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Electrode Supported Biomimetic Membranes: An Electrochemical and Surface Science Approach for Characterizing Biological Cell Membranes

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Highlights

- Biomimetic membranes are built on metal electrode surfaces.
- Static electric field controls membrane processes.
- surface specific techniques provide information about membranes structure and function

Abstract

Planar solid supported lipid bilayers have been developed as simplified biological membranes to model the physical properties of cell membrane processes. Lipid bilayer membranes supported at conductive metal substrates provide a unique opportunity to investigate the effect of the static electric field on the membrane structure and function. The insights gained from this research can be used to develop novel biosensors and biomedical devices. This review summarizes the recent developments in metal supported biomimetic membrane systems. It provides an overview of the various models used to study membrane processes at electrode surfaces, such as metal supported monolayers and bilayers, hybrid, tethered and floating bilayers. The paper discusses the recent advancements in these biomimetic

models and describes the fundamental knowledge about membrane processes that has been extracted from these different platforms. The potential for the design and improvement of biomedical devices using metal supported bilayers is also discussed. Metal supported bilayers allow for the application of a plethora of spectroscopic and surface imaging techniques to obtain information about the voltage dependent properties of biomolecules at the molecular level. The underlying methodology of these analytical techniques and the structural, chemical and kinetic information extracted are reviewed.

Keywords

biomimetic membranes, metal electrode, electrochemical methods, surface sensitive techniques.

Abbreviations

CV: cyclic voltammetry; DC: differential capacity; EIS: electrochemical impedance spectroscopy; sLM: supported lipid monolayer, BLM: bilayer lipid membrane; hBLM: hybrid bilayer lipid membrane; sBLM: supported bilayer lipid membrane; tBLM: tethered bilayer lipid membrane; fBLM: floating bilayer lipid membrane; SAM: self-assembled monolayer; EC-STM: electrochemical scanning tunneling microscopy; AFM: atomic force microscopy; LB: Langmuir-Blodgett; LS: Langmuir-Schaefer; SUVs: small unilamellar vesicles; DMPC: 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPE: 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine DPhPC: 1,2-diphytanyl-*sn*-glycero-3-phosphocholine; DOPC: 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DPTL: 2,3-di-O-phytanyl-*sn*-glycerol-1-tetraethylene glycol-DL-α-lipoic acid ester; PEG: polyethylene glycol; *β*-Tg: 1-thio-*β*-D-glucose; GM1: monosialotetrahexosylganglioside; QCM: quartz crystal microbalance; SPR: surface plasma resonance; SEIRA: surface-enhanced infrared reflection absorption; PM-IRRAS: polarization modulation infrared reflection absorption spectroscopy;

SERS: surface-enhanced Raman spectroscopy, SERRS: surface-enhanced resonance Raman spectroscopy

Introduction

Biological membranes are fundamental components of all living organisms and act as a barrier, which allow selective communication between the interior and exterior of a biological cell. Cell membranes, whose structure is presented in Fig. 1(a), are complex organizations of lipids, cholesterol, carbohydrates and proteins bound together by hydrophobic interactions. Due to the complexity of these biological membranes, simplified biomimetic models with well-defined membrane structures and molecular compositions have been developed to study the relation between the membrane structure and cellular function. The key advantage to these simplified biomimetic systems is that the researcher can exploit specific membrane properties to gain a better understanding about the physical properties and processes that occur within natural biological membranes.

Initial electrochemical studies of biomimetic membranes were performed using black lipid membranes, which have large water reservoirs on both sides of the film [1]. The term “black lipid membrane” refers to the fact that the films appear dark at the desired thickness since the light reflected from the back face of the bilayer destructively interferes with the light reflected from the front face. Black lipid membranes are intact bilayers suspended in solution with large electrical resistances (10^6 ~ 10^8 Ω cm^2) and low capacitances (~1 μF cm^{-2}). These model membranes were popular for studying voltage-gated ion channels formed by proteins and peptides, because the black lipid bilayers are highly sensitive to minute changes in membrane conductance [2]. The major drawback to the black lipid membrane system is that the bilayers are extremely fragile making them susceptible to vibrations and to large electric fields. This reduces the lifetime of the black lipid membrane and restricts their characterization to electrical conductivity measurements. Due to the limited number of experimental methods that can be used to study the membrane properties, further

investigations involving black lipid membranes are beyond the scope of this review.

Biomimetic membranes formed at metal electrode surfaces allow for the application of a plethora of electrochemical techniques (i.e. cyclic voltammetry (CV), differential capacitance (DC), electrochemical impedance spectroscopy (EIS), and chronocoulometry) to characterize the electrochemical properties of biomolecules within the membranes. The solid electrode surface allows for the utilization of surface sensitive techniques, such as spectroscopy, neutron scattering and surface imaging methods, to provide molecular level information for an in-depth interpretation of the electrochemical measurements. Several types of biomimetic membranes can be formed at metal surfaces, which are comprised of supported lipid monolayers (sLMs), hybrid bilayer lipid membranes (hBLMs), supported bilayer lipid membranes (sBLMs), tethered bilayer lipid membranes (tBLMs) and floating bilayer lipid membranes (fBLMs). Cartoon representations of the schematic structures of these biomimetic systems are displayed in Figure 1. A number of excellent literature reviews concerning biomimetic membrane research on metal substrates can be found in the following references [3-15].

In the present review, we will give a brief overview of achievements for each type of membrane and describe the useful knowledge that can be ascertained from the electrochemical and surface specific techniques. This work also describes practical applications of biomimetic membranes for the design and improvement of biosensors and their application in the development and screening of novel drugs. This review will primarily focus on recent developments in this field since this subject has previously been reviewed in detail.

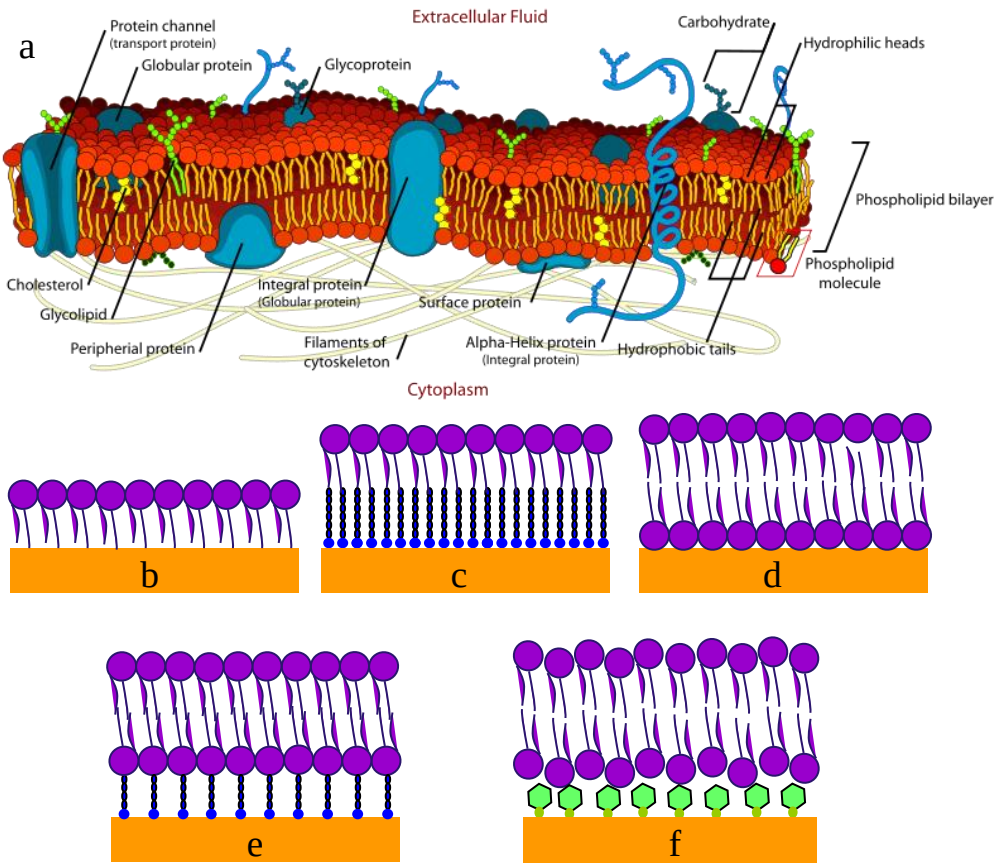

Fig. 1 (a) A detailed diagram of the cell membrane. The figure was taken from https://commons.wikimedia.org/wiki/File:Cell_membrane_detailed_diagram_en.svg. Schematic structure of different biomimetic membranes at the metal surface: (b) supported lipid monolayer (sLM); (c) hybrid bilayer lipid membranes (hBLM); (d) supported bilayer lipid membranes (sBLM); (e) tethered bilayer lipid membranes (tBLM) and (f) floating bilayer lipid membranes (fBLM, this membrane is supported on a hydrophilic monolayer of self-assembled β-Tg represented by green hexagons).

Supported lipid monolayers (sLMs)

The investigations of lipid monolayers at metal surfaces were pioneered by Blank and Miller in 1968 [16]. The fluidity of the liquid mercury electrode surface is compatible with the fluidity of the adsorbed phospholipid monolayers. This allows the phospholipid monolayers to maintain defect-free, self-sealing structures, which creates a barrier that is impermeable to the ions in solution. Nelson's group adopted a modified method to deposit stable and reproducible phospholipid monolayers on mercury surface [17]. The electrode is coated with a monolayer of phospholipids by extruding a mercury drop from the glass capillary of a dropping or hanging mercury

electrode through a monolayer that has been spread at the air-solution interface of the electrochemical cell. Substantial efforts have been made to gain a better understanding about the potential controlled phase transitions in phospholipid monolayers. Stoodley and Bizzotto [18, 19] have made progress in this field by employing epi-fluorescence to visualize the potential induced changes in adsorbed monolayers. More recently, the Nelson research group performed Monte Carlo simulations to model the effect of the electric field on the lipid phase transitions [20] and used AFM to measure potential induced thickness changes in monolayers supported on the Hg electrode [21]. Significant contribution to the studies of phospholipid monolayers at mercury was accomplished by Guidelli and co-workers, who measured the electrochemical properties of monolayers with high precision and accuracy [10] and investigated the reconstitution of antimicrobial peptides into phospholipid monolayers [22-24]. The transport of Tl^+ ions through lipid monolayers containing gramicidin was a popular model to study ion channel properties of peptides in lipid membranes [25-32]. Mercury supported phospholipid monolayers have found several practical applications. For example, they have been used in screening for organic pollutants in water [33-35] and providing information about interactions of model membranes with nanoparticles [36, 37]. Mercury electrodes can also be employed to investigate the effect of electric charge on the adhesion of cells to surfaces using the fusion of vesicles as a model. This was pioneered by Ivosevic et al., who initially studied the spreading of oil droplets [38] and later progressed to phospholipid vesicles [39, 40]. Hernandez and Scholz employed electronucleation theory to describe the kinetics of vesicles fusion on Hg [41-43]. An excellent review of phospholipid monolayers on mercury electrode surface can be found in Ref. [44].

Gold electrodes offer additional opportunities to employ spectroscopic and molecular imaging techniques to study sLMs at electrode surfaces. The EC-STM technique was used by Xu et al. to investigate the fusion of DMPC vesicles at the gold (111) electrode surface [45] and by Sek et al., to monitor the fusion of mixed DMPC/cholesterol vesicles [46, 47]. These studies revealed that the molecules released by the rupture of a vesicle initially self-assemble into a well-ordered

monolayer on the metal surface. When additional molecules accumulate at the surface, the monolayer is transformed into a bilayer. Yamada and coworkers obtained similar STM images of well-ordered phospholipid monolayers supported on hydrophilically modified gold (111) surfaces [48]. Matyszewska et al. [49] performed AFM and STM studies of monolayers of *N*-stearoylglycine and *N*-stearoylvaline ethyl ester. The application of EC-STM has led to significant achievements in the application of phospholipid monolayers where molecular resolution images of ion channel formation were acquired for gramicidin by Sek et al. [50], alamethicin by Pieta et al. [51] and trichogin by Smetanin et al. [52]. These images demonstrated that gramicidin folds into hydrophobic β-helices and creates individual ion channels. In contrast alamethicin and trichogin are amphipathic molecules that aggregate in lipid monolayers to form clusters of ion channels. Several papers have been devoted to spectroscopic characterization of monolayers at gold electrode surfaces. These studies were pioneered by Zamlynny et al. [53] and Zawisza et al. [54] who demonstrated that PM-IRRAS can be used to obtain molecular level information about the potential controlled transformations of organic monolayers. Additionally, PM-IRRAS was used to describe the effect of electrode potential on the structure of a monolayer of GM1 ganglioside [55]. Leitch et al. [56] employed PM-IRRAS to determine the orientation, conformation and water content of a self-assembled monolayer consisting of 2,3-di-O-phytanyl-sn-glycerol-1-tetraethylene glycol-DL-R-lipoic acid ester (DPTL), a thiolipid used in the architecture of tethered lipid bilayers. The tetraethylene glycol moiety of this molecule was designed with the purpose of providing a hydrophilic water rich region that separates diphytanyl chains from the metal. The PM-IRRAS spectra suggested that, in contrast to expectation, a significant fraction of tetraethylene glycol moiety is not hydrated. Junghans and Köper [57] performed electrochemical impedance and neutron reflectivity studies of several thiolipid monolayers, related to DPTL, to measure the resistivity and water content of the hydrophilic spacer and assess the usefulness of these molecules in the design of tethered bilayers.

Raman spectroscopy is another surface specific technique that has been employed by Krysinski et al. [58] to study thiolipid monolayers at gold surface. Monolayers supported at a gold electrode surface have found application in studies involving the interactions of perfluorinated compounds [59] and drugs, such as doxorubicin with model membranes [60, 61]. In addition, thiolipid coated gold nanoparticles are used as drug delivery systems [62]. Gold is not toxic and easy to handle. Presently, most electrochemical sensors employ thin gold films printed on a nonconductive support since these electrodes are inexpensive, reusable and disposable [63].

Hybrid bilayer lipid membranes (hBLMs)

In 1993, Plant proposed that a stable hybrid bilayer lipid model membrane could be created at the surface of a gold electrode by depositing a monolayer of phospholipid molecules on top of an alkanethiol SAM [64]. 1-Octadecanethiol is a typical choice for the hBLM SAM due to its ability to form tightly packed and well-ordered monolayers. The outer leaflet of the hBLM can be deposited on the SAM-covered gold surface by vesicle fusion or a LS touch at the air-water interface of a LB trough. The properties of hBLMs can be tuned by altering the alkanethiols, lipids, and membrane additives, such as sterols and proteins. Altering the lipid chain length of the alkanethiol and/or phospholipid and introducing membrane additives leads to a change in the bilayer thickness, capacitance, phase and uniformity [64]. Furthermore, the addition of ethylene oxide units into the thiol monolayers can result in a spacer region in hBLM between the bilayer and the electrode surface to accommodate transmembrane proteins [65].

The hBLMs are commonly constructed on gold substrates; therefore, electrochemical and surface sensitive techniques can be applied for detection and characterization. Krueger et al. employed neutron reflectometry to describe the structure of the hBLM with a unprecedented detail [66]. Zhan's group built a new family of hBLMs using aromatic thiols and investigated their electrochemical and mechanical properties by EIS and AFM, respectively [67]. Tse et al. [68] embedded

Cu-triazole-based molecules into a hybrid bilayer membrane to study anion transport through lipids by monitoring changes in the oxidation state of the Cu(II)/Cu(I) redox system. This study showed that anion penetration into the hBLM is controlled by anion solubility rather than the presence of defects in the bilayer. The Burgess research group utilized SEIRAS and neutron reflectivity to monitor the behavior of ubiquinone in the hBLM as a function of the applied potential [69]. They reported that the ubiquinone tails are located in the mid-plane of the lipid bilayer and that the molecules do not partition into the distal plane of the bilayer. Nonetheless, the quinone head group is free to move and upon reduction it penetrates into the proximal leaflet assisting in proton transfer across the bilayer. In a related study, Ma et al. [70] employed *in situ* SERS to investigate the reversible interconversion between NADH and NAD^+ mediated by ubiquinone using hBLMs composed of ubiquinone-terminated disulfide SAMs with varying alkyl spacer length. The phospholipid bilayer was then completed by the fusion of unilamellar vesicles. This study provided new insights into the mechanism of biological redox cycling.

Hybrid BLMs are robust, easy to form and offer the possibility of constructing a variety of biomimetic systems by simply changing the proximal SAM or distal phospholipid leaflet. One concern is that the inner alkanethiol monolayer is frequently more crystalline and densely packed than the outer phospholipid leaflet reducing the fluidity of the membrane environment [71]. The restricted mobility of both lipids and proteins in the hBLM makes it difficult to reconstitute transmembrane proteins into these model membranes. Forbrig et al. [72] demonstrated that alamethicin, a short chain antibiotic peptide, cannot be inserted into a hBLM, however, alamethicin molecules can be easily inserted into sparsely tethered lipid membranes.

Supported bilayer lipid membranes (sBLMs)

Supported BLMs are planar lipid bilayer structures adsorbed directly onto the surface of solid substrates [73]. Early attempts to deposit sBLMs onto metal surfaces

were described by Ottova and Tien [74]. In contrast to hBLMs, phospholipid monolayers constitute both the proximal and distant leaflets of the bilayer. sBLMs can be deposited onto a metal surface by the fusion of small unilamellar vesicles (SUVs) or a combination of Langmuir-Blodgett and Langmuir-Schaefer (LB-LS) techniques [4]. The mechanism of sBLM formation by vesicles fusion involves adhesion, followed by deformation, and finally rupture, which leads to the spreading of lipids across the metal surface [75]. Factors affecting the adhesion and rupture of SUVs include vesicle size, temperature, presence of cations, surface charge, surface roughness, ionic strength, solution pH [76] and state (i.e. gel or liquid crystalline) of lipids within the vesicles. Gel state DMPC vesicles readily fuse onto atomically flat, gold (111) surfaces [77, 78], but do not fuse onto gold films that have been evaporated on glass or quartz since the gold film consists of small gold nanoparticles [79, 80]. In contrast, mixed vesicles comprised of DMPC and cholesterol will form stable sBLMs on both of these surfaces [79, 81]. The LB-LS method allows for layer by layer transfer of lipid monolayers from the air-water interface onto solid substrates [78, 82-86]. Lipid monolayers are compressed on the water surface of the Langmuir trough allowing to precisely controlling the physical state and packing density of the film during the transfer. The LB technique can dictate the molecular organization of the film and deposits a homogeneous monolayer over large areas of the substrate surface. In addition, LB-LS transfers enable the construction of asymmetric bilayers where the molecules in the inner leaflet differ from those in the outer leaflet [84, 87-91]. AFM studies of DMPC bilayers at gold (111) electrode surfaces indicate that the bilayer deposited by the LB-LS transfer has fewer defects compared to the bilayer deposited by vesicles fusion [78]. The quality of sBLMs can be improved by incorporation of cholesterol since cholesterol increases the fluidity of the gel state lipids and releases stress within the membrane [82]. The advantage of vesicle fusion over LB-LS is that membrane-active proteins and peptides can be easily incorporated into vesicles and then reconstituted into the lipid bilayers during the fusion. Comprehensive reviews describing sBLM formation on a metal surface are found in Ref. [4, 5].

The static electric field acting on sBLMs assembled on metal electrodes have magnitudes between 10^7 and 10^8 V m^{-1}, which are comparable to the electric fields acting on biological cell membranes. The charge density measurements indicate that the sBLMs are attached to gold surface when the absolute value of the charge at the metal is less than 8 μC cm^{-2}, and detach from the surface when the charge density is more negative than -8 μC cm^{-2} [85]. The neutron reflectivity [92] and SEIRAS [81] measurements have demonstrated that the bilayer is in direct contact with the metal at charge densities between 0 and -4 μC cm^{-2} with small amounts of water present in the head group region. Electroporation of the bilayer occurs when the charge density of metal ranges between -4 and -8 μC cm^{-2} followed by progressive membrane detachment (electrodewetting) at more negative charge densities. At a charge density of ~ -30 μC cm^{-2}, the bilayer is completely detached from the metal surface and floating on a ~1 nm thick cushion of electrolyte.

In situ PM-IRRAS studies have provided molecular level information about the structure, conformation and configuration of lipid molecules and membrane-active proteins in sBLMs as a function of the electrode potential [89]. The conformation and orientation (i.e. tilt angles of acyl chains) of the lipid molecules are quantitatively determined from the measured IR spectra [77, 93, 94]. The hydration of the phospholipid head group region can be estimated from the position of the center of the C=O and the P=O stretching bands [77, 95, 96]. The spectra collected in the lower wavenumber region (850 ~ 1500 cm^{-1}) provide information about the conformation of head group region [95, 96]. PM-IRRAS is a particularly useful technique to study proteins and peptides reconstituted into biomimetic membranes. The amide I absorbance band (1600 ~ 1700 cm^{-1}) of proteins and peptides does not overlap with IR bands of the phospholipids within the bilayer. D_2O is used as the solvent to minimize spectral interference from the overlapping absorbance bands from water. sBLMs provide an *in situ* platform to directly measure the physical properties of membrane-active proteins and peptides, such as myelin-associated glycoprotein [97], gramicidin [98, 99], cholera toxin [88], alamethicin [100], and siglec protein (sialic acid-binding immunoglobulin-type lectins) [101], as a function of the electrode

potential. These studies have contributed unique insight about protein-lipid interactions, the insertion of proteins and peptides into the bilayer and the behavior of voltage-gated ion channels. PM-IRRAS measurements of GM1 incorporated sBLMs have proven that the attachment of cholera toxin and siglec protein to a membrane is facilitated by the presence of the GM1 glycolipid [55, 87]. Short cationic antimicrobial peptides (CAMPs) target negatively charged bacterial cell membranes. Several studies have been devoted to understanding the structure and dynamics of sBLMs composed of negatively charged lipids [96, 102, 103] and determining their interactions with antibiotic peptides [104-106]. An interesting application of the sBLM platform was performed by Matyszewska et al. [59, 107] to study the incorporation and interactions of perfluorinated compounds with model membranes [59, 107].

Metal supported bilayers constitute a subclass of planar supported bilayers, which are perhaps the most popular platforms used to characterize membrane processes [73]. When the bilayer is supported on a metal, a unique opportunity to investigate how the static electric field affects the orientation and conformation of membrane constituents is granted. One issue with the metal sBLMs is that the membrane is exposed to an asymmetric environment since the inner leaflet is physically adsorbed at the metal surface and the outer leaflet is exposed to the electrolyte. PM-IRRAS [84] and SERS [90] studies have demonstrated that the inner leaflet in direct contact with the metal surface is more ordered than the outer leaflet. This arrangement hinders the mobility of the molecules within the sBLM, which in turn affects the physical and kinetic properties of the membrane constituents [108, 109]. A second flaw with the sBLM design is that the close proximity of the metal surface prevents their use in studies involving large transmembrane proteins since they require an aqueous reservoir on the both sides of the membrane [109]. In some cases, even small peptides can be affected by the solid metal support. Laredo et al. demonstrated that the gramicidin helix is stressed when the membrane is physically adsorbed at the gold surface [98]. To overcome these limitations, tethered lipid bilayers were designed to better mimic the natural cell membrane.

Tethered bilayer lipid membranes (tBLMs)

Tethered lipid bilayer membranes are separated from the substrate via hydrophilic spacers to overcome the major disadvantages of sBLMs. The tBLM architecture was first proposed by Lang et al. over 24 years ago [110]. Gold and mercury can be used as the substrate since thiol-based molecules can form Au-S or Hg-S covalent bonds. Tethering molecules are thiolipid derivatives with hydrophilic spacers attached to a hydrophobic tail group and terminated with a thiol or disulfide at the head group. The hydrophilic spacer, such as polyethylene glycol (PEG), lipoglycopolymer or carbohydrate, provides a water rich region between the electrode surface and the hydrophobic region of the bilayer. The thickness of the spacer, determined by neutron reflectivity, is about 1~3 nm [57]. In this architecture, the interaction of the hydrophobic region of the bilayer with the metal surface is minimized and the hydrophilic spacer region allows for reconstitution of transmembrane proteins and peptides into the tBLMs. The inner leaflet of tBLMs is deposited by self-assembling the tethering thiolipid directly onto the gold or mercury surface. The outer leaflet of tBLMs can be formed by fusion of SUVs [57] or multilamellar vesicles [111], rapid solvent exchange [112] or vesicle exchange [113]. Different types of thiolipids used to construct tBLMs are described by Köper and co-workers [57, 114].

PM-IRRAS [56] and neutron reflectivity experiments [115] demonstrated that densely packed hydrophilic fragments of the thiolipid monolayer within the tBLMs consist of insufficient amounts of water. To increase water content, the length of the hydrophilic spacer can be enlarged [57, 116, 117], however, this leads to an overall increase in the number of membrane defects [114]. Cornell et al. [118] and McGillivray et al. [112] proposed the dilution of the long chain thiolipid with short chain hydrophilic thiols to form sparsely packed tethering monolayers. The tBLM can then be completed by the fusion of unilamellar vesicles. The inner layer of the tBLM is composed of a mixture of phospholipids, from vesicle fusion, and the

self-assembled thiolipids. In contrast, the outer layer is strictly composed of the deposited phospholipid molecules. The dilution of the inner tethering monolayer creates a water rich domain between the hydrophobic region of the membrane and the metal surface, which was validated by neutron reflectivity experiments [57, 112, 119]. Furthermore, studies have proven that proteins and peptides insert more readily into sparsely tethered bilayers than densely packed tBLMs [72].

Tethered BLMs are promising tools for the research of the membrane-active proteins and peptides in a quasi-natural environment [120-122]. Electrochemical impedance spectroscopy is the most common electrochemical method used to characterize the electrical properties of tBLMs, specifically the membrane resistance and capacitance, since the electrical parameters of the tBLMs are altered by the reconstitution of functional proteins and peptides into the film. Guidelli and coworkers assembled tBLMs on mercury electrode surfaces and systemically investigated ion channel properties of a series of membrane active peptides, which include valinomycin, gramicidin, alamethicin, and melittin [123-125]. A summary of this research is given in Ref. [126]. Mercury-supported tBLMs have less defects and much higher lateral mobility than gold supported tBLMs, although it has been shown that bilayer defects and electroporation assist in the reconstitution of proteins into model membranes [127, 128]. A major drawback of mercury-supported tBLMs with respect to gold-supported tBLMs is the difficulty to apply surface sensitive techniques to characterize their structure and function at the molecular level.

An EIS model, developed by Valincius et al., was employed to simulate the electrical properties of the tBLM, consisting of membrane defects, deposited on the solid gold electrode [129-131]. In addition to EIS, surface sensitive techniques are widely employed in the research of gold-supported tBLMs. The kinetics of tBLM formation and the incorporation of proteins can be monitored by QCM and SPR techniques [132]. SEIRAS has been utilized in tBLM studies to obtain structural information about the molecular interactions between gramicidin A and various cations on nanostructured gold electrodes [133], the insertion of alamethicin into the phospholipid membrane [72, 134] and the voltage dependent behavior of a

photoreceptor molecules [135]. Wieblack et al. [136] employed SEIRAS and EIS to investigate the bacterial respiratory ubiquinol/cytochrome bo_3 (cyt bo_3) couple incorporated into a tBLM. The functionality of the cyt bo_3 from *E. coli* membrane fragments was preserved in this membrane which allowed investigating cyt bo_3 performance by EIS and SEIRA spectroscopy and by measuring the current of enzymatic O_2 reduction. Knoll group employed SEIRAS [137-139] and SERRS [140, 141] to study the mechanism of electron transfer to RedOx proteins tethered to tBLM. Su et al. employed PM-IRRAS to determine the orientation of gramicidin A in a tBLM [142] and demonstrated that gramicidin inserts into the bilayer with its α-helix assuming the tilt angle of ~29^o at moderate concentrations and adopting a random orientation at high concentrations. AFM imaging was used to determine the surface morphology of tBLMs [143]. The interaction between membrane-active protein/peptide and tBLMs were investigated by the force spectroscopy [144]. There are several excellent reviews that cover various aspects of research with tBLMs [3, 114].

In addition to acting as platforms for biophysical studies of membrane bound proteins and peptides, tBLMs display long term stability, which offers great potential for the development of biosensors [3, 9, 118]. However, thiolipids are neither commercially available nor naturally occurring molecules and require complex synthetic approaches. Another drawback to tBLMs on solid electrodes is their restrictive mobility, despite improvements over the sBLM and hBLM systems [145, 146]. Specifically, the diffusion coefficients of molecules in the proximal leaflet (i.e. leaflet anchored to the substrate) are smaller than the molecules in the distal leaflet exposed to solution. To address this limitation, floating BLMs were designed and are discussed in further detail below.

Floating bilayer lipid membranes (fBLMs)

In order to improve membrane fluidity, attempts were made to separate the bilayer from the solid support by a water rich lubricant layer [147-151]. The lubricant

may be either a water rich polymer [147-150] or hydrogel film [151]. The floating bilayers display good mobility and improved activity of reconstituted proteins. Traditionally, many of these studies were performed on nonconductive substrates. To construct the floating bilayer at the gold-solution interface, Kycia et al. [91] and Su et al. [152], modified the metal surface by first self-assembling a monolayer of β-thioglucose (Tg). A phospholipid bilayer was then deposited on this hydrophilic film using a combination of LB-LS techniques. Ganglioside GM1 [91] or a lipid with a terminal polyethylene glycol chain DMPE-PEG350 [152] were incorporated into the proximal leaflet of the bilayer to increase the thickness of the water rich region, which separates the metal from the bilayer. The AFM images of the DMPC/cholesterol/GM1 fBLM confirm that the acyl chains of the DMPC molecules in fBLM are tightly packed [91]. The PM-IRRAS studies of these fBLMs suggest that the average tilt angles of acyl chains in the fBLMs are smaller than those in the sBLMs. This indicates that the bilayer in fBLM is in fact separated from gold surface by a water rich region and allows the head groups of the phospholipids to pack in a zigzag fashion [152]. The position of the global maximum of the carbonyl stretching band in the fBLMs was shifted to lower wavenumbers in comparison to sBLMs indicating that the C=O group is more hydrated when the film is supported on the Tg surface [152]. The PM-IRRAS results show that the PEG and GM1 spacers provide a desirable, water rich region between the bilayer and the metal surface. An alternative approach was employed by Munro and Frank, who deposited a fBLM onto the gold surface modified by a monolayer of polyethylene glycol thiol [153, 154]. A PEG cushion was tethered to the gold surface though the terminal thiol group and separated the membrane from the metal. This also provided water rich environment for the model membrane allowing for prolonged stability and lipid mobility.

Interesting fBLM architecture was developed for studying redox enzymes and large proteins [155, 156]. Gold electrode surfaces are first modified by a SAM of 4-aminothiophenol (4-ATP). The bilayer is then deposited onto the SAM with the protein either bounded to the distal or to the proximal leaflet. In the latter case, the enzymes are located in the space between the 4-ATP SAM and the membrane. When

a hydrogenase is trapped into the submembrane region, it can directly transfer electrons to the gold electrode and H_2-oxidation catalytic current could be measured [155]. Kriegel et al. [157] applied SEIRAS to study the SAM modified gold electrode attached to an energy-converting, NADH:ubiquinone oxidoreductase, respiratory complex I. The ftBLM was assembled by reconstituting the lipids into the SAM by vesicle fusion. This architecture showed that the activity of the dehydrogenase fragment increases when the complex is imbedded into the bilayer. Independently, the complex I system was investigated in a fBLM by Gutiérrez-Sanz et al. using EIS and AFM [158]. While most of research on fBLM involved a single bilayer an interesting new development was to deposit a stack of multiple bilayers contain redox enzymes [159]. An enhanced RedOx activity of the enzymes was observed in this platform.

In conclusion fBLMs provide improved mobility to the lipid bilayer and better hydration for incorporated proteins. The hydrogel supported fBLMs find applications as inexpensive and versatile biomedical devices [63].

Perspectives and Conclusion

Initially, planar supported bilayers were widely used as platforms for membrane research on nonconductive supports, such as glass or quartz. Metal supported phospholipid bilayers have provided an opportunity to induce biochemical/biological structural changes and monitor membrane processes in response to an applied electric field. This has led to the development of electrochemical biosensors. An excellent overview of challenges in the development of biosensors was recently published by Bizzotto et al. [160]. Noble metal supports, such as gold, have introduced the possibility of employing additional spectroscopic tools that rely on surface selection rules and plasmonic resonances, such as SERS, SPR, SEIRAS and PM-IRRAS to extract the structure, orientation and conformation of the individual molecular components of the model membrane. SEIRAS offers unique opportunity to acquire time resolved spectra and hence to provide molecular level information about kinetics of membrane processes. In addition, conductive supports allow for the application of

STM to provide molecular level imaging of the individual membrane constituents and ion channels formed by peptides within the model membrane. These surface sensitive techniques have significantly contributed to the recent progress achieved in the field of biomimetic research. A wide variety of metal supported biomembrane models have been designed and characterized using a plethora of experimental techniques, which has given us a better understanding about their strengths and limitations. This precious knowledge acts as a toolbox to allow the researcher to select the best platform for further biological/biomedical research. Research involving proteins and peptides reconstituted into metal supported membranes are still at the earlier developmental stage, however, these early works have already demonstrated that biomembranes supported on metal electrode surfaces are ideal platforms for biomedical studies focused on implant biocompatibility, cell adhesion and fusion, drug screening and amyloid plaque formation and are essential for the future development and advancement of biosensors.

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• Paper of special interest.

•• Paper of outstanding interest.

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