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Electrode-supported and free-standing bilayer lipid membranes: Formation and uses in molecular electrochemistry

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Abstract

This short review is aimed at emphasizing the recent most prominent advances in the electrochemical investigation of the interactions of molecules with purely lipid bilayers. The first part which deals with electrode-supported bilayer lipid membranes is articulated around the nature of electrode material with a focus on mercury to celebrate this special issue entitled "100 years from polarography to contemporary electrochemistry". The second part is devoted to suspended or free-standing bilayer lipid membranes that are robust models to investigate the passive transport of molecules. It is notably shown that molecular electrochemistry methods such as cyclic voltammetry, square wave voltammetry, amperometry, electrochemical impedance spectroscopy, or electrochemiluminescence are well-adapted for the investigation of molecules/lipids interactions or the passive transport of molecules. These various techniques bring meaningful information notably on molecule/lipid interactions and transport modes across phospholipids.

KEYWORDS
artificial membranes, bilayer lipid membranes, molecule lipid interaction, molecular electrochemistry, passive transport

1 | INTRODUCTION

Biological membranes are complex architectures essentially composed of lipids, proteins, cholesterol, carbohydrates. Each of these components plays a specific role in the selective communication and regulation of ion or molecules fluxes between the exterior and interior of biological cells. The complexity of real membranes prompted the development of models of simpler but controlled composition allowing the investigation of interactions of molecules with a specific component of membranes.

Within this framework, two main types of model membranes have been developed: (i) suspended "black" lipid membranes (BLM also called free-standing bilayers, in contact with liquid solutions at both faces) and (ii) electrode-supported biomimetic membranes (displaying a single face in contact with the solution, the other being in contact with the electrode) including supported lipid monolayers (sLM), supported bilayer lipid membranes (sBLM), hybrid bilayer lipid membranes (hBLM), tethered bilayer lipid membranes (tBLM), and floating bilayer lipid membranes (fBLM).

Though most of these model membranes have been electrochemically investigated in the presence of embedded proteins to mimic ionic channels and study ion transport, pure lipid bilayers are reliable systems for the exploration

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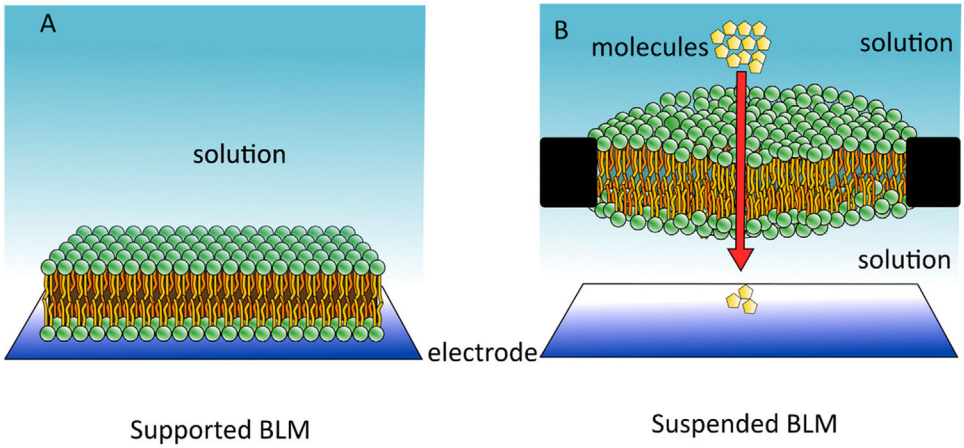

FIGURE 1 (A) Electrode-supported (sBLM) and (B) free-standing, or suspended, bilayer lipid membranes for molecular electrochemistry uses

of molecule/lipid interactions and related passive transport issues of biomolecules, i.e., the major trafficking route for drugs entering cells.

This short review is aimed at emphasizing recent works carried out from the molecular electrochemistry viewpoint to investigate molecule/lipid interactions and passive transport of molecules involving electrode-supported bilayer lipid membranes (i.e., sBLM) or free-standing bilayers by using tools such as cyclic voltammetry, square wave voltammetry, electrochemical impedance spectroscopy (EIS), or electrochemiluminescence. The first paragraph is devoted to electrode-supported bilayer lipid membranes emphasizing the role played by the nature of electrode material with a focus on mercury in keeping with the special issue "100 years from polarography to contemporary electrochemistry". The second paragraph deals with suspended (i.e., free-standing) bilayer lipid membranes that are well adapted to investigate the passive transport of molecules (Figure 1).

Readers interested in the combination of electrochemical techniques with surface characterization to provide molecular-level information on other components than lipids in model membranes,[1] or in the development of biosensors based on lipid membranes platforms[2] are invited to consult the above-cited excellent reviews.

2 | ELECTRODE-SUPPORTED BILAYER LIPID MEMBRANES (sBLM): PREPARATION AND USES

Supported bilayer lipid membranes (sBLM) can be depicted as planar lipid structures adsorbed directly onto the surface of solid substrates.[3] This type of model membrane is well-adapted for the investigation of molecule/lipid interactions provided that they can be prepared with a minimum or even without defects. Importantly, a perfectly covered sBLM electrode prevents electron transfer to take place across the lipid bilayer. Indeed, such an electrode is "blocked" with respect to electron transfer from or to redox species present in solution and a low capacitive current is observed accordingly. However, redox species in solution prone to cross or interact with the lipid bilayer may exhibit faradaic signals or capacitance and resistances changes, thus potentially delivering information on molecule/lipid interactions as well as on the transport features of these redox species through phospholipids.

In this first part, the focus will be made on the main preparation of sBLMs on various electrode materials commonly used in molecular electrochemistry as well as to their most prominent utilizations in view of molecule/lipid interactions studies. In the context of this special issue, and though early attempts to deposit sBLMs onto solid metal surfaces (platinum, silver, stainless steel) were described by Ottova and Tien,[4] mercury, that is a material strongly associated with Jaroslav Heyrovský (Nobel prize 1959), will be first presented.

2.1 | Mercury

Though mercury (Hg) was the first metal surface used for the formation of supported lipid films,[5] essentially monolayers of lipid membranes were investigated only recently. Originally, these membrane models were introduced by I. Miller[6] then adopted in a modified version by A. Nelson.[7] The fluidity of liquid mercury leads to defect-free lipid films with high mechanical stability. Generally, 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

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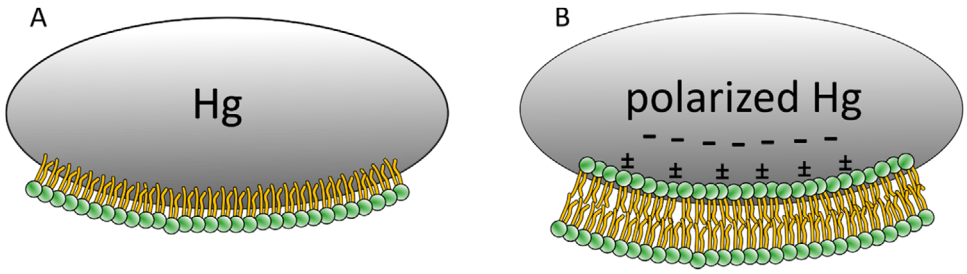

FIGURE 2 Schematic representation of the mono- or bilayer lipid auto-assembly at Hg surfaces as a function of the electrode status: non-polarized and hydrophobic Hg Ref. [8] (A) or polarized at -1 V / -1.3 V vs. Ag/AgCl Ref.[14] (B)

a hanging mercury dropping electrode in the electrolyte. However, owing to the hydrophobicity of mercury the lipid monolayers exhibit hydrocarbon tails directed toward the mercury surface and the polar heads oriented toward the solution, the formation of bilayer films being highly disfavored in polar media such as aqueous solutions (Figure 2(A) 2a). The accessibility and reproducibility of mercury-supported lipid monolayers led to numerous works summarized in Ref. [8] R. Guidelli successfully achieved the formation of tethered bilayer lipid membranes functionalized by sulfur groups in order to bond to mercury electrode surfaces (tBLM). These mercury-tBLMs have been notably prepared to study the ion channel behavior of various membrane-active peptides.[9-11] Such lipid/peptide assemblies are out of the scope of this article, but interested readers are encouraged to consult the excellent summary of this research in Ref.[12]

Actually, the first formation of mercury-supported bilayer lipid membranes has been achieved for the first time quite recently.[13] The strategy relies on the conversion of a mercury-supported monolayer lipid membrane to a bilayer upon application of a specific potential value. Recently, the first electrochemical characterization of dioleoyl phosphatidylcholine (DOPC) bilayers structures on a negatively polarized mercury electrode has been reported.[14] It was notably shown that bilayers were stable on the Hg surface upon polarization in the -1.0 to -1.3 V/Ag/AgCl potential range (Figure (2B)2b). This important result, therefore, opens interesting perspectives in the use of mercury electrodes for the formation of the supported bilayer lipid membrane.

2.2 | Gold

Gold electrode-supported bilayer lipid membranes are generally prepared through either the fusion of vesicles or a combination of Langmuir–Blodgett (LB) and Langmuir–Schaffer (LS) techniques. The use of gold as support for bilayer lipid membranes is particularly interesting when surface characterization techniques such as spectroscopy, neutron scattering and surface imaging methods are envisioned to provide information on the structure of lipids and membrane-active proteins in sBLMs as a function of the electrode potential. A comprehensive review on these aspects can be found in Ref.[1]

An alternative method to obtain lipid bilayers at gold surfaces is the fusion of vesicles, obtained from the successive adhesion, deformation, and rupture of vesicles that lead to a spreading of lipids over the gold surface.[15] Various factors may affect the adhesion and rupture of vesicles: vesicle size, temperature, presence of cations (e.g., Ca^{2+} or Mg^{2+}), surface charge, roughness, ionic strength, solution pH, and order state (i.e. gel or liquid crystalline) of lipids composing the vesicles.[16] For instance, gel-ordered DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine) vesicles readily open and fuse onto atomically flat gold (111) surfaces,[17,18] but not onto gold films that have been evaporated on glass or quartz because in this case the gold film consists of small gold nanoparticles.[19,20] Nevertheless, the presence of cholesterol in DMPC vesicles allows sBLMs formation on both surfaces.[19,21] Indeed, the presence of cholesterol increases the fluidity of gel state lipids.[22] It is noteworthy that the fusion of vesicles enables the preparation of lipid bilayers with pre-incorporated membrane proteins.

The LB/LS (Langmuir–Blodgett/Langmuir–Schaffer) approach allows layer by layer transfer of lipids from the air-water interface onto solid substrates (Figure 3). Lipid monolayers are compressed on the water surface of the Langmuir vessel allowing a precise control of both the physical state and packing density during the transfer on the solid substrate. To form a lipid bilayer on a solid support the first leaflet is transferred by pulling the support through the monolayer (LB technique) and the transfer of the second monolayer is achieved by either LB or LS transfer. The great advantages of this method are the control of the compactness of the layers as well as the possibility to form asymmetric lipid bilayers, i.e., having two leaflets with different lipid composition. Asymmetry

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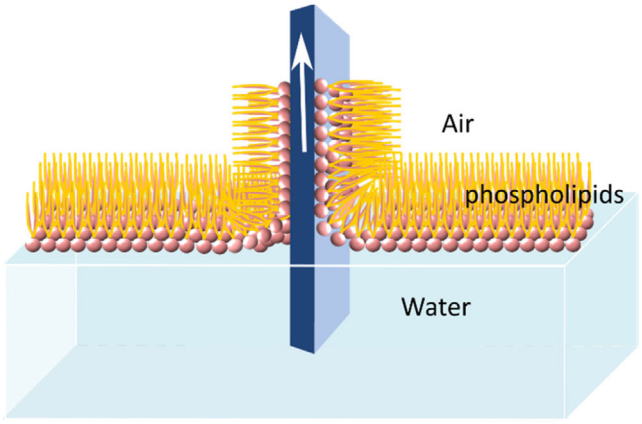

FIGURE 3 Illustration of the Langmuir–Blodgett / Langmuir–Schaffer (LB/LS) technique for the formation of lipid monolayers

is an important property of cellular membranes. A comprehensive review describing sBLM formation on gold surfaces can be found in Ref. [23]

Several interesting applications of gold-supported bilayer membranes were achieved by R. Bilewicz to investigate the incorporation and interactions of perfluorinated compounds by electrochemistry techniques. Indeed, these pollutants may affect the properties of cell membranes and cause developmental and reproductive diseases.

Accordingly, cyclic voltammetry and square wave voltammetry have been used to monitor the incorporation of perfluorinated compounds such as perfluorooctane sulfonic acid (PFOS) in bilayer lipid membranes supported on gold electrodes prepared by the LB/LS techniques.[24,25] More precisely, these investigations relied on the electrostatic interactions between the negatively charged PFOS anions and either a positively charged ruthenium (hexaammineruthenium(III) chloride) or a negatively charged ferricyanide probe, which are compounds prone to access the electrode surface through defects/pinholes present in bilayer membranes.[26] The results demonstrated that PFOS anions efficiently incorporate in pure DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine) bilayers, but can locally generate defects or even lead to a destructuration of the membrane especially at high PFOS concentrations. The presence of PFOS in model membranes was also visualized by electrochemical scanning tunneling microscopy (EC-STM). It was also shown that the introduction of PFOS during the formation of the model membrane (i.e., before their electroanalytical investigations) inhibited the transport of drugs such as doxorubicin, an antibiotic used in cancer treatment. Unexpectedly, electrostatic repulsions were observed between the positively charged drug and the PFOS/DMPC membranes due to changes in the conformation and orientation of the DMPC acyl chains during membrane preparation. These changes in the conformation and orientation of the DMPC acyl chains had previously been confirmed by polarization modulation infrared reflection-absorption spectroscopy (PM-IRRAS) experiments.[27]

Interactions between PFOS compounds and DMPE (1,2-dimyristoyl-sn-glycero-3-phosphoethanoloamine) model membranes were also investigated by cyclic voltammetry for different membrane compactnesses.[28] The accessibility of menadione (an electroactive quinone compound from a group of K vitamins) was investigated in the absence or presence of PFOS compounds. As expected, the "passage" of menadione through pure phospholipid bilayers (i.e., in the absence of PFOS) was more efficient for electrodes modified with DMPE bilayers transferred at a pressure of 3mN/m, the lipid layer exhibiting more defects under these conditions. Interestingly, the presence of PFOS appeared to fill the pinholes (defects), thus limiting the transport of menadione. The passage of menadione was still observed by membrane permeation in agreement with similar electron transfer rate constants found for both types of modified electrodes. This was confirmed using potassium ferricyanide and hexaammineruthenium chloride probes which only access the electrode surface through defects/pinholes present in bilayer membranes.[26]

Supported BLMs made of phospholipid mixtures are also of importance to design lipid raft model membranes.[29] Lipid rafts are microdomains present in the structure of eukaryotic biological membranes. They are involved in many cell functions such as membrane transport, interaction with drugs, and signal transduction. Accordingly, surface and electrochemical properties of lipid raft model membranes prepared from equimolar mixtures of 1,2-dioleoylo-*sn*–glycero-3-phosphocholine (DOPC), cholesterol (Chol), and egg sphingomyelin (SM) were investigated in the presence of cerivastatin, a drug known for lowering blood cholesterol level.[30] Electrochemical impedance spectroscopy established the heterogeneity of sBLM deposited onto Au(111) electrodes. However, incorporation of the statin drug blocked part of the defects and led to increased barrier properties of the film (notably towards water molecules and ions) despite its increased fluidity.

Interestingly, similar effects were also observed with melittin, a cationic antimicrobial peptide, interacting with supported lipid membranes made of negatively charged phospholipids such as 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG) or 1,2- dimyristoyl-*sn*-glycero-3-phosphoserine (DMPS).[31] Accordingly, EIS data featured an increase in resistance and a decrease in capacitance at the electrode/BLM interface. Melittin was also investigated in the presence of zwitterionic choline

lipids BLMs made of DMPC (1,2-dimyristoyl-*sn*-glycero-3 phosphocholine).[32] In this case, the peptide concentration appeared to affect the membrane structure. At high concentrations (10 μM), membranes underwent rapid micellization and subsequent dissolution. At lower concentrations (1 μM), changes were slower. First, melittin adsorbed at the top of the membrane causing fluidization of the lipid film. This was accompanied by reorientation and insertion of the peptide into the bilayer leading to the formation of defects.

Cecropin B, another cationic antimicrobial peptide, was investigated in the presence of zwitterionic and negatively charged lipid bilayers immobilized at gold electrode surfaces.[33] In the presence of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and cholesterol (Chol) at a 7:3 molar ratio, the bilayer remained unchanged in the presence of the peptide. Conversely, the membrane structure was strongly affected when composed of L-α-phosphatidylethanolamine (*E. coli*) (PE), L-α-phosphatidylglycerol sodium salt (*E. coli*) (PG) at an 8:2 molar ratio. Under these conditions, the peptide action involves electrostatically driven adsorption of the cecropin B at the top of the bilayer with simultaneous fluidization and swelling of the membrane. Then, the membrane ruptured through the formation of mixed peptide-lipid aggregates. Similarly, interactions of cationic lipopeptides (amphiphilic compounds combining a peptide moiety and a fatty acid that can be used as antibiotics) with sBLM strongly depend on the net charge of the lipid membrane.[34]

The recent investigation of the effect of a fluorene-based active drug candidate (named K162) on amyloid β peptide aggregation through the formation of Aβ oligomers capable of permeating the cell membrane, appears as a significant advance in the understanding of Alzheimer's disease sequence.[35] In this work, the Au(111)-sBLM was formed from of a mixture of lipids and cholesterol aimed at mimicking aged lipid rafts formed in the membrane from the human frontal cortex cells found in Alzheimer's disease patients' brains. Results obtained by EIS combined with AFM and molecular dynamics showed that K162 prevents lipid membrane poration/permeation by altering the amyloid β peptide aggregation pathway. These investigations described a unique Aβ toxicity inhibition mechanism.

Figure 4 summarizes the nature of the electrochemical information that can be collected on the interactions of molecules and ions with BLMs deposited at gold electrodes.

2.3 | Platinum

Highly stable lipid bilayers, composed of biologically relevant lipids such as phosphatidylcholine, or phosphatidylethanolamine can also be formed on platinum surfaces either from LB techniques or through vesicle fusion.[36]

Alternatively, self-organized supported bilayer lipid membranes based on interactions between a nascent metallic surface and amphipathic lipid molecules can be obtained not only on platinum or gold, but also on Ag, Cu, Ni, stainless steel, or other alloys even if these last ones are not commonly used in molecular electrochemistry.[37–39] Typically, the procedure consists in two consecutive self-assembling steps. In the first step, the substrate on which the sBLM will be formed (e.g., the tip of the Teflon-coated Pt wire with a diameter of 0.1 – 0.5 mm) is immersed in a lipid solution (e.g., 2% phospholipid in *n*-hexadecane, or other organic solvents); then, while still immersed, the tip is cut off with a sharp knife (good reproducibility of sBLMs requires reproducible cutting of the wire) leading thus to a highly hydrophilic surface that attracts the polar groups of the lipid molecules. The second step consists in immersing the lipid layer freshly adsorbed onto the cut end of the metal wire surface into an aqueous solution (e.g., 0.1 – 1 M KCl) where the lipid film spontaneously thinned, forming a self-assembled lipid bilayer. The formation of the sBLM is generally monitored by following its capacitance as a function of time. Generally, the capacitance value rapidly reached a plateau (2-2.5 nF in about 2 minutes) in agreement with the expected thickness of a lipid bilayer. Nevertheless, the main drawback of this method is the instability of sBLM electrical properties and the uncertainty of lipid layer structure at the metal surface. Indeed, monolayers and multilayers could co-exist.[40] Note that on silver, the defects in lipid bilayers structure can be eliminated by application of a negative dc potential during the formation of the sBLM.[41]

Self-organized supported bilayer lipid membranes were also observed at platinum-electrodes after cleaning the metallic surface with a high-power ultrasonic horn.[42] Typically, the Pt surface was first carefully polished with alumina slurries. Then it was cleaned with water and sonicated with a high-power supersonic wave generator in a water bath and acetone bath for 5 minutes in turn. A drop of lipid solution was added to the electrode surface, afterward the electrode was immediately transferred to the electrolyte (0.1 M KCl), allowing its spontaneous thinning and monitoring its capacitance until it fell into the range of BLMs.

This kind of Pt-sBLM was notably used to investigate interaction of Ca^{2+} with phosphatidylcholine/cholesterol or lecithin/cholesterol lipid membranes by cyclic voltammetry and impedance spectroscopy. Experimental results suggested that Ca^{2+} interacted with Pt-sBLM to produce channels or micro-defects, thus making the Pt-electrode surface accessible to charge complexes such as $Fe(CN)_6^{3-}$.[43]

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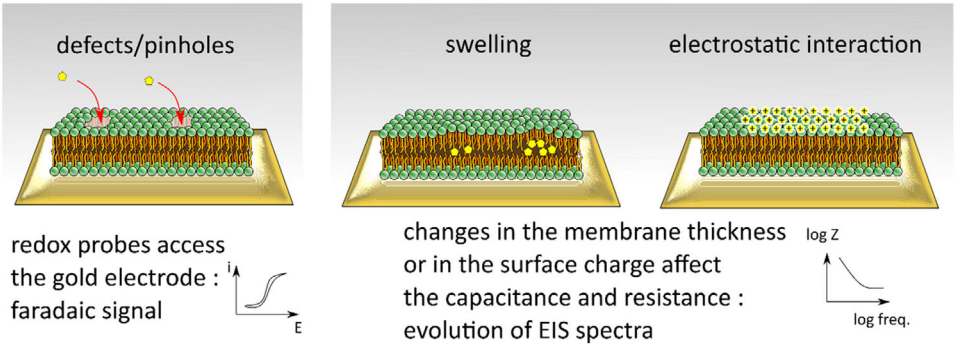

FIGURE 4 Schematic representation of the electrochemical features which are affected upon interaction of molecules with gold-deposited BLMs

Similarly, it was shown that Pt-sBLM made of dimethyldioctadecylammonium bromide (a kind of synthetic lipid) can also act as ion-gate for the permeation of $Ru(bpy)_3^{2+}$ ions triggered by perchlorate anions, very likely thanks to the interactions between the latter and the lipid quaternary ammonium cations. Ion-gate formation could be also evidenced by electrochemiluminescence (ECL) using the $Ru(bpy)_3^{2+}$/oxalate ECL couple. Based on this system the limit of detection for perchlorate anion was 0.1 μM.

The interactions of Pt electrode-sBLM with polyamidoamine (PAMAM) dendrimers (generations 1–7)[44] or polyoxometalates[45] were also investigated by cyclic voltammetry and impedance spectroscopy. In the first case, PAMAM dendrimers caused defects in the sBLM, allowing a complex such as $Ru(NH_3)_6^{3+}$ to reach the electrode surface (same as illustrated in Figure 4 for gold electrodes). In the second case, polyoxometalates promoted access of $Ru(NH_3)_6^{3+}$ or $[Fe(CN)_6]^{3-/4-}$ to the Pt-sBLM electrode surface through pores as suggested by AFM experiments. This behavior was attributed to the specific association of polyoxometalates with lipid phosphatidylcholine phosphate groups.

More recently, interactions of baicalin and baicalein (flavonoids found in *Radix Scutellariae* an essential herb in Chinese medicine) with Pt electrode-sBLM prepared by lipid self-assembling after polarization of the platinum surface were reported.[46] The electrochemical results showed that baicalein interacted more strongly than baicalin with L-α-Phosphatidylcholine (egg PC) bilayer membranes inducing thus more defects in the sBLM as assessed by the faradaic signature of $Fe(CN)_6^{3-}$.

2.4 | Glassy carbon

The inert nature of glassy carbon and its large potential window in electrochemistry, make it a privileged support for sBLM formation.[47] To our knowledge, glassy carbon was originally used for sBLM formation in 1996, along with incorporated single-stranded deoxyribonucleic acid (ss DNA) in view of DNA sensors development.[48] Pure bilayer lipid membranes supported on glassy carbon electrode were initially prepared by E. Wang.[49] The technique is based on the self-lipid organization on a polarized glassy carbon electrode. This approach is still largely used though some variations have been developed. Typically, the glassy carbon electrode (GCE) surface is polished and sonicated for 1 minute in deionized water and acetone, successively. Then, the GCE is immersed in a NaOH solution (0.1 mol/L) and the potential is held at 1500 mV vs. Ag/AgCl for 3 min. The latter is then dried under purified nitrogen and an aliquot of the lipid solution (5 μL) is deposited with a microsyringe on the electrode and immediately transferred into a KCl solution (0.1 mol/L), in which the supported lipid layer forms spontaneously. This is the most widely developed mode of preparation in the works described below.

The effect of the electrochemical conditioning of GCE on GCE-supported bilayer lipid membranes has been investigated by cyclic voltammetry and EIS.[50] In these experiments, it appears that an appropriate positive bias voltage enables the formation of electrically dense membranes. Above a defined voltage threshold, the lacunas on sBLMs increase, and eventually cause the complete oxidation of the lipid membrane. Negative bias voltages could also induce the damage of supported membranes. The changes in the form and balance of graphite oxide on the GCE surface may be responsible for BLM adhesion. However high overpotentials may cause electroporation or damage to deposited BLMs.

As for Pt-sBLM, numerous electrochemical investigations have been achieved to study interactions between GCE-sBLM and biologically-relevant compounds such as nisin, an antibacterial peptide,[51] lanthanide ions,[52] surfactin, an acidic lipopeptide,[53] chlorpromazine, an antipsychotic agent used to treat schizophrenia,[54]

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ibuprofen, a well-known nonsteroidal anti-inflammatory drug,[55] or quercetin, an antioxidant.[56] Accordingly, all these investigations were performed in the presence of ions such as $Ru(NH_3)_6^{3+}$ or $Fe(CN)_6^{4-/3-}$ to probe the membrane integrity and accessibility of the electrode surface (see defects/pinholes represented in Figure 4). At low concentrations, all these molecules induce pore formation whereas the GCE-sBLM progressively exhibits destructuration at high concentrations.

The electrode accessibility was also investigated by scanning electrochemical microscopy in the presence of perchlorate anions known to interact with the lipid quaternary ammonium cations.[57] As already observed for platinum, the presence of perchlorate anions triggers the formation of channels/pores allowing electroactive probes such as $Ru(bpy)_3^{2+}$ ions to access the electrode surface.

Transport, interactions, and reactivity of antitumor ferrocene-based compounds (=ferrocifen) in pure lipid environments have also been investigated electrochemically at GCE modified with a DMPC lipid bilayer film.[58] The apparent loading of the films with ferrocifens strongly suggested that a dramatic structural re-organization of the initial bilipid layer took place. Expectedly, the affinities of the starting neutral complexes for the film were stronger than those of the cationic electrogenerated ferrocenium species which were rapidly expelled from the film. Yet, this latter process was reversible and the film could be replenished with ferrocifen upon reduction of its cation, formally through a CE process limited by the unfavorable partition of the cation in the film.

The interaction of an antimicrobial peptide (recombinant human cathelicidin LL-37)) with GCE-sBLM made of various lipids (zwitterionic such as dipalmitoylphosphatidylcholine (DPPC) or dioleoylphosphatidylcholine (DOPC); negatively charged such as 1,2-Dipalmitoyl-sn-glycero-3-phosphorylglycerol (DPPG)) has been investigated.[59] It was notably shown that LL-37 interacts with negatively charged DPPG lipid head groups.

Very recently, GCE-supported BLM have been used to monitor by cyclic voltammetry the passage of small redox probes such as ferrocene-methanol (Fc-MeOH), $Fe(CN)_6^{3-}$, or $Ru(NH_3)_6^{3+}$ as well as ferrocene-labeled peptides across lipid membranes of an anionic (dioleyl phosphatidyl glycerol - DOPG) or a zwitterionic (globally neutral diphytanoyl phosphatidylcholine (DPhPC)) phospholipid.[60] The behavior of small redox probes at DPhPC-GCE-sBLM features a clear discrimination between ionic forms and neutral ones. Only neutral probes are allowed to penetrate the gel-ordered bilayer of DPhPC, whereas charged probes are expelled from the lipid film. With anionic DOPG all probes display clear electrochemical responses in cyclic voltammetry. The cationic $Ru(NH_3)_6^{3+}$ probe undergoes a strong electrostatic interaction with the anionic lipids which leads to the swelling and de-structuration of the DOPG film. The cyclic voltamogramms (CVs) at DOPG-GCE-sBLM are significantly different from the one observed at DPhPC and DMPC[58] modified electrodes, which display unambiguous behavior towards the probes: neutral species accumulate in the lipid bilayer and exhibit symmetrical "thin layer" CVs while charged ions are blocked or expelled from gel phase phosphatidylcholine (PC) lipid films. The anionic and fluid phase DOPG shows more complex CVs with ferrocene-labeled peptides exhibiting a diffusional shape and an instability upon multiple cycling. The shape and magnitude of the peak currents at DOPG-GCE may therefore account for the slow partition equilibrium of the peptides between the solution and the DOPG film.

2.5 | Indium tin oxide (ITO)

Indium tin oxide (ITO) is an optical transparent semiconductor widely used in molecular electrochemistry especially when combination with microscopy/imaging techniques is envisioned. Though several works described the preparation of ITO-supported BLMs, their use as supports for BLMs is still underexplored.

In the late 1990s, Sackmann showed that ITO-supported BLM could be prepared by vesicle fusion and annealing at elevated temperatures.[61] Moreover, a few mol% of positively charged lipid appeared to be essential. Under these conditions, the positive charge of the membrane favors the fusion onto ITO surfaces that are negatively charged at pH 7.5 resulting in lipid bilayers stable for several weeks.

Pure lipid bilayers can also be prepared on ITO by the LB technique.[62] The ITO surface being hydrophilic, it implies that phospholipids transferred by the LB technique exhibit headgroups directed towards (i.e., in contact with) the material surface.

Recently, the incorporation of ubiquinone (one of the main electron and proton shuttle molecules in biological systems) in ITO-supported DPPC membranes has been investigated by cyclic voltammetry and atomic force microscopy (AFM).[63] It was notably shown that ubiquinone is essentially located in two positions: (a) associated with the DPPC hydrophobic chains, and (b) sandwiched between the two leaflets that form the supported BLM when increasing the ubiquinone concentration (very likely due to the formation of aggregates). Supported-BLM arrays on micro-patterned ITO electrodes have been successfully prepared.[64] In this work, substrates for lipid bilayer arrays were fabricated by patterned deep UV irradiation of trimethoxy(octadecyl) silane (TODS) SAM modified ITO electrodes. The intact TODS-SAM regions (non-irradiated) gave a hydrophobic surface on which a lipid

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monolayer was formed. Then, the irradiated domains became hydrophilic again to support a bilayer.

3 | SUSPENDED/FREE-STANDING BILAYER LIPID MEMBRANES

3.1 | Introduction

Black lipid membranes (also called free-standing or suspended bilayer lipid membranes) were actually the first biomimetic membranes used in electrochemistry. The initial term "black lipid membrane" refers to the fact that the film appears dark at the desired thickness (~4 nm) since the light reflected from the back face of the bilayer destructively interferes with the light reflected from the front face. Compared to their supported analogs, suspended BLM have large water reservoirs on both sides of the film with large electrical resistances (10^6–10^8 Ω cm^2) and low capacitances (~1 μF/cm^-.[65] These model membranes have been widely used to detect single-channel activities of pore-forming peptides or ion channels. However, the short lifetimes and poor mechanical stability of suspended bilayers limit their applications in electrophysiological experiments.[66] Besides, efforts are continuously made to improve the stability of these membranes.[67] Suspended BLM are notably interesting to electrochemically monitor the passive transport of molecules across lipid membranes.

3.2 | Preparation

The preparation of suspended BLMs is regularly reviewed in the literature.[68,69] In this paragraph are summarized the most relevant methods with a focus on those that are developed for electrochemical detection.

To date, the most popular approach to form suspended bilayer lipid membranes (Black Lipid Membranes) remains the "painting" technique.[70] With this technique, the resulting artificial membrane (typically from 50 to 500 μm in diameter) may, however, incorporate solvent molecules which may alter some of the membrane's properties. To reduce the amount of residual organic solvent the "folding" method was proposed.[65] This consists first in spreading a solution of a lipid in a volatile solvent at an air/water interface to form a lipid monolayer at both sides of the membrane support. Then, the water level is increased by injecting an aqueous solution at both sides of the membrane support. Finally, the two lipid monolayers meet at the aperture with the formation of a bilayer architecture. Although a volatile solvent is evaporated during the monolayer formation, a small amount of non-volatile solvent around the aperture is required to assist the bilayer formation. Solvent-free suspended lipid bilayers can be obtained using the LB technique,[71] but the absence of a solvent–lipid annulus at the aperture edge destabilizes the artificial membrane (Figure 5). To overcome this drawback, nanometer-sized apertures have been used to paint stable suspended membranes over anodized porous alumina[72–74] porous polycarbonate membranes,[75] and glass nanopores.[76] These configurations are notably useful for single ion channel recordings after ion-channel proteins are inserted across the artificial membrane. Indeed, small apertures decrease the capacitance and allow precise measurements of resistive variations when ions or molecules pass across the protein channels.[69] Nevertheless, they are not well adapted to electrochemical detection of species crossing the membrane considering the drastic composition changes incurred by the nano volume liquid entrapped between the lipid bilayer and an electrode.[77]

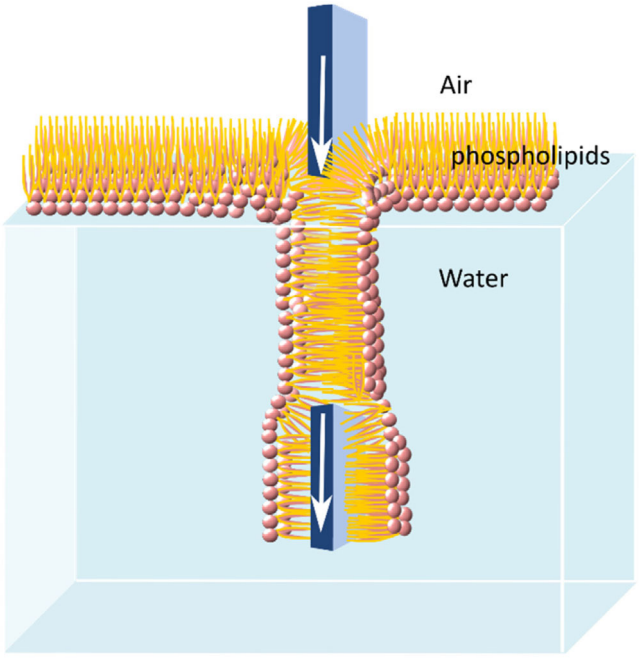

FIGURE 5 Illustration of the preparation of suspended / free-standing bilayer lipid membranes at Teflon apertures. Inspired from Ref. [65]

In terms of membrane stability and electrochemical detection, the formation of lipid bilayers, suspended at glass pipettes with diameters in the 0.5-5 μm range, by the tip–dip method[78,79] is a good compromise. This consists in repeatedly dip a glass pipette onto a lipid monolayer at an air/water interface.

Interestingly, alternative approaches have emerged these last years. Recently, the use of dual-barrel theta capillaries allowed the formation of stable, solvent-free, suspended BLMs. This could be viewed as an evolution of

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the tip-dip method described previously, but in this case the monolayer is formed directly from a lipid solution rather than by moving the pipette out and into a solution that contains a lipid monolayer at the air–water interface. Using this system, the permeation coefficients of a series of aliphatic carboxylic acids that passively permeate across a bilayer could be determined.[80]

Microcavity-suspended lipid bilayers could also be formed from polystyrene sphere-templated polydimethylsiloxane.[81,82] Lipid bilayers are spanned over aqueous buffer-filled, micrometer-sized hemispherical cavities formed from polystyrene sphere templated polydimethylsiloxane (PDMS) rendered hydrophilic by plasma treatment. A combination of LB technique and vesicle fusion techniques was used to form defect-free bilayers spanning the cavities. Lipid bilayers can be reliably spanned across a range of cavity sizes prepared with templating spheres with diameters from 620 nm to 5 micrometers. Moreover, the spanning lipid bilayers remain intact, with reproducible fluidity over several days.

An original method based on the patch-clamp technique has also been recently developed.[83] More precisely, the "inside-out" patch–clamp configuration was successfully used to prepare both solvent- and protein-free lipid bilayers suspended at micro-sized glass pipette tips. In this method, giant unilamellar vesicles (GUVs) were first prepared by electroformation. Then, a patch micro-pipette (1.5-2 μm in diameter) was pressed against the GUV (diameter in the 15 to 40 μm range) allowing the formation of a high resistance seal (1-10 GΩ range) between the glass pipette and the vesicle membrane (giga seal). After the formation of the giga seal, the micropipette was quickly withdrawn from the vesicle, ripping off the sealed patch of lipid bilayer attached to the micropipette. Such suspended lipid bilayers can be characterized by EIS. Compared to the painting or tip-dip methods, the patch-clamp based technique is also well-adapted to prepare suspended real cell membranes. Combination of patch–clamp and amperometric detection allowed the monitoring of molecular fluxes across suspended bilayer lipid membranes (vide infra).

3.3 | Electrochemical investigation and monitoring of the passive transport of molecules

Suspended lipid bilayer membranes free of both solvent and protein are particularly interesting as model cell membranes for the investigation of passive transport of molecules (not ions or water molecules). Within this context, the Overton's rule[84] has been widely applied to describe the permeation coefficient, P, of a molecule across a membrane in terms of the partition coefficient, K, between oily and aqueous phases: $P = KD_m/l$ where D_m is the diffusion coefficient of the molecule in the membrane and l is the membrane thickness. Based on an analogy of aqueous/membrane systems and two-phase oil/water interfaces, K typically changes much more significantly than D (in a bulk hydrocarbon phase) for a homologous series of molecules. It is therefore generally assumed that K largely determines P, and that there is a direct correlation between the two. Consequently, oil/water partition coefficient measurements have become popular in the estimation of permeation coefficients. Yet, only the thermodynamic aspects of the partition equilibrium are described from permeation coefficients. The kinetics of partition and translocation remain unknown. In this context, two electrochemical approaches have been developed to address the passive transport of molecules.

The combination of patch-clamp with amperometric detection at a carbon-fiber microelectrode successfully allowed monitoring and quantifying the passive transport of redox probes across micrometer-sized suspended BLMs.[83] The fluxes of a series of representative redox molecules (ferrocenemethanol, hydroquinone, benzoquinone, $[Fe(CN)_6]^{3-}$, and $[Ru(NH_3)_6]^{3+}$) were investigated in the presence of suspended DPhPC lipid membranes. As expected, only the fluxes of uncharged species (molecules) could be detected, the current dramatically decreasing upon membrane formation, thus reflecting the reduced transport kinetics. Accordingly, benzoquinone was found to cross the DPhPC lipid membrane more efficiently than hydroquinone in agreement with its higher lipophilicity.

A model was proposed to fit the amperometric data, allowing the extraction of the value of KD_m, *i.e.*, the product between the partition coefficient (K) of the species in the membrane and its diffusion coefficient (D_m) as it passes through the same membrane.

Another original method was also developed to quantitatively measure the permeation of weak acids (acetic, butanoic, valeric, hexanoic) through suspended BLMs composed of soybean phosphatidylcholine and prepared by the popular paintbrush method.[85] In this method, an ultramicroelectrode was placed close to one side of the BLM to deliver weak acids by the electrogeneration of protons in the presence of their conjugated base and a small amount of fluorescein, a pH-sensitive fluorophore. Accordingly, a laser confocal scanning microscope was combined with electrochemistry to visualize the electrochemically-induced passive transport. Under these conditions, the resulting steady-state pH distribution in the vicinity of the electrode and on each side of the BLM is highly sensitive to the distribution of the weak acid and the BLM permeation coefficient. Interestingly, it was found that the permeation coefficient of a series of aliphatic weak

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acids does not correlate with the partition coefficient, as generally assumed. Indeed, a trend of decreasing permeation coefficient with increasing acyl tail length for the transport of weak acids across suspended BLM correlated most closely with molecular size (and therefore diffusion coefficient). This trend deviates from Overton's rule which considers that the hydrocarbon/water partition coefficient is the most significant parameter controlling permeation.

Recently, a similar approach was developed, but the suspended BLMs were prepared with the use of dual-barrel theta capillaries. Moreover, the pH gradient was no longer triggered by electrochemistry, but upon permeation of weak acids.[80]

As mentioned previously, microcavity-suspended lipid bilayers (MSLB) could also be formed from polystyrene sphere-templated polydimethylsiloxane.[81,82] These MSLB have been recently used to investigate the interaction of the antibacterial drug vancomycin with various model membrane compositions using non-Faradaic electrochemical impedance spectroscopy. EIS data showed that vancomycin associates at the interface of lipid membranes rather than penetrate and this is promoted by the presence of anionic phospholipids.[86]

The MSLB platform has also been used to investigate the interaction of Miltefosine (the only drug taken orally for the treatment of leishmaniasis) with various model membrane compositions to get further insight on its molecular mechanism action.[87] In this work, the membrane resistance changes in response to Miltefosine were modeled by an empirical Langmuir isotherm binding model to provide estimates of binding saturation and equilibrium association constant.

4 | CONCLUSION

The implementation of molecular electrochemistry approaches (cyclic voltammetry, square wave voltammetry, amperometry), and interfacial electrochemistry methods (essentially electrochemical impedance spectroscopy) on supported or suspended lipid bilayers provide accessible and meaningful model systems to study (i) the supramolecular interactions of molecules with lipids, (ii) the structural changes in the lipid layers, as well as (iii) the complete passage of species across the lipid bilayers. Beyond the individual lipid/molecule interactions, such systems provide information on the mode of transport through phospholipids (e.g. through defects, pores, permeation). Moreover, these investigations can be conducted with any type of molecules such as pollutants, drugs, or antimicrobial peptides providing thus relevant information for targeted applications.

Though most of the sBLM are prepared on gold or glassy carbon materials, the use of transparent and conductive surfaces such as ITO allow combination with luminescence techniques, far more sensitive than amperometry. Such dual electrochemical – optical systems should open avenues in the monitoring of the transmembrane trafficking of biologically active compounds.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Not Applicable

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