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REVIEW

Biomimetic tethered lipid membranes designed for membrane-protein interaction studies

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Abstract The complexity of the biological membranes restricts their direct investigation at the nanoscale. Lipid bilayer membranes have been developed as a model of biological membranes in order to allow the interaction and insertion of peptides and membrane proteins in a functional manner. Promising models have been developed in the past two decades and tethered bilayer design traduces constant improvement of membrane models. The formation of protein free solid tethered membranes can be achieved by direct vesicle fusion, Langmuir–Blodgett, Langmuir–Schaffer transfers, self assembly of various building blocks such as thiol on gold, silane on quartz, grafting of polymers, as well as ligand receptor recognition. In this review, the current state of different tethered bilayer membrane will be described. We will focus on critical analysis of the main advantages/drawbacks of each kind of model construction and their ability to allow protein incorporation in non-denaturing conditions. Some of the current drawbacks encountered in these biomimetic models can be overcome using an innovative tethered bilayer design based on a reliable and fast formation method. The successful protein incorporation of the Adenylate Cyclase produced by *Bordetella pertussis* and the voltage dependent anion channel (VDAC) was demonstrated on this model.

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Abbreviations

AFM	Atomic force microscopy
DSPE-PEG-NHS	1.2-Distearoyl-*sn*-glycero-3-phospho ethanolamine-poly(ethylene-glycol)-*N*-hydroxysuccinimide
EggPC	Egg-phosphatidyl-choline
FRAP	Fluorescence recovery after photobleaching
IS	Impedance spectroscopy
LB	Langmuir Blodgett
MeO-PEG-PDP	Methoxy-poly(ethylene glycol)-2000-N-[3-(2-(pyridyldithio)propionate]
NBD-DPPE	1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-NBD (7-nitro-2,1,3-benzoxadiazol-4-yl)
PEI	Polyethyleneimine
PEG	Polyethylene glycol
POPC	1-Palmitoyl 2-oleoyl phosphatidyl choline
QCM-D	Quartz crystal microbalance with dissipation
SPR	Surface plasmon resonance
VDAC	Voltage dependent anion channel

Introduction

Biological membranes are essential elements for all living organisms; they play crucial roles in cell life. Their central structure, the lipid bilayer, acts as a barrier in order to prevent the exchange of proteins, ions and metabolites between the intracellular and the extra cellular environment. Lipid membranes ensure the individuality of the cell, but at the same time allow the communication and the transport of materials between the intra and extra cellular

matrix. Proteins embedded in the bilayer or interacting with one of the leaflets are responsible for a selective permeability. To accomplish their diverse functions, membranes have a complex organization where physical properties of the bilayer influence protein structure, folding and function and specific interactions with lipid molecules contribute towards the biological activity of some membrane proteins (Findlay and Booth 2006). Because of the complexity of biological membranes, there is a clear need for biomimetic membrane platforms development, in which one or few membrane components can be isolated and studied.

Solid supported membranes have been developed during the past two decades. One of the major advantages of membrane models deposited on or attached to a surface is the wide range of surface sensitive techniques which can be applied to study model characteristics or proteins/membrane interactions. Different constructions and combinations of membrane models on a solid support have been developed since the pioneer work of McConnell group (Brian and McConnell 1984; Tamm and McConnell 1985) such as supported lipid bilayers on hydrophilic surfaces (Brian and McConnell 1984; Nollert et al. 1995; Rädler et al. 1995; Schmidt et al. 1998), hybrid bilayers (Plant 1993; Plant 1999), tethered bilayers (Knoll et al. 2000; Tanaka and Sackmann 2005) and supported vesicle layers (Jung et al. 2000; Nollert et al. 1995; Yoshina-Ishii and Boxer 2003). At present, an important research effort concerns the development of tethered bilayers. Tethered bilayers are made up of a bilayer spaced out from the surface by the use of spacer's molecules or layers which intercalate between the substrate and the bilayer. These tethered constructions solve the proximity problem between the artificial membrane and the solid substrate that can induce frictions between reconstituted transmembrane proteins and the solid surface and by consequence, improve conditions for the study of membrane components within a non-denaturing environment. In the literature, demonstration of transmembrane proteins incorporation into solid supported bilayers is constantly increasing (McConnell et al. 1986; Hinterdorfer et al. 1994; Salafsky et al. 1996; Heyse et al. 1998; Giess et al. 2004; Elie-Caille et al. 2005; Jeuken et al. 2006; Deniaud et al. 2007).

Various strategies for separating the membrane from the substrate are available. The molecular diversity of the lipid bilayer constructions allows to envisage the reconstruction of various protein environments. Assembling strategies mostly depend on the spacer molecules or layers and the nature of the substrate. The choice of the bilayer construction strategy is most of the time influenced both by the surface techniques which have to be used for characterization and by the substrate properties that are required for analyses. Noble metals (gold, silver) are commonly encountered for surface plasmon resonance (SPR) monitoring while transparent surfaces (silica, quartz, glass) are necessary for optical techniques, atomically flat surfaces (mica, silicon, flat gold) being candidates for atomic force microscopy (AFM) imaging, even if certain techniques, such as quartz crystal micro-balance with dissipation (QCM-D), permit a wider panel of substrate nature to be used.

Structural concepts for tethered membranes

Several membrane construction concepts have been developed. They are gathered in Table 1 and schematized in Fig. 1. These different constructions will be described in detail in the following.

- Bilayers on polymer cushions

In the case of lipid bilayers directly deposited on a solid substrate, the substrate nature can strongly influence the biomimetic membrane properties. Hydrophilic materials such as silicon, silica, quartz or mica allow the presence of a 1–2 nm thick water layer between the lipid bilayer and the substrate (Bayerl and Bloom 1990; Beckmann et al. 1998; Johnson et al. 1991) which permits a high diffusion of the membrane lipids (Cezanne et al. 1999; Kalb et al. 1992); conversely, metals or metallic oxides (gold and Indium Tin Oxide (ITO)) have been shown to reduce drastically the mobility of membrane components (Groves et al. 1997; Groves et al. 1998). Surfaces such as oxidized gold, aluminum oxide and titanium oxide even prevent the vesicle fusion to form a continuous membrane (Groves et al. 1998; Reimhult et al. 2003).

To circumvent the influence of the substrate nature on the bilayer formation and its properties, one approach consists in the addition of a soft hydrated polymer (or a polyelectrolyte film) on top of the substrate surface. This polymer or electrolyte layer acts as a deformable, mobile and hydrophilic substrate on which a fluid membrane can be built. Various approaches concerning these polymer-lipid composite films based methods were recently reviewed (Elender et al. 1996; Knoll et al. 2000; Sackmann and Tanaka 2000; Tanaka and Sackmann 2005). Different strategies were envisaged for the formation of the polymer film on the surface. In the case of water soluble polymers such as dextran, a chemical grafting is preferred to avoid dehydration; it can be performed via a reactive self assembled monolayer (Knoll et al. 2000) on the substrate, which allows the chemical linkage of the polymer chains to the surface. On the other hand, physisorption onto the solid surface is used in the case of branched polyethyleneimine (PEI), which sticks to the negatively charged surfaces through electrostatic interactions. Construction of the membrane on top of the polymer cushion can be achieved by Langmuir–Blodgett (LB) transfer, vesicles fusion, a combination of both or by bilayer spreading (Knoll et al. 2000; Sackmann and Tanaka 2000). A comparative study of the

Table 1 Advantages and drawbacks of supported biomimetic membranes

Structural concept	Supporting layer	Advantages	Drawbacks	Lipid diffusion range	References
Bilayers on polymer cushions	Chimisorbed polymer film	The polymer layer acts as a deformable, mobile and hydrophilic substrate	Lack of bilayer stability; exhibits often structural defects	2.5 ± 0.3 μm^2/s	Elender et al. (1996); Knoll et al. (2000)
	Physisorbed polymer film		The quality of the bilayer is dependent of the formation procedure	Not determined	Wong et al. (1999)
	Polyelectrolyte film		The mobility of the lipids is reduced	0.2–0.1 μm^2/s	Zhang et al. (2000); Ma et al. (2003)
Tethered bilayers using functionlized lipid	Supporting layer formed by self-assembly	The high density of the supported layer help to obtain electrical sealing properties of the bilayer	Electrical sealing and incorporation of large protein may not be compatible	0.5 μm^2/s	Naumann et al. (2003); Munro and Franck, (2004)
	Supporting layer formed by Lamgmuir–Blodgett	The density of the supported layer is controlled	Lamgmuir–Blodgett is not an easy route for the Incorporation of labile proteins	0.8–1.2 μm^2/s	Wagner and Tamm, (2000)
	Based on the direct fusion of vesicles containing the anchor molecules	Formation of the bilayer is fast and easy to setup	The presence of the anchor molecule in the outer leaflet of the bilayer	3.0 ± 0.8 μm^2/s	Rossi et al. (2003); Deniaud et al. (2007); Rossi et al. (2007)
Protein-tethered bilayers	none	Help to control the density and the orientation of the incorporated proteins	The mobility of the proteins is drastically reduced	Not determined	Giess et al. 2004

different bilayer formation processes onto PEI layers was detailed by Wong et al. (1999).

As mentioned by Wagner and Tamm (2000), bilayers formed on polymers can often be found to be patchy and to exhibit several structural defects. Uniform and fluid bilayers were obtained on dextran films, their stability was increased using cholesterol incorporation (Elender et al. 1996). The formation of a continuous 1,2-dimyristoyl-sn-glycero-phosphatidylethanolamine (DMPC) bilayer on PEI cushions was found to be dependent of the chosen construction process (Wong et al. 1999). Vesicles deposition lead to the desired fluid and continuous bilayer structure only when fusion was realized on a dried polymer cushion or when the reverse method was used, i.e. polymer adsorption on an already formed bilayer on the quartz substrate (Majewski et al. 1997; Wong et al. 1999). The weak interaction between DMPC lipids and PEI makes delicate the construction using the Langmuir–Blodgett deposition method. A successful bilayer formation using vesicle fusion above a lipid monolayer (deposited by LB transfer) was reported (Wong et al. 1999). An improvement introduced by Seitz et al. (1998) comprised as first step a LB transfer (above the PEI layer) of the inner lipid monolayer containing an isothiocyanate-functionalized 1,2-dimyristoyl-sn-glycero-phosphatidylethanolamine (DMPE), which was followed by vesicle fusion for bilayer completion. The modified lipid polar head group ensured the covalent anchoring of the inner monolayer on the PEI cushion.

Another assembling technique developed for bilayer construction on polymer cushions is to support a mixed bilayer composed by anionic and zwitterionic lipids on alternated polyions multilayers. Then, electrostatic forces ensure the cohesion of the molecular assembly. The interaction kinetics of several peptides such as the peptide wt-20 of the influenza virus, gramicidin peptide (Zhang et al. 2000) and the ion channel forming peptide Protegrin-1 (Ma et al. 2003) with these membrane models were monitored by SPR. One major drawback of such electrostatic based construction is a reduced mobility of lipids in the bilayer due to the electrostatic forces.

- Tethered bilayers using functionalized lipids

An alternative strategy widely used to separate lipid bilayers from the solid substrate is based on lipids with head groups chemically coupled to macromolecules, which act as spacers and linkers. These macromolecules can be built from polymerized ethylene oxide units (Cornell et al. 1997; Lang et al. 1994; Schiller et al. 2003; Tanaka and Sackmann 2005; Wagner and Tamm 2000) or are oligo-peptide based moieties (Bunjes et al. 1997; Naumann et al. 1997; Naumann et al. 1999; Schmidt et al. 1998). The lipid part is commonly a phospholipid, but some examples of cholesterol-polyethyleneglycol molecules, which act at the same time as anchor

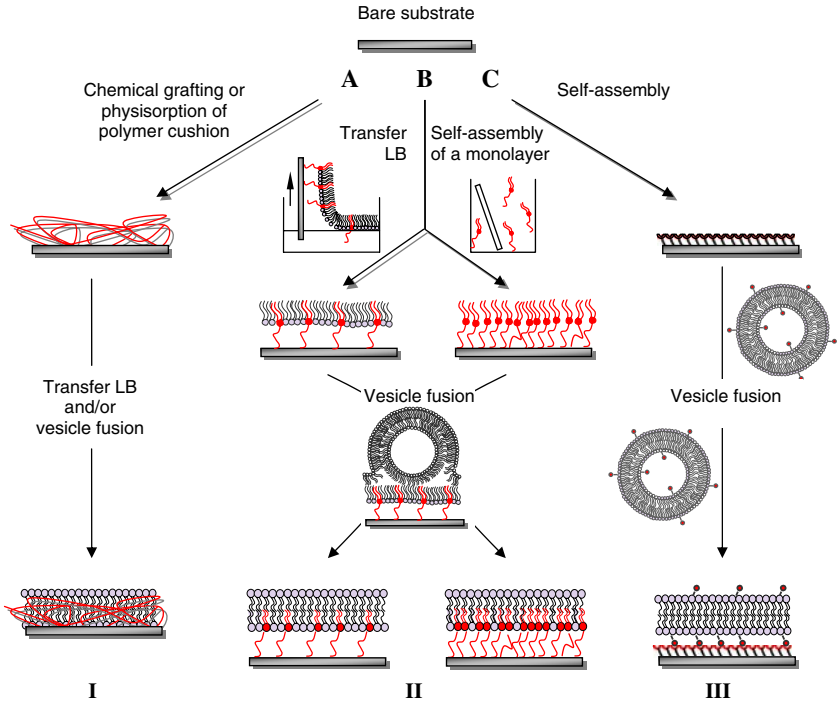

Fig. 1 Different preparation techniques of tethered bilayers. **a** vesicle fusion or Langmuir–Blodgett transfer on a polymer cushion. **b** Langmuir–Blodgett deposition or self-assembling of a lipid monolayer including lipids whose head groups are coupled to the spacer group. The spacer chains are anchored on the substrate and the bilayer is completed with vesicle fusion. **c** Fusion of vesicles containing lipids with a chemically modified head group in order to present a specific recognition site for the functionalized self-assembled monolayer on the top of the substrate

and spacer molecule in phospholipid bilayers, have been described (Cheng et al. 1998; Jeuken et al. 2005).

Vogel and co-workers introduced and extended the "polymer spacer concept" for the construction of polymer supported monolayers using a lipid-ethoxy group chain-disulfide molecule (Lang et al. 1992; Lang et al. 1994) while Knoll's group performed the same approach using a methacrylic terpolymer containing a hydrophilic main-chain spacer (hydrophobic lipid-like parts) and a disulfide termination (Spinke et al. 1992). Since these pioneer works, the commonly synthesized design of functionalized anchor lipids applied in tethered bilayer elaboration is commonly based on a polyethyleneglycol (PEG) chain terminated by a disulfide moiety that ensures chemisorption onto gold surfaces. This preferential choice is explained by the ability of PEG to prevent non-specific adsorption of protein to surface (Prime and Whitesides 1991) and it also provides a soft hydrophilic spacer layer (Sackmann 1996). Thiol and disulfide groups present a high affinity for gold surfaces, which allows fast, stable and reproducible anchorage of molecules (Bain and Whitesides 1989); moreover gold is an inert metal that allows the possible application of a wide panel of surface sensitive techniques such as SPR, impedance spectroscopy (IS), QCM-D and AFM. In addition, optical techniques (adsorption, fluorescence) require transparent surfaces such as silica, quartz or glass, which implies the use of silane, terminated molecules such as PEG-phospholipid ones (Wagner and Tamm 2000) for anchoring the molecular construction on the substrate. The diversity of the surfaces relying on the characterization techniques explains the research efforts towards the development of versatile "tool box" enabling the formation of a molecule design adaptable on several surfaces (Atanasov et al. 2006).

Several techniques are available both for designing and assembling the two leaflets of the membrane. The supported inner lipid monolayer and the outlet lipid layer can be independently assembled either by LB transfer or by a two steps self-organisation procedure such as a first self-assembly followed by vesicle fusion. One of the most versatile approaches appears to be vesicle fusion onto a preformed supported lipid monolayer prepared either by LB transfer (Duschl et al. 1994; Wagner and Tamm 2000) or by self-assembly (Atanasov et al. 2005; Munro and Frank 2004; Naumann et al. 2003a; Raguse et al. 1998; Seitz et al. 2000; Spinke et al. 1992). However, alternative routes were explored. Munro and Frank (2004) described bilayer formation by vesicle fusion on a previously chemisorbed PEG film, composed of 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[PDP(polyethylene glycol)2000] (DSPE- PEG-PDP) mixed with methoxy-poly(ethylene glycol)-2000-N-[3-(2-(pyridyldithio)propionate] (MeO-PEG-PDP); completion of the lipid monolayer was achieved by free lipid deposition in hexane. Cornell et al. (1997) used solvent exchange for bilayer completion; lipids were added as an ethanolic solution onto a previously prepared tethered monolayer which was flush with a brine solution. This method could be seen as an alternative procedure to the vesicle fusion for completion and formation of the bilayer; indeed vesicles fusion was not always spontaneous and required to be induced or initiated by osmotic stress (Atanasov et al. 2005; Munro and Frank 2004; Naumann et al. 2003a; Seitz et al. 2000).

Membranes models developed following such supported lipid bilayer design show very good electrical properties. They are comparable to those of black lipid membranes (resistance larger than 10 MΩ cm² and capacitances smaller than 1 μF/cm² are standard for "good" membranes). Taking advantage of insulating properties of these membranes and the possibility of ion channels incorporation, a functional biosensor was developed where gramicidin was embedded in the membrane and acts as a detection unit (Cornell et al. 1997). Functional insertion of valinomycin in this membrane model proves to be successful (Raguse et al. 1998). Another highly electrically insulating model was developed by Vogel's group in which a polypeptidic molecule comprising a surface anchoring region and channel forming region acts as specific recognition unit for monoclonal antibodies (Tanaka and Sackmann 2005; Terrettaz et al. 2003; Terrettaz et al. 2001). More recently, Knoll and co-workers have synthesized a novel type of tethered lipids. The 2,3-do-O-phytanyl-sn-glycerol-1-tetraethylene glycol-D, L,α-lipoic acid ester lipid (DPTL) allows the construction of tethered bilayers with resistance of up to several MΩ cm² and capacitance less than 1 μF/cm². Valinomycin (Naumann et al. 2003a), gramicidin and cytochrome c oxidase (Naumann et al. 2003a, b) were functionally reconstituted. This membrane model was successfully transposed on silicon oxide surface in which valinomycin and gramidicin were also functionally incorporated; nevertheless, membrane resistance was found to be lower than that of the thiol based system (Atanasov et al. 2005).

Other properties, e.g. the increasing protein mobility, are still under discussion. Wagner and Tamm (2000) have shown that the construction of a tethered bilayer based on a lipid-polymer layer lead to high lateral diffusion coefficient for the lipids and an improved mobility of cytochrome c and annexin V within the bilayer, even if some immobile fraction of the protein are not eliminated. But the increase of protein mobility in these systems is discussed in a recent publication; polymer cushions don't necessarily increase the protein mobility as expected at a first glance (Merzlyakov et al. 2006). Anyhow, Naumann and co-workers have shown by single molecular tracking that high concentration of tethered lipid (Ctether $\geq$ 10 mol%) acts as an obstacle for protein diffusion and modifies the protein diffusion in an anormalous or two-component diffusion (Deverall et al. 2005). Moreover, the percentage of tethered lipid had a strong influence on the lipid diffusion coefficient. In the membrane model described by Wagner and Tamm (2000), the mobile fraction of lipid, at the mushroom-brush transition PEG-lipid percentage, was found to decrease while the lipid diffusion coefficient remained constant. However, a gradual decrease in the lipid diffusion coefficient upon increase of the PEG lipid concentration from 5 to 30% was observed (Naumann et al. 2002).

- Tethered bilayers using receptor/ligand recognition and protein-tethered bilayer lipid membranes

An alternative for the assembly of tethered lipid bilayers on solid substrate is to take advantage of specific ligand/receptor interactions. Such a membrane model is usually composed of a substrate, coated with a self-assembled monolayer or a polymer film that includes a receptor, allowing the immobilisation and the tethering of either the bilayer or the vesicles containing a specific ligand. Coupling or anchoring of the bilayer to the substrate was achieved by divalent cations/chelators lipids coupling. Membranes were built with *N*-nitrilotriacetic acid (NTA) lipid head groups trapping nickel ions to form a complex with histidine residues of tagged proteins (Radler et al. 2000). The use of the biotin/streptavidin complex is also commonly used for this kind of membrane constructions (Elie-Caille et al. 2005; Fisher and Tjarnhage 2000; Proux-Delrouyre et al. 2002; Tanaka and Sackmann 2005).

Brisson's group developed membrane construction systems for the formation of well organized 2D protein layers. A network of streptavidin is formed on top of a mica-supported bilayer containing a biotinylated lipid bilayer. Then, vesicles containing biotinylated lipids anchor and fuse onto this protein network to lead to locally flat lipid bilayers (Reviakine and Brisson 2001). Following this idea, bilayer architectures based on the specific recognition construction can be considered as protein-tethered bilayers. Indeed, spontaneous incorporation of proteins in membrane models leads to a lack of control of their density or orientation. One way to overcome these limitations is to tether the membrane via the incorporated protein. A construction based on a monolayer containing NTA groups and an *N*-terminus His-tagged cytochrome c oxidase was recently described. The reconstitution and reversible binding of cytochrome c oxidase were monitored by SPR and QCM-D. Upon incubation with Cu^{2+} or Ni^{2+} ions, the protein was immobilised on the surface, and flushing with a lipid/detergent solution lead to the protein tethered lipid bilayer structure (Ataka et al. 2004; Friedrich et al. 2004; Giess et al. 2004).

- An easy and versatile model for biomimetic membrane formation

A new concept for membrane construction is based on the direct vesicle fusion that contains both the anchor and spacer molecules on a functionalized surface. This approach combines functionalized/spacer lipids and their use for receptor/ligand binding. This membrane model is expected to overcome different limitations of different constructions developed so far.

In former tethered membrane models, the nature of the substrate governs the reactivity of the terminal end of the anchor molecule which was synthesized on purpose. Indeed, direct anchoring of the spacer molecule to the bare

substrate was ensured by the specific reactive group of the molecule with the substrate. For example, silane was used for mica, glass, silicone and thiol for gold substrates. In the case of "easy and versatile model for membrane formation", the construction of the tethered membrane was achieved by a vesicle deposition on a pre-activated surface. Surface coating was obtained from self-assembled monolayers containing amino groups (amino-thiols or aminosilanes). The bilayer formation step was completed by deposition of vesicles containing a mixture of EggPC and DSPE-PEG_{3400}-NHS, which acts both as a spacer and an anchor molecule. The spacer lipid is terminated by an NHS activated carboxylic acid that can react with any amine coated surface. We performed the construction of the tethered DSPE-PEG-NHS/lipid bilayer on glass and gold (Deniaud et al. 2007; Rossi et al. 2003). Egg PC or POPC/DSPE-PEG-NHS mixture liposomes were injected on the top of an amine grafted surface (cysteamine-coated gold or silanized glass); vesicles were linked to the surface and disrupted leading to the formation of a bilayer (Fig. 2).

The other important advantage of the direct incorporation of the spacer molecule in vesicles is the possibility to control its percentage. This allows to obtain a spacer layer with low density in comparison to very close packed layers of models which are supported by a self-assembled supported lipid monolayer (Atanasov et al. 2005; Munro and Frank 2004; Naumann et al. 2003a; Seitz et al. 2000; Spinke et al. 1992). This high density could constitute a steric limitation and proteins having large extramembrane domains cannot be incorporated appropriately. The construction of this tethered bilayer model was envisaged using a simple and reliable procedure. The challenging part was to determine the experimental conditions that lead to the spontaneous formation of a fluid and homogenous tethered bilayer. A Doehlert experimental design, a second order experimental design, was applied to determine these experimental conditions (Rossi et al.; in press). Four important factors involved in the bilayer formation were studied: the lipid concentration in the vesicles suspension, the mass percentage of anchoring molecules in the vesicles, the time of contact between vesicles and the surface and the time during which the lipidic assembly was left at rest after buffer rinse. A restraint experimental domain, which led to properties in accordance with a bilayer presence, was delimited. The measured properties of the biomimetic were the thickness by SPR on gold substrate and the fluidity by FRAP on gold and glass substrate (Fig. 3,A and B respectively). The tethered bilayer model was characterized by a thickness of 56.5 ± 2.5 Å, a diffusion coefficient of $3.0 \pm 0.8 \times 10^{-8}$ cm²/s and a mobile fraction of $96 \pm 4\%$. AFM imaging was also performed and showed the step by step construction on both glass and smooth gold amine coated substrates (Fig. 3C). The aspect of the membrane formed on the gold and glass substrates looks very similar; the presence of a homogenous flat surface of soft material corresponding to a biomimetic lipid bilayer anchored to the solid substrate was observed. Two different zones can be observed in Fig. 3Cb (gold pathway) and Fig. 3Cd (glass pathway), the brighter one corresponding to the intact tethered membrane and the darker one corresponding to holes showing the bare amine substrate after a limited Triton X-100 solubilization of the membrane. A step height measurement indicated a membrane thickness of 5.4 ± 0.6 nm on the gold substrate and of 5.9 ± 0.4 nm on the glass substrate, which clearly indicates the presence of a lipid bilayer on both the substrates.

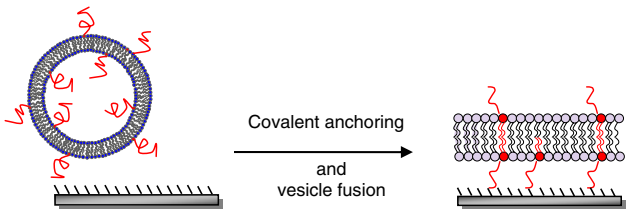

Fig. 2 A versatile tethered bilayer model obtained from vesicle anchorage plus fusion. Self-assembled monolayers of aminopropyldimethylethoxysilane or cysteamine were obtained respectively on glass and on gold. EggPC liposomes containing DSPE-PEG_{3400}-NHS were deposited onto the coated surface, linked to the amine surface, and then disrupted to generate the bilayer

This biomimetic membrane was applied for protein/membrane interaction studies of two different kinds of integral proteins: the bacterial toxin, the adenylate cyclase produced by *Bordetella pertussis* (CyaA) (Ladant and Ullmann 1999) and the mitochondrial outer membrane channel, the voltage dependent anion channel (VDAC) (Colombini et al. 1996). CyaA is able to bind and translocate across the plasma membrane of eukaryotic target cells in a calcium-dependent manner. CyaA presents a hemolytic activity that is due to its ability to form cation-selective channels in lipid bilayers and also a catalytic domain able to translocate across the cell membrane and interact with the endogenous calmodulin to produce supra-physiologic levels of cyclic AMP. Both internalization and hemolytic activities are calcium-dependent. Calcium-bound CyaA should behave as an intrinsic membrane protein, with hydrophobic polypeptide segments putatively inserted within the lipid bilayer. The results obtained by monitoring SPR binding and insertion of CyA to artificial membranes demonstrate a calcium dependent binding, suggesting that CyaA associates with polymer tethered membrane as a result of insertion of its polypeptide chain into the lipid bilayer and therefore behaves as an integral membrane protein (Fig. 4a). The binding properties of CyaA towards a hybrid bilayer and the tethered bilayer in the presence and the absence of calcium were measured. The protein didn't bind or insert to/in the hybrid bilayer even in the presence of calcium; the rigid

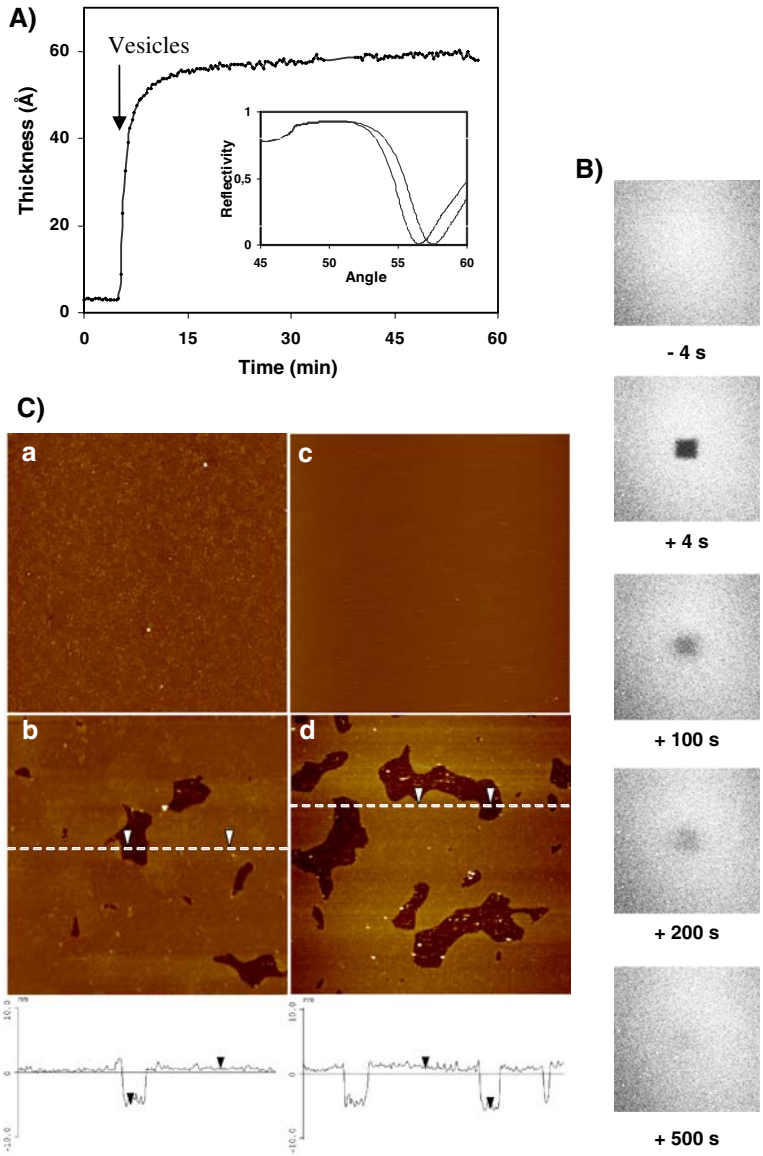

Fig. 3 Characterization of the tethered bilayer model. **A** SPR kinetics of the EggPC/DSPE-PEG3400-NHS on a cysteamine coated gold surface. **B** Images (320 µm × 320 µm) sequence on amino-silane coated glass of fluorescence recovery after photobleaching (FRAP) of tethered bilayer built containing NBD-DPPE as fluorescent probe. **C** Contact mode topographic AFM images (10 µm × 10 µm, *z* scale 20 nm); *a* and *b* are for gold; *c* and *d* are for glass. *a* and *c* were obtained before vesicles addition; *b, d* were obtained after vesicle fusion. Lipid bilayers were treated with Triton X-100 leading to a local solubilization of the membrane, the corresponding height sections are reported below the corresponding images

alkyl monolayer presence prevent the protein insertion in the hemimembrane. Conversely, the protein bind and insert in the tethered bilayer model in a calcium dependent manner (Rossi et al. 2003). VDAC, a central player in mitochondrial biology and apoptosis, was reconstituted into the tethered bilayer using fusion proteoliposomes containing DSPE-PEG-NHS on amine-coated surface (Fig. 4b). VDAC reconstituted in bilayers transported calcium ions efficiently and was blocked by 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid and L-glutamate as known on liposomes and mitochondrial extract (Deniaud et al. 2007).

Conclusion

Due to the complexity of biological membranes and their interactions with intra- and extra cellular networks, direct investigations are difficult and limited to a few examples only. Solid supported phospholipid bilayer model systems have been introduced in order to simplify the complex architecture of the cell membrane and to provide experimental systems to incorporate membrane proteins under native conditions. The formation of protein free solid supported membranes can be achieved by direct vesicle fusion, Langmuir–Blodgett, Langmuir–Schaffer transfers, self assembly of various building blocks such as thiol on gold, silane on quartz, grafting of polymers, as well as ligand receptor recognition. These biomimetic systems allow functional investigations of membrane-spanning proteins and of polyprotein complexes with surface-sensitive techniques such as surface plasmon resonance, impedance spectroscopy, quartz crystal micro-balance with dissipation, fluorescence microscopy and atomic force microscopy (Table 2).

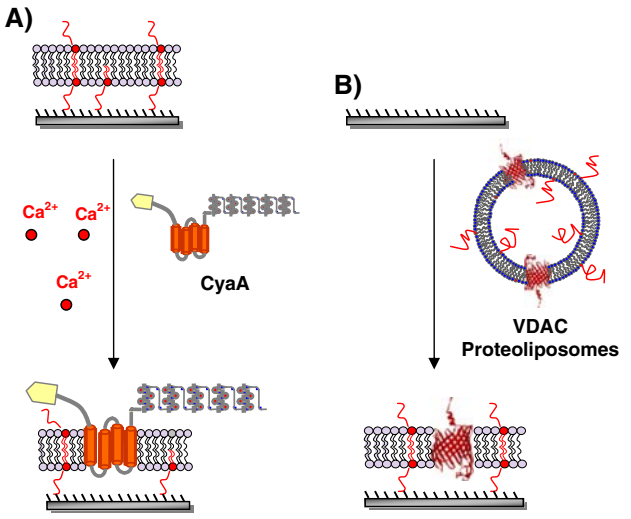

Fig. 4 Schematic illustration of the incorporation of two different integral proteins in a versatile tethered membrane model: **a** CyaA interacts directly with the membrane in the presence of calcium and inserts its hemolytic channels **b** VDAC reconstitution via anchorage and fusion of proteoliposomes containing DSPE-PEG-NHS

The functional assembly of membrane proteins into supported membranes is not a straightforward process (Michalke et al. 2001). Small peptides spontaneously incorporate in lipid bilayers; valinomycin and gramicidin are common candidates for testing supported membranes (Cornell et al. 1997; Schiller et al. 2003). Similarly, urea denatured membrane proteins, such as toxins, spontaneously insert in preformed tethered membranes (Rossi et al. 2003). However, the pore-forming toxin α-hemolysin was only partially reconstituted into supported bilayer membranes (Glazier et al. 2000). Integral membrane proteins have been introduced in lipid membrane using different strategies. His-tagged proteins such as cytochrome c oxidase can be directly coupled to the support using NTA groups in the presence of Cu^{2+} or Ni^{2+}; completion of the bilayer was achieved with addition of phospholipids (Giess et al 2004). Mixed micelles containing proteins can be added to preform tethered bilayers; incorporation of the protein is ensured upon detergent removal (Heyse et al. 1998; Terrettaz et al. 2001, 2003). Detergent removal can be obtained

Table 2 Techniques for surface supported bilayer characterization

Techniques	Bilayer characterization	Principle	Surfaces
Quartz crystal microbalance with dissipation QCM-D	Mass and structural properties of the bilayer. Real time monitoring of the bilayer formation	QCM-D measures the resonance behavior of a quartz crystal oscillator. The quartz oscillation is excited by applying an oscillating electric field to the crystal. The resonance frequency decay curve and the dissipation are measured via the decaying electric field. Adsorption process and structural changes of the bilayer are measured. Coupled water and structural defects can be checked	Gold, SiO_2, mica, metal oxides...
Surface plasmon resonance spectroscopy SPR	Optical thickness of the bilayer. Real time monitoring of the bilayer formation	Surface plasmons are electromagnetic evanescent waves propagating at the surface of a noble metal with a penetration depth of 200 nm. Optical thickness is derived from the difference in refractive index between the adsorbed biomolecules and buffer. Information concerning properties of the deposited material is not available	Noble metals: gold, silver, aluminum
Electrical impedance spectroscopy EIS	Formation and (non-) permeability of the bilayers. Characterization of incorporated ion channels	EIS is performed in a potentiostatic three-electrode configuration by applying an AC potential at different frequencies and measuring the resulting impedance. The resistance and the capacitance of the bilayer/electrolyte system can be determined using a equivalent circuit	Gold, silicon...
Atomic force microscopy AFM	Observation and investigation of the bilayer surface at the nanoscale range. Surface roughness determination	A microscopic probe (tip) is mounted on a soft cantilever. A high precision optical detection (laser) device measures cantilever deflection. The sample is moved using a scanner (piezoelectric elements) and interactions between the tip and the sample are controlled by feedback. So, surface topography and interactions forces between tip and sample can be determined	Atomically flat surfaces: mica, silicon, quartz, flat gold
Fluorescence recovery after phtobleaching FRAP	Mobility characterization of the lipids or proteins (peripheral or integral)	Lipid fluorescent probes or tagged proteins are incorporated in the bilayer. The fluorescence is destroyed in a small area of the bilayer. The fluorescence recovery rate of the bleaching area allows the determination of the diffusion coefficient and the mobile fraction of the probes	Optically transparent but non fluorescent surfaces: glass, silica, silicon, gold

by dilution, by dialysis using specific experimental cells or using polymer gels (biobeads) (Deniaud et al. 2007). Protein rich membranes or proteoliposomes can be the starting material for tethered membrane formation (Elie-Caille et al. 2005). When these protein-containing vesicles were added to a preformed self assembled monolayer, "hydrophobic" fusion ensured the formation of the outer leaflet of the membrane. Proteoliposomes containing anchored lipid molecules can be directly used for the formation of the entire membrane, which is obtained after vesicle anchoring on the surface followed by rupture that leads to the formation of the bilayer. Tethered membrane platforms have been envisaged for the design of biosensors applicable for medical or/and environmental purposes (Cornell et al. 1997; Terrettaz et al. 2001, 2003;). G-protein-coupled receptors (GPCR) and other membrane receptors reconstitution, constitute an important challenging step for fundamental research (signal transduction and cascade) and for applications as pharmaceutical screening or as analytical sensors (Heyse et al. 1998; Robelek et al. 2007). Membrane fusion and vesicle secretion for protein/phospholipid production at the cellular level were studied using these reconstitution approaches (Kiessling and Tamm 2003; Kleinschmidt and Tamm 2002; Tamm et al. 2002, 2003; Wagner and Tamm 2001). Exploring energetic chains (respiratory and photosynthesis), starting from one protein incorporation towards the reconstitution of polyprotein complexes or redox cascades, is a very promising research area (Deniaud et al. 2007; Elie-Caille et al. 2005; Friedrich et al. 2004; Giess et al. 2004; Jeuken et al. 2005, 2006).

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