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Synthesis and characterization of tethered lipid assemblies for membrane protein reconstitution (Review)

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Biological membranes and their related molecular mechanisms are essential for all living organisms. Membranes host numerous proteins and are responsible for the exchange of molecules and ions, cell signaling, and cell compartmentation. Indeed, the plasma membrane delimits the intracellular compartment from the extracellular environment and intracellular membranes. Biological membranes also play a major role in metabolism regulation and cellular physiology (e.g., mitochondrial membranes). The elaboration of membrane based biomimetic systems allows us to reconstitute and investigate, in controlled conditions, biological events occurring at the membrane interface. A whole variety of model membrane systems have been developed in the last few decades. Among these models, supported membranes were developed on various hydrophilic supports. The use of solid supports enables the direct use of surface sensitive techniques (e.g., surface plasmon resonance, quartz crystal microbalance, and atomic force microscopy) to monitor and quantify events occurring at the membrane surface. Tethered bilayer membranes (tBLMs) could be considered as an achievement of the first solid supported membranes described by the McConnell group. Tethered bilayers on solid supports were designed to delimit an inside compartment from an outside one. They were used for measuring interactions with ligands or incorporating large membrane proteins or complexes without interference with the support. In this context, the authors developed an easy concept of versatile tBLMs assembled on amino coated substrates that are formed upon the vesicle fusion rupture process applicable to protein-free vesicles as well as proteoliposomes. The phospholipid bilayer (natural or synthetic lipids) incorporated 5% of 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-poly ethylene glycol-N-hydroxy succinimide to ensure the anchorage of the bilayer to the amino coated surface. The conditions for the formation of tBLMs on amino-coated gold and glass were optimized for protein-free vesicles. This biomimetic membrane delimits an inside “trans” compartment separated from an outside reservoir “cis.” Using this tBLM construction, the authors were interested in deciphering two complex molecular mechanisms involving membrane-associated proteins. The first one concerns two mitochondrial proteins, i.e., the porin voltage dependent anion channel (VDAC) embedded in the outer membrane and the nucleotide transporter (adenine nucleotide translocase) that interacts dynamically during mitochondrial pathophysiology. The purified VDAC porin was first reconstituted in proteoliposomes that were subsequently assembled on an amino coated support to form a biomimetic membrane. As a major result, VDAC was reconstituted in this tBLM and calcium channeling was demonstrated across the lipid bilayer. The same two-compartment biomimetic membrane design

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I. INTRODUCTION

Biological membranes carry out essential cellular functions. The plasma membrane constitutes a barrier that separates cells from the environment, while intracellular membranes of subcellular compartments or organelles are essential for the spatio-temporal organization of metabolism and intracellular trafficking. The design of biomimetic membrane systems constitutes a crucial approach that allows us to reconstitute and investigate biological events occurring at the membrane interface in controlled conditions.

Since the pioneering work by the McConnell group,1,2 a variety of constructions and combinations of membrane models on a solid support have been developed such as supported lipid bilayers on hydrophilic surface supported vesicle layers3–5 and tethered bilayers.6–8 A variety of biomimetic membrane systems on solid supports have been developed during the past three decades.7,9–17 One of the major advantages of membrane models deposited on or attached to a surface is the wide range of surface sensitive techniques that can be applied to study model characteristics or protein/membrane interactions.

Tethered bilayers are made of a lipid bilayer spaced out from the surface by the use of a molecular spacer (e.g., polyethylene glycol) or layers which intercalate between the substrate and the bilayer (e.g., polymers, proteins, and DNA). Tethered lipid bilayers [or tethered bilayer membranes (tBLMs)] have been widely used to characterize the interaction of ligands with membranes, dynamics of membrane proteins, or even more complex receptor/ligand-mediated intercellular contacts. We joined this field fifteen years ago driven by major interest in two different biological processes involving membrane proteins for which the help of tBLM would be required in order to decipher their complex molecular mechanism. Indeed, tools to investigate these proteins were limited, and engineering a novel biomimetic model was a stimulating challenge. The first model involved two mitochondrial proteins that interact dynamically at membrane contact sites during mitochondria pathophysiology; the second one concerned the translocation of a bacterial toxin across the plasma membrane of eukaryotic target cells.

II. PORIN RECONSTITUTION IN TETHERED LIPID BILAYERS

Mitochondria are essential organelles for cellular life; they have important functions such as lipid and energy metabolism, production of reactive oxygen species, and detoxification and cell death. Mitochondria are surrounded by a double membrane architecture presenting different compositions and permeabilities. The presence of the voltage dependent channel (VDAC) or porin in the outer membrane (OM) is responsible for the passage of ions, metabolites, and water fluxes.16 VDAC, the most important integral membrane protein, is comprised of 283 amino acids and spans the OM with 19 beta strands. VDAC plays an important role in the biology of mitochondria and apoptosis and has also been recently shown to be implicated in hypoxia and cancer.19 The inner membrane (IM) constitutes the boundary between the matrix and the intermembrane space with a limited permeability to oxygen, carbon dioxide, and water. Numerous mitochondrial carriers are embedded in this membrane, with adenine nucleotide translocase (ANT) being one of those. ANT is an adenosine triphosphate (ATP)/adenosine diphosphate translocase in normal conditions, but in the presence of ligands, (e.g., Ca2+), it can behave as a channel. The structure of bovine isoform 1 of ANT in the presence of carboxyatractyloside shows a six transmembrane helix structure.20 Coimmunoprecipitation and colocalization approaches have evidenced a possible interaction between VDAC and ANT at contact sites involving OM and IM.21 These structures should facilitate metabolite channeling and/or preclude the formation of a transient structure named the permeability transition pore.22,23

Also, it is of importance to be able to monitor the direct interaction between VDAC and ANT and to identify underlying molecular mechanisms involved in their interaction and their regulation. The use of biomimetic membranes and reconstitution of VDAC constitute a possible approach to achieve this goal. Therefore, as a first step, we have worked out the functional reconstitution of the porin into a tethered biomimetic membrane (tBLM).24 A membrane protein-free supported lipid bilayer can be achieved by the fusion of lipid vesicles on a solid support or by transfer on different supports (gold, quartz, and glass) using the Langmuir–Blodgett method or the Langmuir Schaefer method.25 However, the reconstitution of integral membrane proteins demands the use of a protein micellar suspension or proteoliposomes for the construction of a biomimetic membrane including functional proteins.6,24,26

We developed and optimized an easy and versatile model for biomimetic membrane formation based on the direct vesicle fusion on a preactivated support.24 Gold surfaces are obtained after thermal evaporation of gold onto glass slides or coverslips that are subsequently amino-coated by self-assembly of cysteamine (2-aminoethanethiol) on the gold surface (Fig. 1). The vesicles include 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-poly ethylene glycol-N-hydroxy succinimide (DSPE-PEG3400-NHS) molecules acting as both

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spacers and anchors to the amine functionalized surface. This approach combines functionalized/spacer lipids that are used for receptor/ligand binding to the surface. This membrane model was developed to overcome the limitations arising with the use of different constructions such as the need for specific synthesis of molecules adapted to the nature of support chemistry. The DSPE-PEG_{3400}-NHS molecule is used to anchor the vesicles onto the amine grafted surface. This amine activated surface can be obtained on silica using silanes or on gold using the self-assembling properties of thiols on gold. The formation of a supported tethered lipid bilayer can be followed by optical techniques such as fluorescence recovery after photobleaching (FRAP) using glass substrates or by surface plasmon resonance (SPR) monitoring using gold slides. The optimized experimental conditions leading to the formation of a tBLM are as follows: a lipid concentration from 0.1 to 1 mg/ml, 4%–8% of anchoring molecules in the vesicles, and 1–4 h of contact time between vesicles and the surface followed by 21–24 h of resting time after buffer rinse. Using these conditions, a membrane having an optical thickness between 54.5 ± 5.0 and 59.0 ± 10 Å, a diffusion coefficient between 2.5 ± 0.3 × 10^-8 and 3.60 ± 0.5 × 10^-8 cm^2/s, and a mobile lipid fraction between 94 ± 2 and 99 ± 1% was routinely obtained. The presence of a continuous and homogeneous bilayer was confirmed by atomic force microscopy (AFM) observations.24

The transition from vesicles anchored at the surface to the formation of a fluid and continuous biomimetic membrane was optimized for protein-free vesicles. The same procedure was adapted to VDAC proteoliposomes. VDAC was purified from the mouse liver in Triton X-100 micelles using well-established procedures. Its channeling activity was ascertained by electrophysiological measurements performed in black lipid membranes. The classical signature of the porin is in a fully open state at low potentials (below 30 mV) with a main conductance of 4000 pS in 1 M KCl and closure at high voltages and with numerous subconductance states. The VDAC containing proteoliposomes were obtained after the addition of a 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC)/DSPE-PEG_{3400}-NHS (5% w/w) lipid mixture to the VDAC micellar suspension until solubilization of the lipids. Biobeads (Bio-Rad) were added for the detergent removal, leading to a micellar–proteovesicle transition attested by the formation of proteoliposomes. The extrusion of this suspension through polycarbonate membranes ensures the formation of calibrated proteoliposomes having diameters of 90 ± 10 nm. For practical reasons, the dihydronicotinamide adenine dinucleotide-ferricyanide activity of VDAC was monitored for optimization of the process of reconstitution of active VDAC-liposomes. The optimized protocol for proteoliposome reconstitution involved the use of vesicles with a lipid:protein ratio of 100:1. The transition from VDAC proteoliposomes anchored at the surface to the formation of a fluid and continuous biomimetic membrane was followed by SPR (Fig. 2).

SPR measurements led us to determine an optical thickness of 57 ± 3 Å for tBLM. A lateral diffusion coefficient of 3.0 ± 0.2 μm^2 s^-1 and a mobile fraction higher than 95%
were determined from FRAP analysis. The results obtained by FRAP and SPR were confirmed by imaging the tethered lipid bilayer using contact mode AFM. Altogether, these analyses agree with the formation of a continuous biomimetic bilayer including the protein VDAC. Demonstrating the functionality of a protein reconstituted in a tethered lipid bilayer is not a trivial task; for example, electrical measurements on channel proteins require gigaohm seals on membranes that are very difficult to obtain with the liposome fusion procedure. In the case of VDAC, we took benefit of its channel activity and we measured the specific calcium uptake by the porin using a fluorescent dextran calcium sensitive probe, Rhod-dextran [molecular weight (MW) 10000; 552/581 nm]. The calcium probe was encapsulated in the aqueous compartment of the proteoliposomes or was inserted in the "trans" compartment of the POPC tethered bilayer containing VDAC. When trapped, the probe was able to measure the calcium fluxes. The calcium uptake by VDAC was efficient when VDAC was in proteoliposome or when reconstituted in the planar anchored biomimetic membrane (Fig. 3). As proof of activity, the calcium uptake was inhibited by two molecules known to block the channel activity of VDAC [4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) and L-glutamate]. The membrane thickness, fluidity, and continuity were ascertained by SPR, fluorescence microscopy, and atomic force microscopy measurements. VDAC reconstituted in tBLM efficiently transported calcium ions, and this transport was modulated by two channel blockers. This novel setup was further used for the study of the assembly of a polyprotein complex centered on VDAC and its role in mitochondrial biology, calcium fluxes, and apoptosis.

III. tBLM BASED TOOLKIT AS AN IN VITRO TRANSLOCATION DEVICE

The transport of proteins across membrane is a process that usually involves complex translocation machineries. 

![Fig. 2. Characterization of biomimetic tBLM-VDAC formation.](image)

![Fig. 3. Functional reconstitution of VDAC in tBLM.](image)
Many protein toxins from poisonous plants or from pathogenic bacteria are able to penetrate into the cytosol of their target cells where they exert their toxic effects. These toxins may exploit the endogenous cellular machinery of endocytosis and intracellular sorting to gain access to the cell cytosol, while others carry their own translocation apparatus. The latter offer a unique opportunity to analyze the molecular mechanisms and the physico-chemical principles underlying polypeptide transport across biological membranes.

Among bacterial toxins, the adenylyl cyclase toxin (CyaA), secreted by the causative agent of whooping cough Bordetella pertussis, is a pertinent example. CyaA intoxicates eukaryotic cells by a direct translocation of its N-terminal catalytic domain across the plasma membrane of target cells.\textsuperscript{44} The translocation of the adenylate cyclase (AC) domain across the plasma membrane is efficient even in the absence of the specific αMβ2 integrin cell receptor (CD11b/CD18) that helps to recruit CyaA on the cell surface.\textsuperscript{45–47} The CyaA toxin is a 1706 amino acid (aa) protein made of several structural and functional domains. The CaM activated AC domain corresponds to the first 400 N-terminal aa,\textsuperscript{48} followed by the translocation domain (TR, 400–500),\textsuperscript{49,50} and the 1206 carboxy-terminus residues are responsible for the hemolytic properties of the bacteria.\textsuperscript{51,52} After membrane insertion [Fig. 4(a)], CyaA transfers its AC domain directly into the cytosol. This event called the translocation process occurs at temperatures above 15°C (Ref.\textsuperscript{45}), is dependent on the presence of a cell membrane potential,\textsuperscript{52–55} and requires the presence of calcium in the millimolar range.\textsuperscript{45,56,57} Once translocated, the AC domain is activated by intracellular calmodulin (CaM) which binds with high affinity to AC (K_D below 1 nM) and stimulates its enzymatic activity more than 1000 fold to reach a high catalytic rate (k_{cat} > 2000 s^{-1}).\textsuperscript{58} This results in a marked increase in intracellular cyclic adenosine monophosphate (cAMP) concentrations and triggers cell death. The original protein translocation mechanism offers the opportunity to design and set up a tBLM based biomimetic construction that can be elaborated and tested using the unique properties of this protein.

The tethered membrane model that was previously developed and used for VDAC reconstitution delimits two compartments: an external “cis” compartment corresponding to the extracellular compartment and an internal trans compartment that can be considered to mimic cell cytoplasm. We addressed the possibility of including CaM in the trans compartment. CaM is indeed an intracellular ubiquitous marker and is also the activator of the CyaA toxin that could be used as a reporter for monitoring the translocation of the AC domain. The first step was to ensure the formation of an impermeable tBLM above a functional CaM layer [Fig. 4(b)].

CaM immobilization was performed using 1-ethyl-3-(3-dimethylaminopropyl)carbo diimide activation of carboxylates from amide bond formation with the amino coated surface in the presence of calcium that stabilizes the protein in its active conformation. SPR spectroscopy was used to monitor CaM layer formation and stability. The quantity of CaM covalently bound on the surface increased with the

![Fig. 4. CyaA binding and translocation assay on the tBLM/CaM assembly. (a) Structural organization of the functional domains of the 1706 residue-long CyaA toxin. AC: adenylate cyclase, calmodulin-activated catalytic domain, H: hydrophobic segments, and RD: calcium binding repeat in toxin domain. H and RD domains are responsible for the binding of the toxin, the hemolytic activity, and the translocation of the AC catalytic domain across cell membranes. (b) Scheme depicting the assay developed to monitor binding and translocation of CyaA toxin across the tBLM/CaM assembly using SPR and enzymatic activity measurements. (c) Scheme of the SPR cell used to validate the assembly of the tBLM/CaM system and monitoring the binding and translocation of CyaA toxin. The SPR cell was implemented with two electrodes (cis and trans) in order to apply an electrical potential across the membrane.](https://example.com/fig4)
concentration of injected CaM until a complete coverage of the surface for a density of 120 ng/cm² (corresponding to about 40,000 protein molecules/μm²) is reached. In a second step, the tBLM was assembled on the amine-coated surface derivatized with CaM. Lipid vesicles composed of 95% EggPC and 5% DSPE-PEG3400-NHS were deposited on the CaM covered surface. After 1 h, the surface was flushed with buffer to favor lipid vesicle rupture and fusion, leading to the formation of a continuous planar bilayer. The formation of the membrane on the surface was monitored by SPR, and its fluidity and continuity were characterized by FRAP. The formation of lipid structures on top of the CaM layer was found to be dependent on the CaM coverage densities. For CaM coverages below 60 ng/cm², the optical thickness and the mobility of the fluorescent lipid probes of the tethered membrane were similar to those obtained for tBLM and the mobility of the fluorescent lipid probes of the tethered membrane were similar to those obtained for tBLM reconstituted on amine layers alone. The assemblies obtained for higher CaM densities were heterogeneous as attested by optical thickness and FRAP measurements. According to these results, further biomimetic membrane assemblies were formed over the CaM layer with densities of about 40 ng/cm².

The main goal here was to setup a two distinct compartment biomimetic assembly, and therefore, the next step was to test the efficacy of the membrane boundary between the outside (cis) and inside (trans) compartments (containing CaM). To validate the membrane impermeability, we used enzymatic assay to monitor AC activation upon binding to the CaM layer in the presence of calcium. Experiments were performed with the isolated AC domain in the absence and in the presence of the membrane. In all these experiments, the enzymatic activity served as a reporter of the presence of active AC species. The amount of CaM and AC bound was monitored by SPR, while the enzymatic production of cAMP was measured using an indirect Pi colorimetric assay. Indeed, AC converts ATP into cAMP and PPI that can be further hydrolyzed by pyrophosphatase into two Pi. We first verified that AC was activated by the immobilized CaM layer (coverage density about 40 ng/cm²). As the detergent Triton X-100 is used to remove the membrane (see below), we also verify that Triton X-100 had no effect neither on the binding of AC to CaM nor on the AC activity. Finally, the insulating properties of the tBLM were tested by measuring AC activity for four different surface constructions: the amine layer, the CaM layer (about 40 ng/cm²), the tBLM over the CaM layer, and the tBLM over the CaM layer + Triton X-100 washing. In all cases, the last step before measuring AC activity consists of an extensive wash with 0.1% Triton X-100. After incubation of these four surface constructions with AC (48 and 240 nM), no activity was detected neither on the amine layer nor on the biomimetic membrane construction, while AC activity was measured similarly on the CaM layer and the Triton X-100 washed membrane construction. These experiments demonstrate that the tethered membrane assembled over the CaM layer is an effective barrier separating the bulk medium from the underlying compartment. The biomimetic design comprised of a tBLM assembled over a surface derivatized with CaM thus creates a protein impermeable lipid barrier, which separates two distinct compartments. This model was then challenged for monitoring the in vitro translocation of the full-length CyaA toxin.

The biomimetic membrane design tBLM/CaM was built in a homemade SPR cell with a 1 ml teflon chamber having inlet and outlet tubing, and the chamber was sealed by a gold coated glass slide on one side and a glass side on the other [Fig. 4(c)]. A silver wire (+) was introduced in the Teflon chamber, while the gold surface was used as the negative electrode. This cell was mounted on an optical SPR optical bench using the Kretschmann configuration [Fig. 4(c)]. As demonstrated in a preliminary study, when CyaA was injected on the top of the tBLM in the presence of calcium, it binds to tBLM as monitored by SPR measurements (Table I). After an extensive wash with a buffer containing the calcium chelator ethylene glycol bis(2-aminoethyl ether)tetraacetic acid, a large fraction of the bound CyaA remained attached to the tBLM (about 110 ng/cm²), likely due to the insertion of its hydrophobic segments in the bilayer. An extensive washing with 0.1% Triton X-100 removed almost all the membrane and associated protein as confirmed by the recovery of the ground levels of AC enzymatic activity that were measured in these conditions. This indicated that, in these experimental conditions, the AC domain of CyaA could not associate with the immobilized CaM, and thus, the membrane constitutes an impermeable barrier.

| TABLE I. tBLM/CaM construction and CyaA binding and translocation monitored by SPR. |
|------------------------------|----------------|----------------|----------------|
| **Experimental conditions**  | **1-CaM + CyaA** | **2-tBLM + TX-100 wash + CyaA** | **3-tBLM + CyaA** |
| CaM/tBLM assembly           | 34 ± 10        | 35 ± 5         | 35 ± 4         |
| tBLM mass (ng/cm²)          | N/A            | 540 ± 20       | 540 ± 10       |
| tBLM thickness (Å)          | N/A            | 54 ± 2         | 54 ± 1         |
| Triton X-100 wash (total mass ng/cm²) | 34 ± 10 | 60 ± 12 | N/A |
| CyaA binding                |                |                |                |
| Triton X-100 wash (total mass ng/cm²) | 236 ± 8 | 257 ± 15 | 684 ± 6 |
| Total mass (ng/cm²)         | 202 ± 8        | 197 ± 15       | 109 ± 6        |
| CyaA bound (ng/cm²)         | 232 ± 3        | 190 ± 12       | 77 ± 15        |

The binding of CaM and CyaA toxin (ng/cm²), the lipid anchoring (ng/cm²), and the membrane thickness (in Å) were monitored using SPR measurements. The optical thickness is registered between each step of the tBLM/CaM construction, the CyaA binding, and after Triton X-100 (TX-100) wash. The values presented are the mean ± standard error of the mean from at least three independent measurements.
In another set of experiments, CyaA was similarly injected over the tBLM/CaM construction in the presence of calcium. After binding/insertion into the tBLM as above and extensive washing to remove the unbound protein, an electrical potential of $-80 \text{ mV}$ was applied for 5 min between the two electrodes (gold and an Ag reference electrode were connected to the ground following convention, and the bulk medium potential was set to 0 mV). The membrane was removed with Triton X-100, and the SPR signal revealed a mass of about 200 ng/cm$^2$, compared to about 80 ng/cm$^2$ when the same experiment was performed in the absence of the application of electrical potential. This indicated that the CyaA protein was now bound to the immobilized CaM layer, after having crossed the membrane to reach the trans compartment. This was further confirmed by enzymatic measurements that detect a high adenylyl cyclase activity when a negative potential had been applied after CyaA binding to the tBLM/CaM. The application of a negative potential across the biomimetic membrane favors the translocation of the catalytic domain of CyaA that could interact with the immobilized CaM to acquire its enzymatically active form.

Using this well-defined experimental procedure, we investigated the efficacy of the translocation process as a function of the membrane potential. After binding of CyaA to the membrane, potentials from $-80$ to $+50 \text{ mV}$ were applied across the membrane for 5 min. The enzymatic activity bound to CaM was strictly dependent on the application of a negative potential across the bilayer. These data are in agreement with the voltage dependence of the CyaA intoxication of myocytes reported by Otero et al. These results attest that the in vitro translocation assay reproduces the fundamental properties, i.e., calcium and voltage dependence governing CyaA intoxication of targeted cells.

To evaluate and validate the in vitro translocation assay, different control experiments were realized.

1. We first applied the proteolytic protection assay commonly used to measure CyaA translocation in erythrocytes and other cells to our in vitro translocation assay. After the CyaA binding and application of the same range of voltages previously used ($-80$ to $+50 \text{ mV}$) on the trans side of the tBLM followed by a trypsin treatment, the membrane was removed using Triton X-100 and the adenylyl cyclase activity was measured. As the control experiment, trypsin was also added before the application of a potential to ensure the accessibility of the AC domain before translocation. The results obtained confirm that after membrane binding of CyaA, the AC domain was exposed to the cis compartment, accessible to protease. After the application of negative voltage, AC activity was measured, demonstrating that the AC domain became inaccessible to protease digestion after translocation across the lipid bilayer. This protease sensitivity assay also confirmed the bilayer integrity for voltage between $-80$ and $+50 \text{ mV}$ (as the protease had no access to the underlying compartment). When higher voltages ($>+50 \text{ mV}$) were applied, defects in the lipid bilayer started to be apparent as the protease could now get access to the CaM layer and inactivate AC.

2. Next, we used a blocking monoclonal antibody (mAb 3D1) that is known to recognize an epitope located between the residues 373 and 399 of the CyaA toxin and to inhibit the delivery of the catalytic domain in the cytosol of targeted cells. While binding of CyaA on our tBLM was not affected by coincubation with mAb 3D1, the AC activity measured after the application of a negative potential in the translocation assay was significantly diminished. These results are similar to those observed previously in vivo and demonstrate the potential of our model to study this specific translocation mechanism.

3. CyaA toxicity is also dependent on the acylation of K860 and K983; indeed, the nonacylated protein proCyaA is known to be unable to perform AC translocation. To further validate our lipid bilayer model, we then tested proCyaA using the in vitro translocation assay. When compared to the results obtained with CyaA, the AC activities measured for proCyaA in translocation conditions were in the background as previously observed in vivo.

These three types of control experiments confirm the validity and the robustness of the in vitro translocation assay that we developed using the tBLM/CaM, two-compartment setup. In this setup, AC translocation across the tBLM (designed to mimic the plasma membrane) was clearly demonstrated to be dependent only on the presence of calcium ions and upon application of a negative potential without requiring additional components. Moreover, the CaM protein layer present in the trans compartment is able to stimulate AC catalytic activity. This tBLM/CaM multilayered biomimetic assembly exhibits the fundamental characteristics of an authentic biological membrane in creating a continuous yet fluid phospholipidic barrier between two distinct compartments: a cis side corresponding to the extracellular milieu and a trans side marked by CaM, a ubiquitous cytosolic signaling protein. This modular in vitro biomimetic system offers the advantage of permitting the analysis of protein translocation across the tethered membrane under highly controlled conditions. To our knowledge, this is the first in vitro system ever reported for the characterization of protein translocation across synthetic tethered bilayer membranes. This strategy may be used for the functional and biophysical characterization of the molecular mechanisms governing the translocation of various toxins and proteins.

**IV. SUMMARY AND CONCLUSIONS**

In the last few decades, the study of molecular mechanisms associated with biological membranes is benefited from the development of various biomimetic membrane models that help for the investigation of these machineries. These models, ranging from a simple lipid monolayer to a supported lipid bilayer and a more complex tethered lipid bilayer, have been widely used to study membrane protein
and protein complex reconstitution, toxin/membrane interactions, and ion channels, among other. However, despite the development of numerous biomimetic membrane models, these are in general designed for a particular application and only a few of them are versatile enough to be easily modified to study different systems.

Here, to overcome the limitations of the existing models, we have developed a versatile tBLM model that can be applied to study a large number of membrane proteins and that can be adapted to study complex mechanisms like protein translocation across membranes. This tBLM model was assembled on various supports (e.g., glass and gold surface) modified with an amine layer for lipid bilayer reconstitution using vesicles containing anchoring lipids. This system was then modified to insert a protein layer below the lipid membrane in order to mimic the intracellular compartment. The lipid bilayer thus reconstituted has been fully characterized and shows the characteristics of a continuous lipid bilayer membrane.

To demonstrate the utility and the versatility of this biomimetic model, we reconstituted and studied two different molecular mechanisms. In a first example, the direct reconstitution of a voltage dependent protein channel, VDAC, was performed using proteoliposomes containing the purified protein to assemble the lipid bilayer. The VDAC protein reconstituted was shown to be fully functional and able to transport calcium ions for 3 h. Moreover, the VDAC channel activity could be modulated by two known in vivo inhibitors. In a second example, the tBLM system was further developed to integrate a protein marker in the internal compartment, namely, calmodulin, a ubiquitous intracellular marker. After demonstrating the impermeability of the lipid bilayer assembled above the CaM layer, we demonstrated the possibility to study and monitor the toxin interaction with the membrane and to reconstitute the translocation mechanism of the CyaA toxin. Therefore, we have shown that our system reproduced the conditions required in vivo for the translocation of the toxin CyaA (membrane potential and the presence of calcium ions in the millimolar range).

With this new modular tBLM system, we develop further the promise of the biomimetic membrane model by proposing an easy solution to study a large panel of membrane proteins and the mechanism associated in a minimal and extremely controlled environment. This system could be easily modified and adapted to the study of other proteins and toxins by simply changing the reporter protein layer and adapting the lipid composition.

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