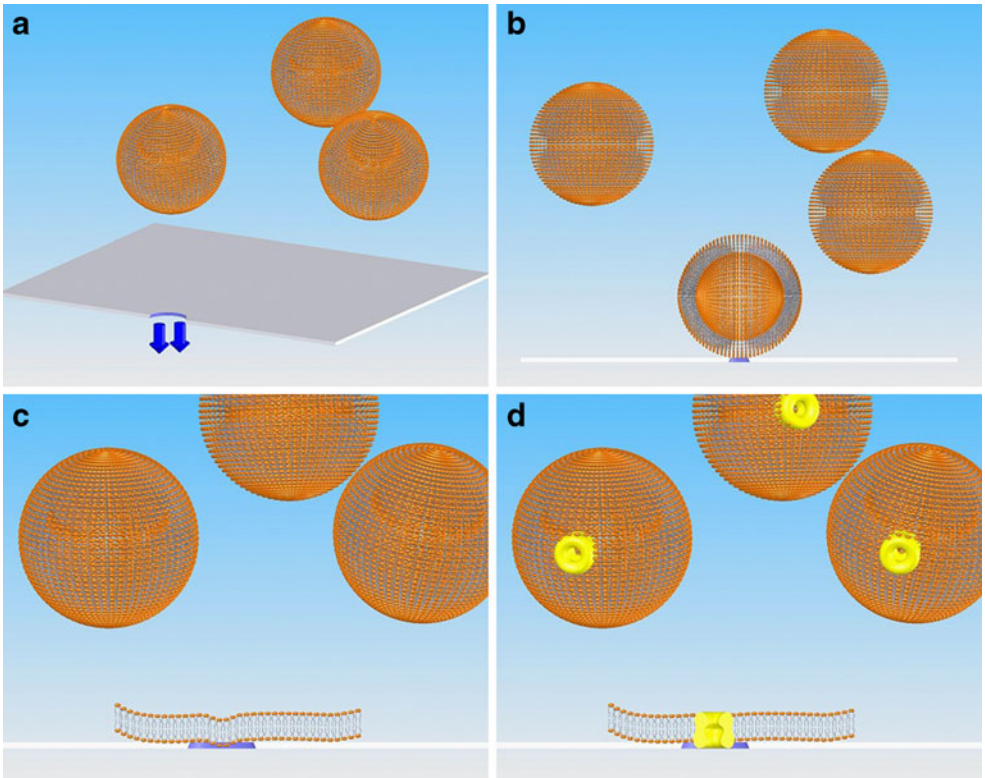

Fig. 5a–d When GUVs touch the glass surface of the chip (**a**), they burst (**b**) and form planar bilayers with the formation of a gigaseal (**c**). When proteoliposomes are used, a planar lipid bilayer is obtained with the reconstituted protein already present (**d**), so that patch clamp recording can start right away

GUVs can be prepared using the electroformation method described in [139, 142, 143].

Techniques to measure ion channel activity

The most important technique for studying ion channel activity and measuring the kinetics of ion channels is the patch clamp method, as pioneered by Neher and Sakmann [144]. This method allows the quantification of ions that translocate through a cell membrane. The membrane is controlled (clamped) by a feedback amplifier, so the voltage potential across the membrane is kept constant. This electrochemical method can record the translocation of the ions through the whole membrane or through a small membrane area. In their experiments, Neher and Sakmann were able to observe single-channel activities by pressing a small pipette against the membrane of a cell. They achieved high resistivity (gigaseal) and very low background noise by patching the membrane surface, which allows the measurement of an extremely small current (on the order of few picoamperes, pA). This high resistance increases the resolution of the ion channel recording, so a few or even single open channels can be directly observed.

Automated patch-clamp techniques

Although the traditional patch-clamp technique is the most important tool used in ion channel studies and has a very high resolution, for practical applications it is an expensive method that is difficult to use for large-scale screening (e.g., drug screening, environmental control, etc.) and requires very good patch-clamping skills. To overcome the low efficiency of this technique and to facilitate the automation and parallelization of ion channel recording, patch-clamp devices have been developed that increase the low throughput of conventional patch clamp by a factor of more than 1000. Moreover, due to automation, the reproducibility of the data has increased and the manual handling involved with the traditional patch clamp has been replaced with computer-controlled fluid handling. This allows large-scale ion channel screening with high throughput, high information content, and a lower price per data point. One of the key steps was replacing the patch-clamp pipettes with chip sensors with small holes that separate two electrolyte-containing compartments. Recent advances allow the design and fabrication of chips that are able to mimic the patch-clamp pipette tip in two different configurations: planar and lateral. In the planar configuration, the patch pore is etched in a horizontal substrate that divides the top cell compartment from the recording electrode compartment. Compared with the planar architecture, the lateral configuration keeps both the pipette and bath electrolytes on one side of the substrate. This configuration allows easier fluidic integration and has the advantage of simultaneous electrical and optical recordings [140, 144]. The process of sealing

has been handed over to automated suction and software control systems. Such single chips are successfully used to array format and record from multiple membranes simultaneously. The planar patch-clamp chip substrate contains a micron-sized aperture, where the membrane is captured (in the case of cells) or formed (in the case of artificial bilayers). Few of these chips are capable of gigaseal recordings, while the seal resistance is lower in other cases, on the order of a megaseal.

Patch-clamp chips have been developed using different substrate types: quartz [134], glass [147, 148], polymer (e.g., silicon oxide coated nitride membranes) [146, 147], silicon elastomers [137], or polyimide films [149].

Commercial automated patch-clamp systems include the Ionworks HT and Quattro (Molecular Devices Corporation, now MDS Analytical Technologies, Sunnyvale, CA, USA), Port-a-Patch, Patchliner, and the SyncroPatch 96 platform (Nanion Technologies GmbH, Munich, Germany), Dynaflow HT (Cellectricon, Gothenburg, Sweden), PatchXpress (developed by Axon Instrument, now MDS Analytical Technologies), QPatch (Sophion Bioscience, Copenhagen, Denmark), CytoPatch (Cytocentrics, Rostock, Germany), and Flyscreen (Flyion GmbH, Tübingen, Germany). Real gigaseal patch-clamp recording platforms are the Port-a-Patch, Patchliner, PatchXpress, SyncroPatch 96 platform, Dynaflow HT, Flyscreen and the QPatch, and CytoPatch.

IonWorks HT and IonWorks Quattro

The Ionworks HT system is an automated, high-throughput planar patch-clamp that uses a 384-well PatchPlate based on polyimide substrate. Each well of the PatchPlate substrate contains a small 1–2 μm hole. Using the Ionworks HT system, the success rates of the experiments vary between 50 and 90%. In contrast, the innovative Quattro technology, released onto the market in 2006, raised the success rate to >95%. The system records the activity from multiple membranes in each well, and achieves at least one successful measurement per compound. This technique utilizes a new PatchPlate substrate with the Population Patch Clamp (PPC) technology [150]. The setup contains a switchable amplifier that permits the use of either the original single-hole PatchPlate substrate or the new PatchPlate (PPC) substrate. The PPC substrate has 64 apertures in each well, which is a big advantage over the single aperture in each well in the case of the Ionworks HT. Compared with the Ionworks HT, the throughput of the Quattro system fourfold greater. In addition, the cost per data point is significantly reduced by about 50%. Ionworks Quattro allows parallel measurements for a few hundred wells, so it facilitates the highest throughput and is widely used in screening. A major drawback of the system is that it does not achieve gigaseal resistance. The low seal resistance decreases the data quality due to a lower signal-to-noise ratio [151].

Port-a-Patch, Patchliner, and the SyncroPatch 96 platform

Nanion introduced the Port-a-Patch in 2003. It is a miniaturized patch-clamp setup supporting gigaseal recording from either whole cells or artificial bilayers. The basic configuration of the Port-a-Patch minimizes the sample volume of the solution required. In this system, the traditional pipette is replaced by a planar glass chip with a simple hole (1 μm diameter). The chips (NPC-1, Nanion) are composed of borosilicate glass, which has a low capacitance, thereby reducing background noise [133]. The miniaturized Port-a-Patch setup is shown in Fig. 6 [152].

In 2006, the Patchliner was launched, which is a fully automated patch-clamp workstation supporting gigaseal recordings from cells (or from artificial membranes) simultaneously. The Patchliner platform uses borosilicate glass chips as recording substrate. Gigaseal is achieved with a high success rate. Solutions are automatically manipulated into 48 chambers without the use of a microscope or micromanipulator. The Port-a-Patch has an approximate data throughput of 50 data points per day, whereas the Patchliner is capable of 500 data points per day [153].

The SyncroPatch 96 platform has been available on the market since 2009. This system uses a 96 well plate patch-clamp substrate (glass), enabling full dose–response measurements with the addition of multiple drug (or other analyte) concentrations to each of the 96 cells. The disposable borosilicate glass substrate used for seal formation ensures high-quality data and a throughput of 5000 data points per day. Currently, the SyncroPatch 96 is one of the most important tools for efficient, accurate screening and safety testing [154].

Dynaflow HT

The Dynaflow system offers multichannel perfusion that can be combined with conventional patch clamp. The system uses microfluidic channels made from silicone elastomer to enable rapid perfusion and increased throughput in conventional patch-clamp analysis [155].

PatchXpress

PatchXpress was the first automated patch-clamp device supporting gigaseal recordings to enter the market [156]. The system uses a perforated glass substrate in a linear 16-well format, and the data throughput for the machine is 250–300 data points per day.

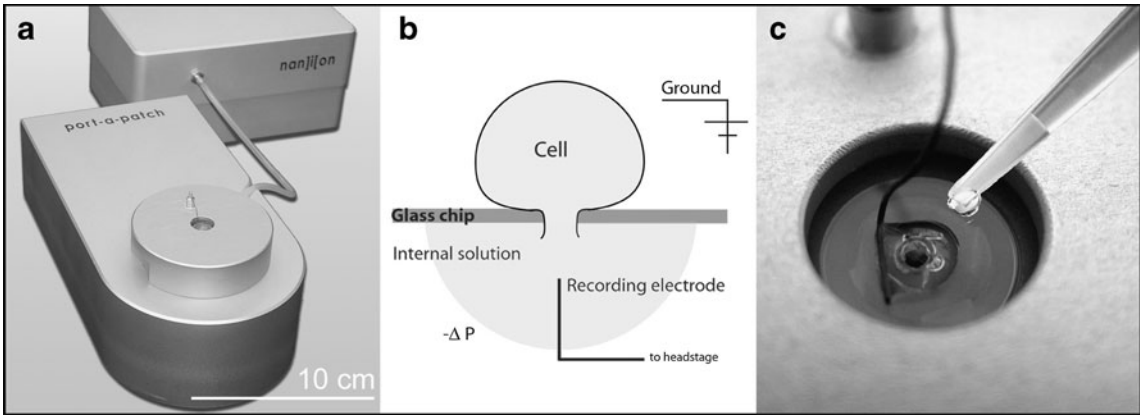

Fig. 6a–c The Port-a-Patch system. **a** The Port-a-Patch is a miniaturized setup for high-quality patch-clamp recordings. **b** A cell is automatically sealed to borosilicate glass. **c** Drug application can be performed manually (as shown in the figure) or via a special perfusion system. Figure reprinted with permission from [152]

QPatch

The QPatch 16 (16 channels) system from Sophion Biosciences [157] was commercialized in 2004 and allows gigaseal recordings. Gigaseal is achieved using a fabricated planar patch-clamp silicon chip coated with SiO_2 [158]. The system contains a 16-channel electrode array (called the QPlate) that includes glass-coated microfluidic channels. The QPlates are made from silicon structures with plastic channels for solution administration. The data throughput for the system is 250–1200 data points per day. The 48-channel QPatch HT extends the throughput of the QPatch 16 by about threefold with high data quality. The QPlate technology uses the same QPatch 16 plate repeated three times, which allows parallel recordings of 48 cells at a time. Its throughput is 750–3500 data points per day.

CytoPatch

The key component to the CytoPatch Instrument is the microfabricated CytoPatch chip. The novelty in the design of the CytoPatch chip is a glass pipette, which mimics the properties of a conventional patch-clamp electrode. The 3D patch-clamp electrode array construction is based on silicon substrate with a SiO_2 layer, and contains an aperture in quartz glass (SiO_2). The combination of two access channels makes the method unique compared to all other automated patch-clamp techniques, and allows robust gigaseal recordings [159].

Flyscreen

Flyscreen recordings are performed in "flip tips" or "chip tips," which are patch clamp glass pipettes where the cells are patch clamped on the inside rim of the pipette tip, rather than on the outside rim of the glass pipette as in a conventional patch clamp [160]. The system contains three or six channels. When parallel recording is performed, a throughput of 100–500 data points per day can be achieved [154].

The development of automated patch-clamp systems has had a significant impact on bioanalytical applications, but the automated technologies need to be developed further in order to provide devices with higher throughput and higher sensitivity. Despite the reduced prices per data point achieved by the newest available devices, costs are still high. Therefore, the development of techniques that lead to decreased costs remains an important issue.

Other techniques

Fluorescence-based assays (FLIPR assay, FluxOR assay)

Fluorescence imaging plate reader (FLIPR) assays are often used in drug discovery (e.g., for pharmacologically active substances that modulate voltage-sensitive ion channels) and research environments. Such membrane potential assay kits detect ion channel modulation by increasing or decreasing the fluorescent signal as the cellular membrane potential changes. The fluorescent signal intensity increases or decreases as the dye follows the positively charged ions into or out from the cell [161, 162].

Compared with planar patch-clamp systems, fluorescence assays have the advantage of higher throughput in parallelized multi-well formats.

The fluorescent assay FluxOR was developed to sense potassium-selective channels. The basis for this assay is the bright fluorescence of the FluxOR dye upon binding to thallium ions, which serve as surrogate ions for potassium. The fluorescent signal is proportional to the potassium channel activity. Drugs that inhibit potassium channels diminish the response, while drugs that activate the

channels increase it. The FluxOR dye does not exhibit significant affinity for calcium, which would interfere with measurements using other thallium-sensitive dyes. Off-target effects from other ion channels in the cells do not interfere with the signal. Because the FluxOR assay is cell based, the channels are observed in an intact biological system. The FluxOR platform also reports the activities of modulator compounds that control potassium ion channel activity, and not just those that close the pore.

One major drawback of fluorescence assays compared with patch-clamp automated technologies is that fluorescence assays cannot be used in all types of ion channels (e.g., sensors based on membrane voltage).

Optical techniques

Optical biosensors based on surface plasmon resonance (SPR) exploit the total internal reflection of light at a surface–solution interface to produce an electromagnetic field, or an evanescent wave extending a short distance (usually about 200–300 nm) into the solution. These techniques allow the user to study the interaction between immobilized receptors and analytes in solution, in real time, and without labeling the analyte [35]. Observed binding rates and binding levels can be interpreted in different ways to provide information on the amount of bound analyte (e.g., drug), its affinity, and its association and dissociation kinetics. Binding affinities and kinetics can be determined using very low amounts of compound without the need for prior chemical or radiolabeling [163].

Electrochemical impedance spectroscopy (EIS) based assay

This surface-sensitive technique is a powerful tool for characterizing the degree of integrity of the lipid bilayer (tethered bilayer). Many arrays of biosensors based on tethered bilayers have been developed, and these show great promise in many biotechnological applications, including disease diagnosis and environmental monitoring. In an impedance spectral measurement, a small amplitude sinusoidal voltage is applied between the two electrodes at successive frequencies and the current response is measured [164].

Transistor-based assay

The receptor cell transistor (RCT) uses a silicon chip with integrated field-effect transistors. Cells with the ion channels of interest are cultured on the chip, and activating channels causes an electrical signal in the cell that can be detected by the transistor gate as extracellular voltage under the cell. The RCT system has applications in drug discovery, high-throughput screening, and in the discovery of toxins [165].

Ion channels as recognition elements

Natural, artificial ion channels and pores are very important tools for developing bioanalytical assays. They are relevant in the fields of drug screening, medical diagnostics, biology, environmental control, etc. The applications vary greatly in terms of the conditions involved. For a better overview, the applications described in this section are summarized in Table 1.

Ion channel sensors for safety screening

Aptamer-encoded artificial nanopores were used to quantify the bioterrorist agent ricin in solution at single-molecule resolution [166]. A ricin A chain-targeted aptamer was immobilized on the inner surface of a 56 nm glass nanopore. The current trace indicated the presence of 100 nM of a ricin A chain protein in the external solution. In this system, the sensing signal comprises a series of stepwise, discrete current blocks that are attributed to individual target molecules that are sequentially captured by the immobilized aptamer in the nanopore. This aptamer-encoded nanopore is advantageous over other systems. Firstly, it performs rapid label-free target detection (ricin) with high sensitivity and selectivity. Secondly, the nanopore electrical detection is much simpler than other optical and mass spectroscopic methods, making it more suitable for real-time detection. The digital signal produced by individual ricin molecules distinguishes them from the analogous background signal, thus greatly enhancing the signal/noise ratio. This is particularly useful for real-time detection, because one can still identify discrete single molecule events, even when the background current dynamically drifts or fluctuates with the environment. Thirdly, the aptamer-encoded nanopore can distinguish between transient current blockades caused by nonspecific molecules passing through the nanopore and much longer blocks resulting from the target binding. Applications of this nanopore sensor technique currently focus on bioterrorist materials because of their importance to homeland security, but this invention actually has high potential for a broad range of applications. Aptamer-based nanopore sensors can be used for environmental monitoring, to detect pollution or contaminating materials in water. It can be applied to medical diagnoses by quantifying biomarkers in blood samples. Using DNA aptamers is also advantageous because they are more durable than most protein receptors, resisting most denaturing and degrading conditions, including immobilization, and they are simple to synthesize, modify, and immobilize using low-cost methods. Compared with the DNA aptamer based assay, the stability of protein-based biosensors is always claimed to be greater in practical use. However, DNA aptamers are chemically stable. They are stable between pH values of 2 to 12, and are thermally renaturable [167].

Table 1 Types of biosensors and techniques

	Type of biosensor	Type of matrix / bilayers	Type of technique / instrumentation	References
Safety screening	Bioterrorism agent ricin	Glass nanopore (solid state nanopore)	Aptamer-based nanopore	[166–168]
	Detection of explosives using α-hemolysin	Black lipid membrane (BLM)	Patch clamp (conventional) / Port-a-Patch	[169]
Drug screening	Safety pharmacology	Cell membrane	Automated patch clamp	[134, 150, 151, 153–160, 171–173]
			Fluorescence-based assays (FluxOR)	[173–175]
		Droplet interface bilayer (DIB)	Two-droplet system	[177]
	Based on receptor cell transistor	Cell membrane on silicon chip	Receptor cell transistor (RCT)	[165]
Sensing narcotic drugs	Nanopore on a chip	Free-standing membrane	Conventional bilayer technique / automated patch clamp (Port-a-Patch)	[47]
Detection of small organic molecules, ions, and enantiomers	Based on engineered α-hemolysin pores	BLM / DIB / free-standing membrane	Conventional bilayer technique / automated patch clamp (Port-a-Patch) / DIB	[60, 178–181]
	Based on artificial pore		Conventional bilayer technique / automated patch clamp (Port-a-Patch)	[64, 183–188]
Detection of bacteria, viruses	Based on natural and artificial ion channels	Tethered lipid bilayers	Electrochemical technique (impedance)	[51, 192]
Detection of antibodies			SPR technique, electrochemical technique (impedance)	[54, 193–195, 197]
Detection of DNA, RNA		Black lipid membrane (BLM)	Conventional bilayer technique / Port-a-Patch / automated patch clamp / DIB	[60, 190, 191, 194–198]

DNA aptamers are more resistant to denaturation and degradation, their binding affinities and specificities can easily be manipulated and improved by rational design or by molecular evolution techniques, and they can be modified with functional groups or tags that allow covalent, directed immobilization on biochips, resulting in highly ordered receptor layers. Moreover, DNA aptamers will maintain their structure, and will not dissociate or otherwise change their characteristics, which can be a problem with protein-based assays [168].

Heaving already created a nanopore detector for ricin, the next aim is to develop a nanopore sensor that will use an aptamer to detect anthrax spores, which are well known to be a bioterrorist threat. Scientists already have developed an anthrax aptamer that can be used in future designs of such biosensors [60].

Engineered versions of the transmembrane protein pore of α-hemolysin (α-HL) can be used as stochastic sensing elements for the identification and quantification of a wide variety of nitroaromatic analytes at the single-molecule level. The binding sites for nitroaromatics were built within the lumen of the (α-HL) pore from simple rings with seven aromatic amino acid chains (Phe, Tyr, or Trp). By monitoring the ionic current through a single pore at a fixed applied potential, various nitroaromatics were distinguished by evaluating the amplitude and duration of individual current-blocking events. Rings of less than seven aromatics bind the analytes more weakly. This suggests that direct aromatic–aromatic interactions are involved. Such quick tests that use engineered pores are intended for use in the detection of explosives [169].

Ion channel sensors for drug screening, narcotics, small organic molecules, ions, and enantiomers

Drug screening

Many genes encoding ion-channel subunits play critical roles in diseases, either directly or indirectly. The big challenges of ion-channel screening are to find more specific drugs that are able to selectively block ion-channel subtypes or ion-channel mutants and to allow the development of new and safer pharmacotherapies [13]. For a long time, due to the lack of high-throughput techniques, drug screening on ion channels was a slow process. In the last decade, high-throughput automated electrophysiological platforms have been developed that are more suited to large drug detection, such as those used in the modern pharmaceutical industry [170]. This development is expected to accelerate the individuation and lead optimization of drug candidates. With voltage-gated channel assays, changes in voltage across the membrane act as a stimulus to open the channel proteins, allowing the flow of ions across the membrane. Specialized patch clamp amplifiers provide these voltage stimuli. On the other hand, ligand-gated assays require at least one additional compound (i.e., agonist or agonist plus antagonist). Multiple compound additions necessitate a sophisticated fluidics system [157].

In the case of chloride ion channels, a number of assays have been performed for drug screening and to develop treatments of, for example, insomnia, convulsions, and other diseases based on disorders of the target neuronal chloride channels [171, 172]. Many of these monitoring methods use high-throughput patch-clamp electrophysiology, and some of them are based on radioactive and fluorescence assays [173].

Potassium ion channels attract huge attention as targets for therapeutic indications and for safety profiling. In order to facilitate the pharmaceutical development of potassium channel modulators, high-throughput potassium-specific optical assays are critical. A major challenge in designing such assays is the shortage of K^+-specific fluorescent indicators capable of detecting narrow physiological variations in the extracellular K^+ concentration. K^+-conductive channels are also permeable to other ions such as thallium. Therefore, due to the lack of K^+-specific fluorescent indicators, the flux of the thallium surrogate ions is used to measure the activity of the K^+ ion channels. Using Tl^+-sensitive fluorescent probes [174], the florescence assay can measure the activity of the K^+ channel. Along with the efflux of K^+ ions (e.g., from cells), the activation of K^+ channels allows the influx of Tl^+ ions (e.g., from the extracellular solution through open channels into cells). Thus, Tl^+-sensitive fluorescent dyes inside cells can detect the activity of K^+ channels and their modulation by drugs.

The activity of K^+-selective channels can be successful monitored using the fluorescent assay FluxOR. This is a universal K^+ channel assay that produces relevant pharmacological data using standard fluorescence microplate readers. The basis of this sensor is the bright fluorescence of the FluxO dye upon binding Tl^+ ions (the surrogate ions for K^+). FluxOR is cell based, so the channels can be observed in an intact biological system. The fluorescent signal is proportional to the activity of the K^+ channels. Drugs that inhibit K^+ channels diminish the response, while drugs that activate the channels increase it. The FluxOR platform also reports the activities of modulator compounds on K^+ ion channels, not just those that close the pore [175].

A no-wash assay for K^+ channels was introduced using ThalKal, a Tl^-sensitive dye, along with a quencher. This assay offers an alternative way to perform safety profiling and the primary screening of modulators of ligand- and voltage-gated K^+ channels. Unfortunately, this kit has several characteristics that could adversely affect its performance; for example, the potential interference of quencher molecules with drug compounds or limited detection sensitivity due to nonspecific background fluxes. Improvements addressing these shortcomings could take this fluorescence-based assay to the next level [176].

The viral potassium channel (Kcv) can screen for blockers of ion channels using a droplet interface bilayer (DIB) array. Electrodes embedded within the droplets allow the measurement of transmembrane ionic currents through individual channels and pores [177].

Serotonin-gated ion channels and voltage-gated ion channels are successfully used in biosensors based on receptor cell transition (RCT). Cells with ion channels are cultured on a silicon chip. This method has already been implemented for 5HT3A channels (serotonin-gated channels) and for Kv1.3 (voltage-gated K^+ channels). Clinically, the serotonin receptor is blocked during cancer therapy to inhibit emesis (vomiting). The Kv1.3 channel has been intensely discussed as a target for autoimmune diseases like multiple sclerosis due to its role during T-cell activation. The activation of channels causes an electrical signal in the cell that can be detected by the transistor gate as extracellular voltage under the cell [165].

Narcotic drugs

Analytical techniques for the rapid detection of drugs are important in border-control situations or in drug investigations. This method permits rapid and highly selective detection using the transmembrane toxin α-hemolysin (α-HL) combined with a DNA aptamer with a long tail. DNA aptamers have specific recognition properties for small molecules, and the conformation of aptamers changes after ligand binding. Aptamers selectively recognize, for example, cocaine at low concentrations (300 ng/mL cocaine, the drug test cutoff limit) within 60 s using parylene nanopores embedded in a microchip [47].

The DNA aptamer is single stranded before binding to the target molecule. The single-stranded DNA (ss-DNA) can pass through the RHL pore because the constricted region of the channel (1.5 nm) is larger than the diameter of the ss-DNA (~1 nm), as depicted in the left panel of Fig. 1a. In contrast, the size of a ligand-bound aptamer does not allow it to pass through the pore (Fig. 7a, right). The presence of the ligand-bound aptamer retained in the pore can be clearly observed because of the large difference between the channel currents for the translocated and captured states. Recognition of a target molecule is clearly achieved when the aptamer is retained in the pore, and the cocaine concentration can be determined by measuring the time until capture.

Detection in the microfluidic device (Fig. 7b–d) indicated that cocaine at a concentration of 3 μg/mL could be sensed in just 25 s with high selectivity. The detection times ranged from 60 s at 300 ng/mL to 5 s at 30 μg/mL. Such systems where the cocaine at a concentration equivalent to that of the drug test cutoff limit was recognized within 1 min could be applied to not only cocaine but also a wide variety of targets, as several types of aptamers have already been developed. This method is expected to aid in the development of real-

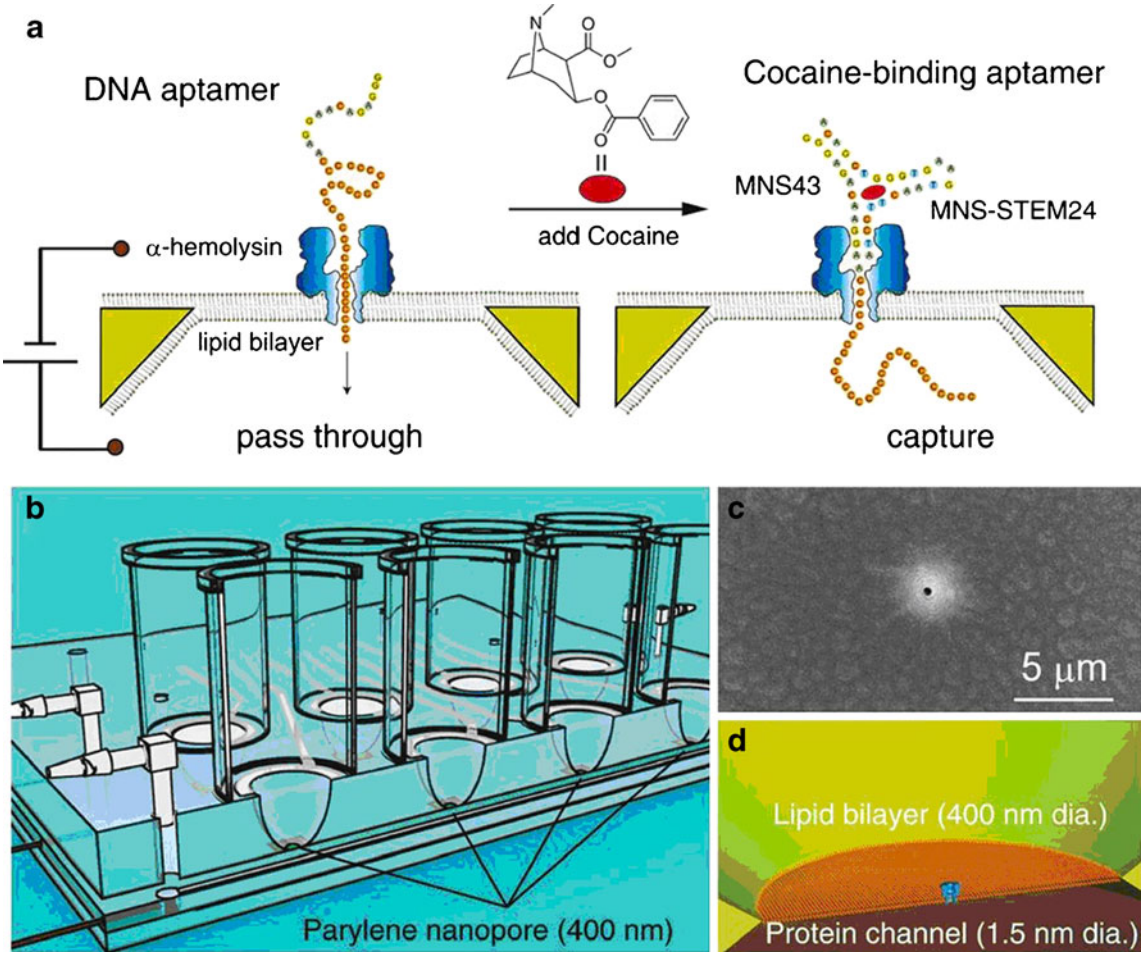

Fig. 7 **a** Schematic overview of detection using the cocaine-binding aptamer (CBA). The DNA aptamer can pass through the pore in the absence of cocaine (*left*). However, in the presence of cocaine, the CBA cannot pass through and is captured in the pore (*right*). **b** Microfluidic device with nanopores (parylene). **c** Scanning electron microscopy image of a nanopore. **d** Lipid bilayer membrane with a small area (~400 nm) formed in the nanopore. Figure reprinted with permission from [47]

time drug testing technology and the rapid sensing of small molecules using biosensors on a chip.

Small organic molecules, ions, and enantiomers

Stochastic sensing of organic molecules is based on engineered α-HL channels. These channels are equipped with an internal, noncovalently bound molecular "adapter" that mediates channel blocking by the analyte. Cyclodextrins have been used as adaptors because they fit comfortably inside the pore for periods that are long enough to observe host–guest interactions, and they present a hydrophobic cavity suitable for binding a variety of organic analytes [60].

Biosensors based on engineered α-HL pores can be used for the detection of many types of small organic molecules, metal ions, and enantiomers [55, 58].

For example, when α-cyclodextrin is lodged in the pore, it remains capable of binding the same organic molecules that it binds when free in solution. Moreover, a single sensing element of this sort can be used to analyze a mixture of organic molecules with different binding characteristics. The binding events cause current fluctuations that permit the quantification and identification of the molecules [178]. Multiple molecules can be detected concurrently with the same cyclodextrin, and the system is highly flexible and combinatorial. Moreover, additional adapters can be also used to target specific organic molecules (for example cyclic peptides) [179].

The α-HL pore was successfully used to sense different metal ions. A tetrahistidine motif, genetically engineered on one of the seven subunits in the lumen of the β-barrel, can reversibly capture single divalent metal ions such as Zn^{2+}, Cd^{2+}, and Co^{2+}, enabling one pore to detect metal ions at nanomolar concentrations in the mixture. Moreover, concentrations of two or more divalent metal ions in solution can be determined simultaneously with a single sensor element [180].

A ring-shaped molecule such as cyclodextrin or a cyclic peptide can be lodged in the α-HL hemolysin pore lumen,

where it acts as a molecular adaptor to discriminate between structurally similar compounds (e.g., drugs) [178] and enantiomers (e.g., ibuprofen enatiomers) [181].

Chiral compounds interacting with cyclodextrin can be separated based on their block durations and current amplitudes [60].

Artificial pores can be used as recognition elements in diagnostics by sensing multiple analytes in complex matrices such as foods, or biological fluids such as blood [182].

For example, using a rigid-rod β-barrel architecture with the peptide sequence LKLHL, a pore for sugar sensing has been successfully constructed. Diagonally positioned lysines and histidines at the inner surface make these pores more responsive to blockage by adenosine 5′-triphosphate (ATP) than by adenosine 5′-diphosphate (ADP). Using this important ATP/ADP discrimination for sugar sensing, soft drinks such as Coca Cola or Red Bull were tested, and the consumption of the ATP blocker during phosphorylation was optically transduced as fluorogenic pore opening [183]. Sensing of the lactose level in milk was also targeted with synthetic pores (also utilizing a β-barrel architecture) with minor changes. Selectivity was achieved by replacing invertase with β-galactosidase, which is sensitive to lactose. The β-barrel architecture was also used to sense the acetate in vinegar or citrate in orange juice [184].

A nanoporous H_2O_2 sensor based on single conical polymer nanochannels has been developed. The walls of this nanopore are functionalized with the horseradish peroxidase (HRP) enzyme [64]. Monitoring the redox reactions via changes in the nanochannel transport properties offers an easy method to detect H_2O_2. It has been recognized that the quantitative monitoring of hydrogen peroxide is important for medical diagnostics. This is because accumulation of H_2O_2 in the mitochondria, for example, can lead to cancer as well as neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's.

Single conical nanochannels were fabricated in 12 μm thick films of polyethylene terephthalate by the track-etching technique. This technique is based on first irradiating films with single swift heavy ions, followed by the selective chemical etching of the damage trails caused by the ions along their trajectories. The conical shape of the channels resulted from the asymmetric development of the damaged tracks in a concentrated sodium hydroxide solution. The immobilized enzyme remains active in redox reactions that occur inside the single nanochannel in the presence of even nanomolar concentrations of H_2O_2. The function of the immobilized enzyme in a single nanochannel was confirmed by studying products of the redox reactions occurring in the presence of substrates based on 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) and at various concentrations of H_2O_2 [64]. The cationic radical ABTS reduced the ion current in the nanochannel in a voltage-dependent manner, consistent with voltage-dependent concentrations of ions in conical nanochannels. The magnitude of the current blockage was correlated with the H_2O_2 concentration in the solution. The current response scaled linearly over a wide range of H_2O_2 concentrations, from micromolar to nanomolar. The sensor sensitivity is predicted to reach ~10 nM, which is superior to those of many existing H_2O_2 sensors [185, 186], but still lower than the best electrochemical sensing devices known (~10 pM) [187, 188]. However, the nanoporous system is simple to prepare, not susceptible to fouling, and can be used multiple times without losing the sensing signal. Covalent binding prevents the horseradish peroxidase (HRP) enzyme from leaching. Future expectations are that narrower pores will lower the detection limit and improve the sensitivity.

Biosensors for the detection of bacteria, viruses, antibodies, DNA, and RNA

Ion-channel devices used for the detection of bacteria, viruses, antibodies, DNA, and RNA are very often stochastic ion channel sensors in which the conductance of a population of natural ion channels is switched by the recognition event. Such devices are based on an artificial membrane packed with gramicidin A. The binding of the target molecule splits the gramicidin A dimers into nonconducting monomers. By varying the nature and type of receptors in the stochastic sensor, this technique has been applied to blood typing, the detection of bacteria, viruses, etc. The receptors include antibodies, enzymes, DNA, antibody-binding proteins, and synthetic ligands. Highly sensitive analyte detection is achieved due to the large flux of ions transmitted through this ion channel [51].

When used as a rapid point-of-care test, the assay based on ion channels has comparable levels of sensitivity and reproducibility to a virus culture (the "gold standard") or a chromatographic immunoassay, but without the need for chemical or other pretreatments of the sample and on-board calibrator. The rapid stochastic ion-channel test provides an objective readout within 10 min of specimen inoculation into the chamber wells. Moreover, engineering the stochastic ion-channel biosensor with specific antibodies enables the rapid detection and identification of influenza A subtypes. Recently, researchers generated a panel of influenza A H5N1 monoclonal antibodies based on an electrode array switch that can potentially be used to build an H5N1-specific biosensor. Incorporating appropriate antibodies into the ion channel will make this biosensor a diagnostic device enabling the rapid monitoring of potential pandemic and influenza outbreaks [189].

Immunosensing of antibodies is classically performed by enzyme-linked immunosorbent assay (ELISA) and radio-immunoassay techniques, which require time-consuming preparation steps, including the use of fluorescently, radioactively, or enzyme-labeled antibodies. Direct immunoassays without sample preparation would improve the speed and reliability of point-of-care diagnostics. Label-free biosensors based on ion channels were developed to meet this target. The transducers use surface-sensitive techniques that allow the detection of antibodies in blood (e.g., with SPR), and in blood serum (e.g., with impedance spectroscopy).

Tethered lipid bilayers containing a transmembrane synthetic ligand-gated ion channel (SLIC) have been formed on gold surfaces. The SLIC was designed as a highly selective receptor to detect antibodies in whole blood. These antibodies are important in malaria diagnosis. The specific binding of the antibody to the sensor surface was monitored on-line either optically by SPR in whole blood or electrically by measuring the channel activity of SLIC in blood serum. The SLIC comprises two functional elements: an extramembranous site, which serves as a receptor to selectively recognize and strongly bind the analyte (for example an antibody), and a transmembrane amphipathic four-helix bundle forming an ion channel whose activity is gated by the binding of the analyte to the receptor part [54].

These techniques demonstrate the feasibility of a highly sensitive and easily applicable whole-blood biosensor on the basis of simple commercially available components. The sensor might find application in the field of infectious diseases, such as in point-of-care diagnostics of malaria, in the high content quality control of blood samples from donors, or in the monitoring of vaccination efficacy. Such simple and robust biosensors are urgently needed to detect infectious diseases (for example in the populations of developing countries), during vaccine development to monitor the antibodies in the blood of vaccinated test subjects, or for high content quality control of blood samples from donors. The biosensor can be produced using presently available technologies and components, such as automated high-throughput instruments [190, 191] or miniaturized portable SPR devices [192–194].

α-HL pores were successfully used for the detection of biomacromolecules like RNA or DNA. Single-stranded RNA or DNA molecules can pass through the wild-type α-HL pore in an elongated conformation (cf. Fig. 7). The transit time and extent of current block reveal information about the length of the nucleic acid and its base composition [195, 196].

In certain cases, the magnitude of the conductance changes within each blocking event. This communicates additional details about the nucleic acid structure, such as the compositions of stretches of ~50 bases [197], or the presence of mismatches in DNA hairpins [198].

Such nanopores may also be used as next-generation technology for DNA analysis. The characteristic current modulations permit individual base species to be discriminated and thus an entire DNA sequence to be obtained [60].

Conclusions

The performance of patch-clamp instrumentation has developed such that highly parallel measurement is now achieved. In addition to this instrumental development, advances in bilayer design and in the engineering of nanopores have led to reductions in the size and cost of planar patch-clamp systems. This permits high-throughput screening, and has led to increased interest in major industries such as the pharmaceutical and food industries. The potential of instrumentation based on ion-channel sensor elements has been demonstrated for safety controls and for drug tests.

Automated electrophysiological assays based on voltage-gated ion channels have some advantages over assays based on ligand-gated ion channels. Unlike voltage-gated ion channels, ligand-gated ion channels require an agonist–receptor interaction to open. With voltage-gated channel assays, changes in voltage across the membrane act as the stimulus to open the channel proteins, allowing ions to flow across the membrane. Ligand-gated assays require at least one additional compound and several wash steps to avoid desensitization and/or internalization of the receptors. The development of high-throughput techniques must address these challenges. Multiple compound additions necessitate a sophisticated fluidics system, and high throughput has been limited by the timescale resolution of translocation assays thus far. The great advantage of ligand-gated channels is their intrinsic gain factor of over a million. Due to the structures of ligand-gated channels, their functions can be modulated by other compounds in the sample. This can be considered either a disadvantage or an advantage, because it provides access to a variety of analytes and processes beyond the ligand itself.

One of the big issues is to develop sensors that can be used for longer, by replacing the fragile artificial lipid bilayer with more stable types. Nanotechnology, further technical achievements, and focused research work will play major roles and in improving the designs of new biosensors based on ion channels. Taking advantage of modern technologies and exploiting emerging systems, the next biosensing platforms will exhibit high resolutions, fast responses, high throughputs, and low costs. In the present paper, we have reviewed potential features of such platforms and described their applications.

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