applied sciences

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Review

Why Do Tethered-Bilayer Lipid Membranes Suit for Functional Membrane Protein Reincorporation?

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Abstract: Membrane proteins (MPs) are essential for cellular functions. Understanding the functions of MPs is crucial as they constitute an important class of drug targets. However, MPs are a challenging class of biomolecules to analyze because they cannot be studied outside their native environment. Their structure, function and activity are highly dependent on the local lipid environment, and these properties are compromised when the protein does not reside in the cell membrane. Mammalian cell membranes are complex and composed of different lipid species. Model membranes have been developed to provide an adequate environment to envisage MP reconstitution. Among them, tethered-Bilayer Lipid Membranes (tBLMs) appear as the best model because they allow the lipid bilayer to be decoupled from the support. Thus, they provide a sufficient aqueous space to envisage the proper accommodation of large extra-membranous domains of MPs, extending outside. Additionally, as the bilayer remains attached to tethers covalently fixed to the solid support, they can be investigated by a wide variety of surface-sensitive analytical techniques. This review provides an overview of the different approaches developed over the last two decades to achieve sophisticated tBLMs, with a more and more complex lipid composition and adapted for functional MP reconstitution.

Keywords: biomimetic membranes; tethered-Bilayer Lipid Membranes; membrane proteins

Citation: Girard-Egrot, A.P.; Maniti, O. Why Do Tethered-Bilayer Lipid Membranes Suit for Functional Membrane Protein Reincorporation?. *Appl. Sci.* **2021**, *11*, 4876. https://doi.org/10.3390/app11114876

Academic Editor: Domenico Lombardo

Received: 27 April 2021
Accepted: 22 May 2021
Published: 26 May 2021

1. Introduction

Cellular membranes, and more particularly the plasma membrane, are of upmost importance in the living cells. Hosting a vast plethora of proteins, plasma membrane not only serves as a physical boundary, but also mediates exchange processes between the cell and the extracellular matrix. Cellular membranes are also essential inside the cell. They aid the different organelles to carry out their cellular functions. Furthermore, many vital biochemical processes essential for cell life are managed by the biological membranes.

Only a few nanometers thick, biological membranes are very complex in terms of composition but exhibit a perfect organization at the molecular level [1]. Lipids, held together by hydrophobic interactions, play a structural role by forming a continuous self-assembled bilayer acting as a passive diffusion barrier. Proteins associated with the membrane, either transmembrane proteins or peripheral membrane proteins, respectively embedded within the lipid bilayer or transiently associated with it, represent ~30% of the open reading frames in complex organisms [2]. Due to their abundance, they are the key factors of the cell metabolism, involving cell–cell communication, cell adhesion, nutrient import, signal transduction, biocatalysis processes, energy production and others [3]. As a result, membrane receptors are currently the target of over 60% of medicinal drugs [4,5].

Nowadays, cell membranes are no longer considered as a simple double lipid layer but as a set of complex and dynamic protein–lipid structures and segregated microdomains, that serve as functional spatiotemporal platforms for the interaction of lipids and proteins involved in cellular signaling pathways [6–10]. The membrane composition, and therefore

the overall function of the cell membranes, is altered in a wide range of human diseases, including cancer, neurodegenerative disorders, cardiovascular pathologies, obesity, etc. A lipid alteration can affect the localization and activity of transmembrane proteins and thus impact on the intracellular cell signaling. From this belief, a new concept of membrane lipid therapy (MLT) has emerged [11,12] with the idea that lipid components of biological membranes can also be selectively targeted to induce membrane disorder and reverse the malfunction [13]. This approach now represents a target of choice for pharmaceutical companies [14]. Hence, investigating membranes and membrane proteins (MPs), including lipid–lipid, protein–lipid or ligand–protein receptor interactions, is of critical importance. However, due to their complexity, in situ investigations to unlock the secret of the biological membranes remains a great challenge. In this respect, the development of artificial models that mimic the cell membrane by constitution and composition, is an asset to study biological membrane properties.

In the plasma membrane, hundreds of different lipid species can be found. For instance, some of them have a negative charge, which can promote interactions with positively charged amino acids in proteins [11]. Depending on the size of their polar head group, certain lipids allow docking of bulky protein lipid anchors or form tightly packed areas to help some membrane proteins to bind to regions where these lipids are abundant. It is now well-accepted that the membrane lipid composition may have a profound role in membrane functioning and cell signaling [13]. In this respect, the crucial role of non-bilayer lipids present in large amounts in biological membranes on the MP activities must be underlined [15,16]. Conversely, reconstitution of functional membrane proteins after in vitro production or purification is challenging. Due to their amphiphilic nature, they are prone to early denaturation during in vitro handling. To properly evaluate their functionality, they require a native lipid environment. Ideally, MPs should be reconstituted in natural lipid extracts as it is now well-known that lipids in the immediate vicinity of membrane proteins influence their activity [13,15,17]. As a result, there is a great need to develop biomimetic membrane platforms, in which, not only one but several purified membrane lipid components can be used for in vitro reconstitution, and in which reincorporated membrane proteins can retain their structural integrity and functional activity.

Different types of models have been developed through the years to mimic cell membranes as well as possible and reproduce the basic functions of cell membranes. These models are solid-supported lipid membranes [18,19], polymer-cushioned membranes [20–22], hybrid lipid bilayers [23–25], free-standing lipid layers or suspended-lipid bilayers [26–28] and tethered-bilayer lipid membranes or tBLMs [29–39]. All these models are suitable for systematic studies of different types of membrane-related processes and provide the lipid environment required for the study of membrane-associated proteins. They correspond to models of planar membranes confined to a solid support and localized at the bulk interface, allowing the application of a manifold of surface-sensitive techniques for their own characterization [40] or biosensing applications [41–43].

Besides all these advantages, tBLMs appear as very attractive platforms for the reinsertion of transmembrane proteins. Because they are lifted from the support, they best mimic the cellular environment, and transmembrane proteins with protrudant domains extending outside the membrane can “comfortably” reinsert into the bilayer without steric hindrance or loss of mobility due to a close contact of the membrane with the support [44].

The current review focuses on the design of tBLMs suitable for reinsertion and characterization of MPs. After a brief overview of all the techniques of characterization, we will describe the different models of tBLMs including polymer-tBLMs, anchorlipid-tBLMs, peptides-tBLMs, and other modes of attachment like avidin–biotin systems. Then, we will focus on the attached-protein-tBLMs, which allow the protein to be oriented in a lipid bilayer, to finally finish on the advantages of cell-free expression for reinsertion of membrane proteins before or after formation of tBLMs.

2. Design of Tethered-Bilayer Lipid Membranes

tBLMs are a natural progression from the planar supported lipid bilayers (SLBs), first reported by McConnell et al. [19]. SLBs, classically obtained by the spreading of small unilamellar vesicles on hydrophilic solid supports [45–47], including glass, mica, titanium and silicon oxides, or gold (for recent reviews see articles by Lind & Cardenas 2016 [48] and Clifton et al. [40]), consist in a lipid bilayer deposited and separated from the solid substrate by an ultrathin film of water (0.5–2 nm) [45,49–51]. This aqueous layer acts as a lubricant and confers to SLBs the fluidity required for lateral diffusion in 2D space [45]. In this model, lateral and rotational mobility of individual lipids are preserved and anything linked to the phospholipids or glycolipids in the upper leaflet retains this mobility [52]. Given this key feature, SLBs have been used extensively over the past decades to study the spatially and temporally regulated lipid–lipid or lipid–protein lateral interactions [53–56], lipid segregation [57–59], protein clustering and cell adhesion [60–63] and membrane dynamics [64–66].

However, the close proximity of SLBs to the substrate affects the diffusion of lipids and proteins, which is more than twice slower than in free-standing bilayers under the same conditions [67]. This limitation is due to the fact that the substrate exerts a greater influence on the behavior of the proximal leaflet than the distal leaflet of the SLBs, due to its closer proximity to the surface [40,68,69]. The roughness of the substrate and the complementarity between the surface and lipid charge will determine the magnitude of this surface influence [50]. Furthermore, SLBs suffer from the crucial drawback of not possessing a large hydration reservoir on both sides of the membranes, which limit examination of transmembrane proteins. The fundamental requirement for a membrane protein to function properly is to be surrounded by buffered-saline solution on both sides of membranes [70]. In SLBs, hydration layer is often not thick enough for proper folding of large extra-membranous domains of transmembrane proteins, which can extend to several tens of nanometers far out from the bilayer [18]. The limited membrane-substrate distance, which can lead to strong non-physiological interactions with the solid support [18,20], can cause both a loss of protein dynamics and a partial loss of its functionality, or even complete denaturation of the protein [71–73]. In addition, anionic substrates (such as quartz, mica, silica, silicon oxide) may hinder (in the absence of divalent cations) the formation of SLBs enriched in negatively charged lipids in the proximal leaflet, due to electrostatic repulsions [46,66,74]. However, negatively charged lipids, like phosphatidylglycerol (PG), phosphatidylinositol (PI), cardiolipin (CL) or even lipopolysaccharide (LPS) in the Gram-negative bacteria membrane, are important signaling lipids which can trigger membrane protein association and affect membrane-regulated pathways. They are essential in membrane function, and studies of the membrane phenomenon regulated by these lipids are becoming crucial for a realistic understanding of membrane-related events. The use of mimetic sample systems with ever greater biological precision in lipid composition is now required.

For all these reasons, more advanced planar model membranes are currently in development with the aim to create more accurate biomimetic systems adapted for integral (trans-)membrane protein characterization, where the substrate interactions are minimized and large solution reservoirs on both sides of the bilayer are provided. In tBLMs, the lipid bilayer is separated from the surface of the substrate by insertion of a soft and flexible hydrophilic layer of "tethering" molecules that anchor the proximal leaflet [31,34,71,72,75,76]. This layer solves the substrate proximity by lifting the membrane off the surface and provides a reservoir underneath the bilayer in which the membrane proteins can fold into a native-like conformation, while keeping the membrane anchored to the support (Figure 1).

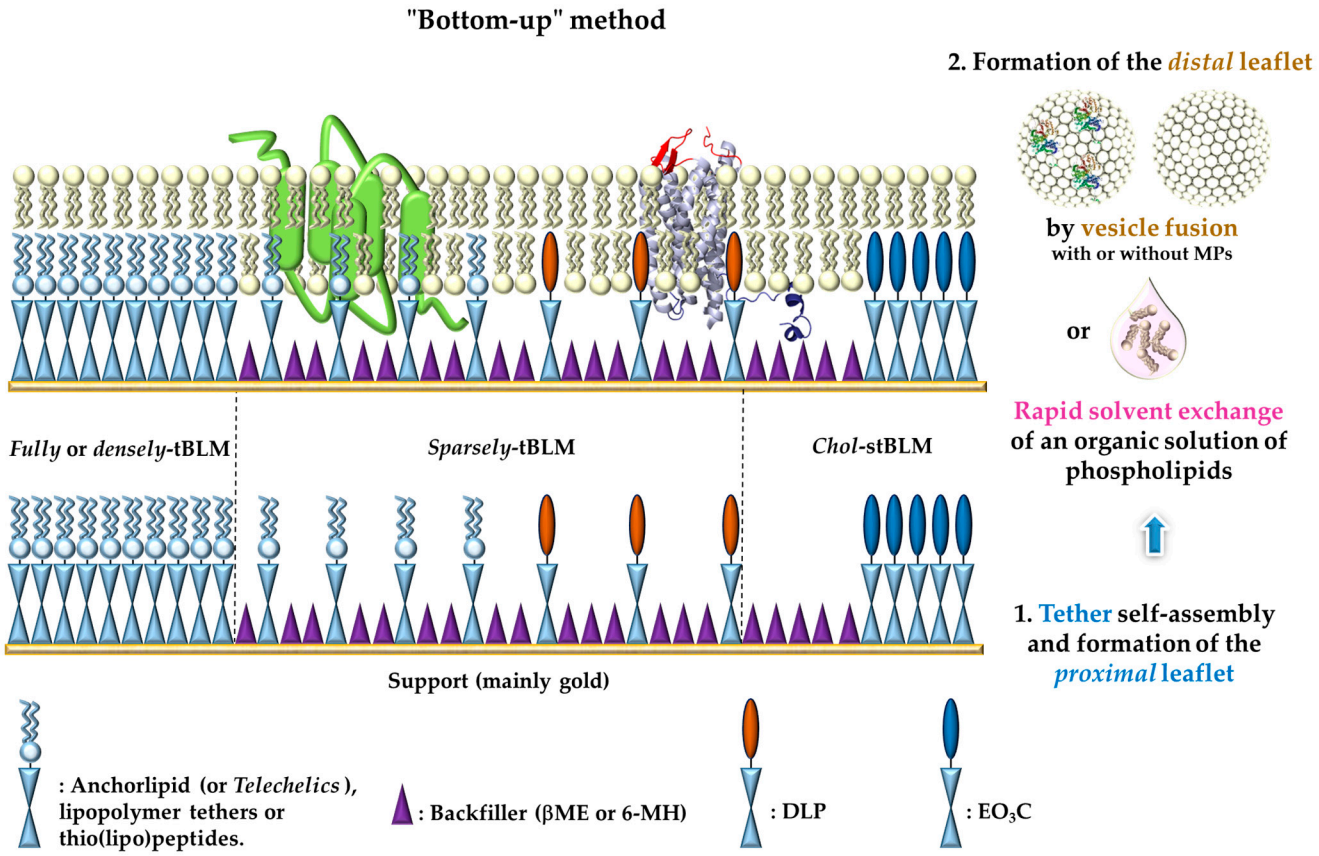

Figure 1. Different types of *tethered-Bilayer Lipid Membranes* (or tBLMs) obtained by a "bottom-up" approach. : represents either anchorlipid (i.e., Telechelics) mainly 2,3-di-*O*-phytanyl-*sn*-glycerol-1-tetraethylene glycol-D,L-α-lipoic acid ester lipid (DPhyTL), lipopolymer tethers or thio(lipo)peptides; : Backfillers, mainly β-mercaptoethaonl (βME) or 6-mercaptohexanol (6-MH); : benzyl-disulfide (tetra-ethyleneglycol)$_{n=2}$ C20-phytanyl tether (or DLP); : ethyleneoxy-linked cholesterol (or EO_3C).

The large variety of assembling molecules capable of forming a tethering layer offers multiple possibilities for fine-tuning the properties of tBLMs [37]. Whatever its proper nature, the role of the anchor (spacer group) is multiple. It should at the same time (i) maintain bilayer fluidity and provide a sufficient well-hydrated sub-membrane space to accommodate incorporated proteins, (ii) cover small surface roughness features in order to reduce the hydrophobic influence of the metal surface and the unfavorable frictions to the support, (iii) provide a hydrated reservoir between the substrate and the membrane, and (iv) supply ample space to harbor membrane protein ectodomains. The different types of tBLMs vary mainly in the chemical structure of the tethers and in their density, two factors significantly influencing the structural characteristics of the bilayer as well as the functional reincorporation of membrane components [51]. Ideally, and in order to mimic a natural membrane, a tBLM should have a high electrical impedance and a low capacitance—to be sure that transport across the membrane is mainly due to the function of the embedded protein, as well as high fluidity and high sub-membrane hydration—to ensure protein function. However, increases in membrane hydration and fluidity are generally accompanied by a reduction of the electrical sealing properties, resulting from a higher defect density [77,78]. Subsequently, applying ultrasensitive surface imaging techniques allowing the direct characterization of all the steps of the tBLM formation

with a high lateral resolution could lead to an optimization, step-by-step during the tBLM building, in order to reduce the number of defects.

3. Characterization of Tethered-Bilayer Lipid Membranes

Because they are firmly held in place, tBLMs are considerably more robust than supported lipid bilayers such as black or bilayer lipid membranes (BLMs) [79,80], also renamed free-standing lipid layer or suspended-lipid bilayers, which are originally formed across on a microsized-aperture, and more recently, on nanopores [26–28]. Generally speaking, tBLMs typically show a high robustness and long-term stability and hence, they are accessible to a portfolio of different analytical tools operating at a bulk interface [42]. They include imaging techniques, like *atomic force microscopy* (AFM) [30,81–87] and *fluorescence microscopy* (FM) [34,87–89], *fluorescence recovering after photobleaching* (FRAP) [20,35,81,87,90–93], *neutron reflectometry* (NR) [77,94–101] and *X-ray photoelectron spectroscopy* (XPS) [20,82,86,87,102,103], spectroscopic techniques such as *ellipsometry* [94,104], *infrared reflection absorption spectroscopy* (IRRAS) [96,105,106] or *surface-enhanced infrared absorption spectroscopy* (SEIRAS) [107–110], *surface plasmon resonance* (SPR) [30,34,35,37,39,111–113] or *quartz crystal micro-balance with dissipation monitoring* (QCM-D) [30,39,85,112], as well as electrochemical methods such as *electrochemical impedance spectroscopy* (EIS) [24,32,41,88,94,108,109,114–117] and current-voltage (CV) analysis [118–123]. These techniques are sensitive to net changes in packing or interfacial mass (QCM-D or ellipsometry), bilayer morphology (AFM, FM), the presence of chemical groups (IR, XPS), structure and composition (NR) have been used so far to evaluate the full picture of the lipid membranes (i.e., structure, composition and functional properties) and represent a very powerful combination to unravel the mechanism of biomolecular interactions.

While SPR and QCM-D allow real-time monitoring of the tBLM formation in a label-free format, fluorescence microscopy (FM) and FRAP investigate domain morphology and membrane dynamics with the measurement of the lateral diffusion of lipids, respectively. AFM has been used to gather surface details in terms of occurrence of peculiar structure and defects. One of the unique features of this latter technique is that it can measure surface forces with a nanometer lateral resolution. Recently, single-molecule force spectroscopy (FS) measurements have provided in-depth insight to assess the orientation of reconstituted transmembrane proteins in tBLMs [86]. NR also provides high resolution structural information on lipid bilayer stacking and internal distribution of components after interaction between intrinsic proteins and disordered membrane [124]. EIS is an excellent tool to characterize the electric properties of membrane including resistance and capacitance.

This large panel allows a fine characterization of tBLMs during and after their formation, in terms of structure, (optical or acoustic) thickness, fluidity and sealing [125]. It shows that the chemical nature of the sub-membrane space has a significant impact on both the structure of the lipid bilayer and the functional incorporation of membrane components [51]. The possible combination of multiple complementary measurements with biologically accurate samples is key for a realistic understanding of membrane related phenomena. Only through the use of complementary techniques, such as the ones hereby mentioned, does it become a realistic aim to resolve the relative position, orientation and distribution of the membrane components to obtain detailed information on molecular mechanisms by which peptides, proteins or other chemical compounds (e.g., drugs) interact with biomembranes. Table 1, adapted from Rossi and Chopineau [126], Sondhi et al. [70], Clifton et al. [40], presents a synoptic of the characteristics of all the techniques useful for the study of supported planar membrane models, including tBLMs. For more details, see the recent review by Clifton et al. [40], which presents the main information that can be deduced from model membranes due to the different surface-sensitive techniques listed above.

Table 1. Characteristics of main surface-sensitive analytical techniques useful for investigation of supported planar model membranes included tBLMs (adapted from [40,70,126]).

Techniques	Bilayer Characterization	Surfaces
Surface plasmon resonance (SPR) imaging	Optical thickness of the bilayer, highly sensitive real-time monitoring of interactions without labeling of the analytes or the ligand, real-time monitoring of bilayer formation	Gold, silver, aluminum
Quartz crystal microbalance with dissipation (QCM-D)	Interfacial wet mass determination and viscoelasticity (dissipation sensitive to viscoelastic properties of the adsorbed material), (acoustic) film thickness, real-time monitoring of bilayer formation	Gold, SiO_2, mica, metal oxides
Imaging ellipsometry (IE)	Indirect quantitative characterization of structural and functional properties of bilayers such as thickness and dry adsorbed mass (i.e., lipids in the adsorbed layer), anisotropy (lateral uniformity and phase separation), molecular area, and receptor-protein interaction affinities. Real-time large area imaging with high sensitivity	Oxide (silicon) substrates
Fluorescence recovery after photobleaching (FRAP)	Dynamics, fluidity, and mobility characterisation of lipids and proteins (peripheral or integral), intergrity of artificial membranes	Optically transparent substrates: glass, silica, silcon, gold
Electrochemical impedance spectroscopy (EIS)	Electrical properties (resistance and capacitance) of lipid bilayer membranes, formation process in real-time, stability of the membrane, characterization of incorporated ion channels	Gold, silicon
Atomic force microscopy (AFM)	In-plane structure and morphology: surface roughness determination, investigation of bilayer surface at the nanoscale range in real-time and in aqueous environment, direct measure of physical properties at high spatial resolution, phase separation (domain formation) and quantification of bilayer thickness	Atomically flat surfaces: mica, silicon, quartz, flat gold
(AFM) single-molecule Force Spectroscopy (FS)	Membrane stiffness and mechanical stability on the nanometer length scale, in-depth insight of the orientation of reconstituted transmembrane proteins	Mica, silicon, quartz, flat gold
Neutron Reflectometry (NR)	Non-damaging technique giving high structural information on lipid bilayer and internal distribution of components (lipid or protein) within the bilayer (thickness of stratified layers normal to the interface), roughness and interaction with inserted proteins (easy differentiation of lipid and polypeptide components across the membrane structure after interaction)	Gold, silicon
X-ray photoelectron spectroscopy (XPS)	Provides quantitaive analysis of elemental composition of a surface and its chemical state	Quartz
Grazing incidence small angle neutron or X-ray scattering (GISANS and GISAXS)	Non-destructive method for the structural investigation of biomembranes and mixed lipids systems with different topologies	Performed in quartz glass

4. Different Types of Tethered-Bilayer Lipid Membranes

As seen above, the membrane–substrate distance in SLBs is usually not sufficiently large to avoid direct contact between transmembrane proteins incorporated in the membrane and the solid surface. This problem is particularly serious when working with membrane receptors, whose functional extra- and intra-cellular domains can extend to several tens of nanometers.

Several strategies have been used to achieve "perfect" tBLMs. Their difference is based on the nature of the tethering molecules which are polymer cushion [39,127–130], anchor-lipids [32,38,101], peptides [71,131] (Figure 1) or proteins [81,112,132–134] (see Section 4.6). Several excellent recent reviews have already described these different tethered mod-

els [42,51,125]. In the present contribution, we will discuss on specific advantages and disadvantages of each of them in the field of membrane protein reconstitution.

4.1. Polymer-tBLMs

To overcome the problem of the close proximity between membrane and support, polymer-cushioned membranes have been developed, pioneered by Sackmann's group [21]. This model allows separating membrane from the solid substrate using a soft polymeric material that rests on the substrate and supports the membrane [127]. The hydrated polymer used to "cushion" or "tether" the supported membrane acts as a lubricating layer between the membrane and the substrate. This approach significantly reduces the frictional coupling between the transmembrane-incorporated proteins and the solid support, and hence the risk of protein denaturation. Depending on the type of fabrication method and polymer system used, the addition of the polymer layer can result in bilayer-substrate distances of 5–100 nm, making these model membranes suitable for the analysis of transmembrane proteins.

Several different strategies for assembling lipid bilayers supported by a polymer have been previously pursued [127,128]. One relatively straightforward fabrication approach relies on the application of polyelectrolytes as cushion materials and the subsequent addition of a bilayer containing a certain fraction of lipids with oppositely charged headgroups. The attachment of the bilayer on the surface of polyelectrolytes using attractive electrostatic interactions can be combined with the established method of layer-by-layer (LbL) deposition of polyelectrolytes, which provides flexibility in terms of accessible polymer cushion thicknesses. However, the presence of polyelectrolytes in electrostatically stabilized polymer-supported lipid bilayers may have a disturbing effect on the properties of reconstituted membrane proteins [60].

An alternative method has emerged consisting of the stable attachment of the lipid bilayer to the polymer cushion by tethering. In the resulting supramolecular assembly, referred to as *polymer-tethered lipid bilayers*, lipids with macromolecular head groups, so-called *lipopolymer tethers*, are incorporated into the lipid bilayer [33,34]. In this system, the proximal lipopolymer layer is generally first attached to support by chemisorption and the bilayer is completed with a distal lipid leaflet, formed either by vesicle fusion or Langmuir-Schaefer transfer, in a two-step preparation process also called the "bottom-up" method (for details, see [60,128]). The polymer head groups act as spacers that control the substrate–membrane distance and, in common with polymer cushions, prevent direct contact between extra-membranous transmembrane protein subunits and the bare substrate surface [60]. The ability to flexibly adjust both the length of the polymer spacer and its lateral density allows fine adjustment of the membrane-substrate distance and the viscosity of the polymer layer, both of which control the lateral diffusivity and function of transmembrane proteins. Thus, the separation distance in polymer-tBLMs is enough to host transmembrane proteins [20]. Additionally, it was shown using *polarization modulation-infrared reflection absorption spectroscopy* (PM-IRRAS), a surface sensitive technique applicable to thin film [135], that polyethylene glycol (PEG), conventionally used as the tethered-polymer, adopts an amorphous, disordered conformation, which promotes hydration of the inner layer essential for the proper functioning of reinserted transmembrane proteins. It forms a water-rich region between the bilayer and the metallic surface with a water content up to 90 $\pm$ 3% as determined by NR [97]. However, the nature and the density of the attached lipopolymer may significantly impact the membrane properties. In particular, the lateral diffusion of both free lipids and embedded membrane proteins can be hindered by a tethering density greater than 4–5% to 10% depending on the nature of the lipopolymer [20,91,136], with the possible presence of motionless fractions [90]. This slowing-down effect of the immobilized tethers on the dynamics in the tethered bilayers has been confirmed by molecular dynamics simulation that revealed that the lateral diffusivities of both the proximal and distal layers are lower than for freestanding bilayers with PEG [78]. In addition, the lipid bilayer (membranes) formed on polymers often contains

holes and defects [91]. A high grafting density and a long tether length induce undesired conformations, such as undulated tBLM systems, tether detachment, and even pores in the bilayer [78], which makes polymer-tBLMs generally less suitable for use as bio-detection platforms: the polymer acts as a passivation layer, which can greatly degrade the detection sensitivity on a noble metal such as gold [137], widely used in surface analysis methods (see Table 1), and the lipid membrane does not have sufficient electrical sealing properties for the investigation of ion transport process and quantitative characterization of ion channel activities [42,51]. For these reasons and despite their potential attractiveness, only a few successful reincorporation of integral MPs has been achieved in polymer-tBLMs formed by "bottom-up" approach (i.e., using proteoliposomes for fusion on preformed proximal lipopolymer layers during bilayer formation). They mainly relate to *cytochrome* b_5 [20], *t-SNARE receptor*, a central player in membrane fusion in exocytosis and intracellular vesicle transport [138,139], *bacteriorhodopsin* [136], *human platelet integrin receptor* for modelling of cell adhesion processes [140], and the multi-drug resistance protein 1 (MDR1) for which drug interaction in vitro assays (pravastatin) have been carried out [141]. It is likely that the two-step process of forming, one after the other, the two membrane leaflets is unfavorable for the preservation of functional MP structure during bilayer formation and hence, a new "top-down" approach have been developed (see below Section 4.5).

On the other side, polymer-tBLMs are able to respect the deformability or the natural fluctuation of the membrane and they can provide a more natural and cell-like surrounding than a solid surface [97]. By modifying the lipopolymer into "lipoglycopolymer", it becomes possible to reproduce by mimicking, the cytosol or cytoskeleton of the cell [142]. For these reasons, polymer-cushioned and polymer-tBLMs have been widely used, as models of the extracellular matrix or the cell surface glycocalyx [127]. More recently, they have been further developed in *polymer-tethered lipid multi-bilayers* formed by a stack of three or four bilayers separated from each other by covalently attached PEG molecules in surface of each membrane. This multi-bilayer stacking results in a lesser influence of the tethered-lipopolymer than in polymer-tBLMs. Bilayers within stacks are free of optically visible defects and show a good stability. Lateral diffusion can be adjusted by the number of bilayer in the stack, with a correlated gradual increase of lipid diffusion with the degree of bilayer stacking [60]. This tunable diffusion and mechanical properties made such multi-bilayers systems cell surface-mimicking materials with well-defined surface properties for the analysis of cellular mechanosensitivity with advanced breakthroughs in the understanding of cell adhesion and cell migration, as recently shown for polymer-tethered lipid multi-bilayers functionalized with N-cadherin linkers [143] (for details see [60]). However, such applications of polymer-tBLMs is out of the scope of this review focused on the membrane reincorporation of functional integral membrane proteins and therefore they will not be discussed further.

4.2. Anchorlipid-tBLMs

Anchorlipids, also called *telechelics*, were the first to be used for tethering a lipid membrane [31]. The most widely used correspond to thiolipids. *Telechelics* are composed of three distinct molecular segments: (i) an amphiphile that becomes part of the proximal monolayer of the final bilayer structure (i.e., anchorlipid), (ii) an hydrophilic spacer unit, the "tether" that decouples the bilayer from the substrate, (iii) a substrate-specific head group (i.e., a chemical linker to surface), for instance based on thiol, disulfides, lipoic acid, silane, or alike for grafting on gold, silver, silicon oxide, metal supports or mercury electrode [24,32,71,72,76]. Anchorlipids are mainly archaea analogues thiolipids with two phytanoyl chains that improve the fluidic (hence, sealing) character of the hydrophobic core and a polyethylene glycol as spacer [30,38,77,84,87,101,102,104–106,144–151]. The hydrophobic moiety of the anchor can also be either saturated C14 (myristoyl) [94,124,152,153] or C16 (palmitoyl) [85,154], or unsaturated C18:1 (oleoyl) acyl chains [96]. As it is also an essential component of mammalian membranes, cholesterol-based anchors have been

synthesized [89,155–158]. The chemical structure of each of the different anchorlipids mentioned above is detailed in a recent review [125].

The fabrication of an *anchorlipid-tBLMs* is in most cases a two-step process, i.e., a "bottom-up" method: it begins with the formation of a self-assembled *telechelic* lipid derivative, covalently linked to the support, thus forming the proximal layer of the final architecture and the second step comprises the formation of the phospholipid bilayer by adding the distal layer on the top of the hydrophobic surface exposed to the bulk aqueous phase. The latter step can be achieved in several ways, such as the well-known Langmuir–Blodgett technique [159], rapid solvent exchange of an organic solution of phospholipids [41,77,88,94,99,154] or self-directed fusion of vesicles upon interaction with the substrate [30,32,84,95,101,104,144]. The last mentioned is the most commonly used technique as it is best suited to handle protein-containing vesicles. It is further expected to allow the incorporation of MPs by fusion of vesicles that might contain reconstituted proteins.

In another approach, Becucci and Guidelli's group has proposed to form the distal phospholipid monolayer by simply immersing a thiolipid-coated mercury drop in an aqueous electrolyte on the surface of which a lipid film has been previously spread. Thanks to the hydrophobic interactions between the alkyl chains of the thiolipid and those of the lipid, this simple procedure gives rise to a lipid bilayer anchored to the mercury surface via the hydrophilic spacer moiety of the thiolipid. By avoiding the use of vesicles, this procedure excludes any artifacts due to partially fused vesicles and guarantees good seaming properties to the bilayer [146,148]. This approach has been intensively used to study spontaneous interaction/incorporation from the aqueous solution, of diverse membrane-active peptides presenting phytotoxic, antifungal and anti-bacterial activities [147,160–163].

In contrast to polymer supported membranes, thiolipid anchored-tBLMs typically exhibit a high long-term stability and a high electrical resistance value [51] because the proximal leaflet consists entirely of anchor molecules, i.e., it contains no (or minimal) free phospholipids [88]. For these reasons, they have been extensively used to study small membrane-active peptides, such as antimicrobial peptides [149,150], pore-forming toxins such as gramicidin [41,104,123,164] or large oligomeric α-hemolysin [165,166], ion channels [167], such as valinomycin [95,104,106,114,164,166,168,169] or ligand-gated ion channels [170]. They have been also used as a bilayer platform to study the structure of the *human immunodeficiency virus type* 1 (HIV-1) *Gag protein* as well as its interaction with lipid bilayers [171], to mimic the outer membrane of Gram-negative bacteria which can cause some of the most dangerous multi-drug resistant infections [150,151,172], to investigate membrane incorporation from aqueous solution of bulky proteins such as *OmpF* porin from *E. coli* [173] and micelles of Triton X-100 solubilized *human ether-a-go-go-related gene* (hERG) *potassium channel* [174], or for biosensing applications [41,42,166].

Nonetheless, in most cases, the chemical structure of the tethers has a significant impact on the structure of the resulting lipid bilayer [166] and the functional incorporation of membrane components. For the important class of anchorlipids, i.e., *telechelics*, neutron scattering [32,77] and PM-IRRAS studies [105] have revealed a poorly hydrated spacer segment despite polyethylene glycol (PEG) being water soluble, due to a coiled (helical) conformation exposing the hydrophobic carbon segments of the ethylene glycol moieties to the surroundings when assembled into tBLMs, excluding water from the sub-membrane space [105]. Sub-membrane hydration can be changed by modifying the spacer segment and increasing the number of ethylene glycol units. However, increasing the length of the tethers also leads to a significantly higher number of defects [77]. In addition, the tether density affects the resulting bilayer properties [102]. The high packing density of the tethers results in a densely tethered proximal leaflet, which, in addition to limiting hydration in the surrounding medium, imposes spatial constraints which may have an impact on the structural adaptation of the sub-membrane protein subunit. Since the incorporation of proteins with bulky extra-membrane domains requires space and significant hydration environment, thiolipid anchored-tBLMs from *telechelics*, due to their high packing density, have been shown to be detrimental to harbor large trans-membrane proteins [71,175]. As

recently confirmed by molecular dynamics calculations, the tether density of thiolipid anchors affects the hydration level, bilayer fluidity, defect density, free energy of lipid flip-flop, lateral mobility and membrane dielectric permittivity [176]. Therefore, it has been found that pure thiolipid anchored-tBLMs, also named *densely* or *fully tethered-BLMs*, partially respond to the challenge of loss of protein diffusion or induction of protein denaturation due to friction with the fully tethered proximal lipid layer, which can hamper functional incorporation of bulky trans-membrane proteins.

4.3. Sparsely-tBLMs or stBLMs

In order to address issues of space and hydration of the sub-membrane compartment to better facilitate protein incorporation into tBLMs, *sparsely tethered-bilayer lipid membranes* or stBLMs have been developed [125]. The density of the tethered anchor thiolipid can be reduced, either by using a longer and bulkier disulfide (or lipoic acid) surface anchoring segment instead of thiol [77,101], or by diluting the anchor thiolipid forming the proximal leaflet with a second shorter surface-active backfilling molecule such as tetraethylene glycol lipoic acid ester [159], thiol terminated tetraethylene glycol molecules [85] or β-mercaptoethanol (βME), which competes for space by co-adsorption on the substrate [94,102]. For sparsely tethered membranes diluted with βME, a hydration level of up to 75 vol% of the submembrane compartment has been achieved, depending on the tethering density of the distal leaflet and the type of lipid used to complete with the outer leaflet [94]. However, the sealing properties of the resulting lipid membrane are significantly reduced with the increase of molar % of backfillers, likely due to the high level of hydration leading to a higher propensity for defect formation [94]. Additionally, the exact composition of its inner leaflet, depending on the relative solubility of the compounds (i.e., anchor lipids and backfiller molecules) and their kinetic binding, is no longer known [125], which is not the case when using a thiolipid with a modified anchoring group [77,101]. However, highly hydrated systems that are stBLMs formed by dilution strategy appear optimal for the study of membrane properties that are not related to its electrical sealing properties, because a very fluid (or disordered) bilayer is created with high hydration levels [88], suggesting the presence of significant submembrane space for protein incorporation. Indeed, it has been demonstrated that stBLMs are a better option for protein reconstitution [124] than *densely tethered-BLMs*, presumably because of a higher flexibility of the membrane due to the possible presence of free phospholipids in the proximal layer [88,151].

To assemble stBLM, new class of thiolipid anchors have been prepared by changing the two branched chains (phytanoyl) by saturated (C14 *n*-alkyl [94] or C16 *n*-alkyl [154] chains) or unsaturated acyl chains (C18:1 [96]. Coadsorbed with a short-chain backfiller (i.e., βME), this variety of chemical structures forming the distal leaflet makes it possible to refine the properties of the resulting membrane architecture with the possibility of supplementing the distal leaflet with various phospholipids (e.g., DPhyPC: 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine, DOPC: 1,2-dioleoyl-*sn*-glycero-3-phosphocholine, POPC: 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, and DMPC: 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine [94], including charged lipids such as DOPG: 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol [154] or mixed Cholesterol/DOPC) [153,177]. In addition to supplying a more spacious sub-membrane compartment containing water, stBLMs completed with a distal leaflet composed of unsaturated phospholipids (DOPC) have been shown to be more fluid than *fully tethered membranes*, with diffusion coefficients in the distal leaflet, approaching values similar to the natural cell membranes (diffusion in the proximal leaflet is hindered by the presence of the anchorlipids) [88]. Likewise, stBLMs formed with an anchor thiolipid containing double bonds in the alkyl segment exhibit higher lipid diffusivity relative to those formed with saturated alkyl chains. This increased membrane fluidity is due to the more disordered bilayer structure caused by the more flexible unsaturated alkyl chains [96]. This further demonstrates the high flexibility with which membrane properties can be fine-tuned depending on the desired application.

stBMs, composed of diluted thio-anchorlipids mentioned above, have served as good membrane models to improve knowledge of several membrane phenomena. For instance, ion transporters and ion channels are incorporated more effectively and possibly, exhibit improved function when they are incorporated into a stBLM. Compared to dense tBLMs, the functionality of pore-forming toxins such as α-hemolysin able to spontaneously penetrate from aqueous solution in the membrane after formation of the lipid bilayer, was improved upon insertion into stBLMs, whether formed with an anchor lipid having a larger anchoring group [165] or inserted into bilayers diluted with βME [124], due to, in both cases, a better sub-membrane hydration. In the latter case, EIS has shown that α-hemolysin channels in sparsely tethered membranes have the same properties as those formed in free-standing bilayer lipid membranes. Structural information from NR allowed a precise localization of the protein within the stBLM and revealed that the interaction between the channel and the disordered membrane was consistent with the predictions based on the channel's X-ray crystal structure [124]. stBLMs have also been employed to develop models mimicking the complex outer membrane of Gram-negative bacteria with adaptable properties and to achieve membrane platforms for screening of potential antimicrobial agents, like *colistin*, an antibiotic belonging to the polymixin family [151]. Associated with EIS, they have been intensively used to investigate the functional mechanism of β-barrel pore-forming toxins, mainly produced by Gram-positive bacteria, belonging to the family of cholesterol-dependent cytolysins (CDCs), like *pyolisin*, the most important actively secreted virulence factor of *Trueperella pyogenes*, an opportunistic pathogen associated with suppurative infections, such as mastitis, septic arthritis, liver abscessation, pneumonia and endometritis [178]. They have been helpful to understand how the specific interaction that exists at the lipid membrane level, between α-synuclein—a Parkinson disease-related protein—and the glucocerebrosidase—the enzyme deficient in Gaucher disease—results in an inhibition of glucocerebrosidase, and provides a molecular connection between Parkinson and Gaucher diseases [98]. Very recently, Penkauskas et al. have used stBLMs to investigate the pleiotropic effects of statins attributed to mechanisms independent on the cholesterol-lowering effect like anti-inflammatory, antioxidant, immunomodulatory and anti-proliferative properties. They showed that hydrophobic statin (i.e., simvastatin), by interacting with pre-formed membrane containing 40% of cholesterol in the outer leaflet, prevents pore formation by a CDC pore-forming toxin, namely *pneumolysin*, the virulent factor of *Streptococcus pneumoniae* responsible for pneumonia, meningitis and septicemia; simvastatin by enhancing clustering of cholesterol-enriched lipid microdomains, would prevent *pneumolysin* pore formation [179]. The pH-dependent action mechanism of two CDC toxins, *inerolysin* and *vaginolysin*, respectively produced by vaginal bacteria, *Lactobacillus iners* and *Gardnerella vaginalis*, and involved in vaginal dysbiosis, has been also elucidated by using preformed cholesterol-rich stBLMs with perspective application in medical diagnostics [153,180]. Thanks to EIS and NR studies, stBLMs have also shown that amyloid β-oligomers, well-known to reduce the membrane barrier to ion transport, fully insert into the bilayer and affect both membrane leaflets and membrane properties by inducing lateral heterogeneity. These effects induce ion conductances across artificial membranes, depending on their lipid composition [181]. As neural networks strongly depend on electrochemical gradients to function, these findings shed new light on the development and progression of neurodegenerative diseases, offering stBLMs as a valid platform for the study of the effects of Aβ oligomers. Finally, stBLMs have been used as sensing elements of biochips for the detection of lethal factor (LF) secreted by *Bacillus anthracis*, after self-insertion of *Bacillus anthracis* PA63 ion channels, an octameric pore which bonds LF, in preformed stBLMs [152]. Zhou et al. [87] have developed a versatile and simple "bottom-up" approach to create stBLMs on the coating of oxidized surfaces of most nanoelectronic devices, using a covalent tether attachment chemistry based on silane functionalization, followed by step-by-step stacking of two other functional molecular building blocks, oligo-PEG and phospholipid (i.e., DOPE: 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine). In this process, oligo-PEG serves as both hydrophilic spacer and tether for the grafting of

DOPE; standard vesicle fusion process was used to complete the bilayer formation. These stBLMs have been formed on three common classes of (nano)electronic biosensor devices (i.e., indium-tin oxide-coated glass, silicon nanoribbon devices, and high-density single-walled carbon nanotubes (SWNT) networks on glass). The fluidity of the stBLMs has been checked by FRAP and *alamethicin* has been used to record real-time single ion channel activities with a high sensitivity and spatial resolution, demonstrating the successful integration of nanoelectronic devices with electrophysiology [87].

The need for a diluted sub-membrane space and a consequential increase in sub-membrane hydration has also been demonstrated using a cholesterol-tethered membrane with 6-mercaptohexanol (6MH) acting as a diluting agent [155,157]. In this case, the anchorlipid is also constituted of three structural elements: a thiol group, as the surface chemical linker, connected to a triethylene glycol as hydrophilic spacer unit, itself linked to cholesterol as membrane lipid anchor (i.e., ethyleneoxy-linked cholesterol or EO_3C). Membrane formed with a mixture of anchorlipid EO_3C and 6MH have revealed good electrical resistance properties, and hydration of the sub-membrane compartment has been ascribed to a phase separation creating nanodomains of 6MH allowing formation of spacious aqueous reservoirs (large spaces) underneath the bilayer tethered by the ancholipid EO_3C (Figure 1) [82]. EO_3-cholesterol anchorlipid and derivatives have been successfully used to study transmembrane transport mediated by small peptide ionophores like *valinomycin* or *gramicidin* [156,157,182], to scrutinize the conformational changes during the transition from the "open" to the "closed" state of the human transmembrane voltage-dependent anion channel (hVDAC) [108], or to investigate the enzyme activity of transmembrane proteins like *cytochrome* bo_3, an ubiquinol oxidase [109,119,183,184] or *CymA*, a menaquinol-7 dehydrogenase of *Shewanella oneidensis* [185], all functionally reinserted into cholesterol-stBLMs by using proteoliposome fusion to form the distal layer. Cholesterol anchored bilayers have also been used to create an in vitro mimic of the cytoskeletal scaffold after reinsertion of *ponticulin*, a protein of mold (*Dictyostelium discoideum*), able to bind F-actin network of cytoskeleton and serving as nucleation site for actin polymerization [186]. These membrane models were useful to investigate interaction of antibiotics, like *vancomycin* or *ramoplanin*, with stBLMs inserting two precursors to peptidoglycan formation in order to mimic the membrane of Gram-positive bacteria for addressing questions related to antibiotic action and resistance [187]. Finally, sparsely cholesterol anchored bilayers have been successfully used to scrutinize the process by which *alamethicin*, an antimicrobial peptide, absorbs to and then integrates into a lipid bilayer. *Alamethicin* acts by assembling in pores made up of four to six individual peptide strands. While *alamethicin* is not able to penetrate *fully tethered lipid bilayers*, in sparsely tethered ones, individual peptides may first adsorb to the membrane surface and then aggregate before inserting into the bilayer to form pores [110]. An interesting review introduced the different applications of numerous cholesterol-based anchors and tethers, even beyond the field of membrane protein reconstitution (see [158]). More recently, Eicher-loka et al. have synthesized a series of new bifunctional cholesterol compounds with cyclic sulfide as surface linker chemical group [89]. Interestingly, they proposed cholesterol derivatives with an electroactive group to study electron transfer processes, or fluorescently-labeled to monitor cholesterol trafficking in membranes.

Another tethering chemistry has also been explored to form sparsely-tBLMs. The tethering monolayer (i.e., proximal leaflet) is prepared with two benzyl disulphide families, one being a tethering group comprising an eleven oxygen–ethylene glycol linker group with a single C20 hydrophobic phytanyl chain as the hydrophobic tether, named as DLP ("double-length" reservoir phytanyl lipid (DLP) [188]), and a second being a spacer molecule containing a four oxygen-ethylene glycol spacer, terminated with an OH group as diluting molecules [116], generally used in a 1:9 ratio, respectively. The bilayer is then completed by rapid solvent exchange of an organic solution of phospholipids, which allows to tailor the lipid composition as desired to mimic natural cell membranes [99,100,189]. These types of stBLMs have revealed good sealing properties [24] and provide a large ionic reservoir and space for transmembrane protein insertion [188]. They appear particularly

suited for EIS investigations. Hence, they have been intensively used (i) to study ion channel activity or conductance, like *alamethicin* a small peptide voltage-gated ion channel [190], a synthetic ligand-gated ion channel (SLIC) [24], small peptide ionophores, like *valinomycin* or *gramicidin* [117], the *chloride intracellular ion channel* (CLIC1) a metamorphic protein able to insert into phospholipid bilayers enriched in membrane sterols from their soluble state [191], or (ii) to identify the mechanism of the antimicrobial peptides forming different types of pores in lipid membranes [192], like *Kalata B1* and *Kalata B2*, cyclic antimicrobial and insecticidal cyclotides [193], or *Melimine* [194]. They have been also used as harboring lipid supports for cell-free expression (see Section 5) and functional reconstitution of recombinant *OprF* porin as target for antibacterial drugs, and the subsequent structural study by NR [195]. More recently, they have been employed to investigate the effect of the heat transfer between cell membranes and gold nanoparticles (GNPs) used in laser-targeted treatment for infections, tumors and for the controlled released of drug in situ [196]. They also represent a platform as substrate for phospholipase A (PLA) catalytic assay [197] or for toxicity examination of food cyanobacteria toxins like *micricystins*, suspected to compromise the cell membrane integrity [198].

Finally, with the goal of avoiding the cumbersome chemistry of anchor thiolipids synthesis, Squillace et al. have recently developed a versatile approach based on wet chemistry via aryldiazonium sulfonic acid, for grafting PEG as cushion mixed with a commercial surfactant, i.e., Brij58 as alkyl chain harpoon, for subsequent formation of tBLMs by adsorption and spontaneous fusion of lipid vesicles [103]. In this stBLM, the lipid bilayer was stabilized by molecular coatings composed of diluted anchor-harpoon surfactants that grab the membrane with an alkyl chain out of a PEGylated-hydrogel layer, which acts as a soft hydration cushion [199].

4.4. Peptide-tBLMs or Pep-BLMs

Another strategy to improve space and hydration in the sub-membrane compartment is based on the self-assembly of thio-polar peptides as spacers to form *peptide-tethered-bilayer lipid membranes* (pep-tBLMs). These pep-tBLMs have been shown to be suitable for membrane protein reincorporation by providing a biocompatible sub-membrane environment in which extra-membranous subunits can fold into a native-like conformation [71]. Peptides are actually of the same nature as the cytoskeleton inside or the extracellular matrix outside the cell with which the protuberance of the membrane proteins can interact in real life.

The main advantage of using peptides as tethers is that their length, their secondary structure and their hydrophilic properties can be easily tuned by changing their aminoacid sequences. This enables a flexible adjustment of both membrane–substrate separation and viscosity of the tether units, which are important to ensure sufficient lateral membrane diffusivity for functional protein incorporation [200]. Thus, pep-tBLMs are well-designed to preserve the integrity and functionality of membrane proteins. On the contrary, the resistance of pure pep-tBLM is rather low, so these membranes are not particularly suitable for electrochemical and EIS measurements [71].

Classically, peptides used as tethers to form pep-tBLMs are prepared from synthetic or native thiopeptides, or thiolipopeptides (Table 2, [71]), used without backfilling molecules (no dilution). These peptides are functionalized at their N-terminus by a sulfur group such as a cysteine, or a lipoic acid designed for self-assembly on gold, and except for LP12 which can be used without additional chemistry, their *C*-terminus extremity is chemically activated afterwards for the coupling of the amino polar headgroup of different phosphatidylethanolamine (PE), thus forming the proximal lipid monolayer [37], which acts as the anchor of the membrane by forming the proximal leaflet of the bilayer [111,115,131,201,202]. As for anchorlipids, pep-tBLMs have been firstly formed by "bottom-up" approach [34]. The first peptide layer was built via self-assembly process from a dilute solution of tether molecules. The bond with the substrate provided stable anchoring of the proximal leaflet. After chemical activation and grafting of PE via an ester

bond, the distal layer was generally formed by fusion of liposomes with or without the reconstituted protein of interest onto the hydrophobic self-assembled proximal layer [72].

Table 2. Amino acid sequence of thio- and thiolipo-peptides used for pep-tBLMs formation (Reprinted from Publication [71] Copyright (2009), with permission from Elsevier and adapted from [200]).

Name	Amino Acid Sequence
P5	Lip-Ala-Ala-Ala-Ala-Ala-COOH [1]
P7	HS-$(CH_2)_2$-Ala-Ser-Ser-Ala-Ala-Ser-Ala-COOH
LP12 [2]	HS-Cys-Ala-Ser-Ala-Ala-Ser-Ser-Ala-Pro-Ser-Ser-Lys(Myr)-Myr [1]
P19 [3]	HS-Cys-Ser-Arg-Ala-Arg-Lys-Gln-Ala-Ala-Ser-Ile-Lys-Val-Ala-Val-Ser-Ala-Asp-Arg-COOH
P19-4H	HS-Cys-Ser-Arg-Ala-Arg-Lys-Gln-Ala-Ala-Ser-Ile-Lys-Val-Ala-Val-Ser-Ala-Asp-Arg-His-His-His-His-COOH

[1] Abbreviations: 'Lip' for lipoic acid; 'Myr' for myristoic acid (C14:0). [2] LP12 is used like this, without additional chemistry. [3] Natural peptide corresponding to a part of α-subunit of *laminin*, a complex glycoprotein of the extracellular matrix, consisting of three chains α, β and γ able to interact with cellular receptors (e.g., *integrins*) and implied in the cell attachment, differentiation, cell shape and motility [71].

Different integral membrane proteins e.g., cytochrome c oxidase [131], cytochrome bo3 ubiquinol oxidase [203], H^+-ATPases (from chloroplasts and E. coli) [115,201,202,204], dimer of nicotinic acetylcholine receptor from Torpedo californica [111], hERG potassium channel [205], integrins [206] or channel-forming peptides like gramicidin or alamethicin [207] have been successfully reincorporated in pep-tBLMs formed with functionalized thio(lipo)peptides.

Pep-tBLMs have also been developed by using combined Langmuir-Blodgett and Langmuir-Schaefer transfers [208]. In this approach, a monolayer of 1,2-dimyristoyl-sn-glycero-3-phospho-*ethanolamine* (DMPE) is directly transferred by vertical dipping onto activated carboxyl groups of a P19 self-assembled monolayer formed on gold by strong S-Au interaction. The distal lipid monolayer is obtained by Langmuir-Schaefer transfer. Owing to this stepwise assembly, it is easy to change the lipid composition of the distal layer. By this way, the adsorption of the amyloid β-peptide (Aβ40) with several lipid mixtures has been analyzed. The Aβ adsorption was found critically depending on the lipid composition of the membranes, with Aβ specifically binding to membranes containing sphingomyelin. Further, this preferential adsorption was markedly amplified by the addition of sterols.

Becucci et al. [207] have incorporated channel-forming peptides, *gramicidin* and *alamethicin*, in a mercury-supported lipid bilayer composed of a tethered thiolipid monolayer with a self-assembled DOPC monolayer on top of it. The thiolipid consists of a hexapeptide chain with a high tendency to form a 3_{10}-helical structure, which terminates at the N-terminus end by a sulfydryl group for anchoring to the metal [209], while the *C*-terminus end is covalently linked to the polar head of DMPE. The hexapeptide moiety has two triethyleneoxy side chains that impart a satisfactory hydrophilicity and are intended to keep the anchored thiopeptide chains sufficiently apart, so as to accommodate water molecules and inorganic ions, and to create a suitable environment for the incorporation of integral proteins.

Nevertheless, in the conventional way of preparing tBLMs, both leaflets of the bilayer are formed independently through a "bottom-up" approach: first proximal layer by self-assembly process, and second distal layer completed (the most often) by fusion of vesicles. The building-up of pep-tBLMs has been mainly followed by SPR spectroscopy and FRAP [113,210], and characterized by AFM, EIS, FM [210]. These studies report that a true lipid bilayer is not always fully obtained and that the films are not fluid on the micrometer length scale [210]. Actually, the step-by-step procedure of the "bottom-up" method sometimes leads to lateral heterogeneities or discontinuities in tethered lipid bilayers due to the partial surface coverage with some undisrupted liposomes adsorbed onto the solid surface [37,71,210]. Indeed, depending on their composition, vesicles could have a low propensity to fuse on the hydrophobic surface of the proximal layer exposed to aqueous solution, especially if they incorporate an integral protein [167]. In addition, the leaflet-by-leaflet formation process could lead, despite the successful reincorporation

mentioned above, to a misfolding of the transmembrane proteins which require a lipid core to maintain their function; these latter can be denatured, even partially, during the coverage of the hydrophobic proximal layer by the distal monolayer and thus, lose their functional activity. Indeed, from a biochemical point of view, the only physical insertion of proteins, transmembrane or not, in a lipid bilayer is not a guarantee of the functional folding of the protein, and only a few report the functionality of large transmembrane proteins reinserted into tBLMs (It should be emphasized here that most of the reinsertion studies carried out with densely tBLM or stBLM are obtained from proteins which insert spontaneously into the preformed membrane from an aqueous solution) [115,131,185,204,206]. A possible explanation of this failure may be the small amount of reincorporated proteins [203], which does not allow to measure any protein activity nor quantify it. Lastly, the functionalization of the thiopeptides by the lipid anchor to generate thiolipopeptides requires a synthetic chemistry, often complicated, which limits the type of lipid anchor which can be added, classically dimyristoylphosphatidylethanolamine (DMPE) [37]. It is the same situation with anchorlipids mainly constituted of two phytanoyl chains. Hence, the proximal layer is monospecific and only formed by one lipid species, which could restrict both the dynamic behavior of the tethered lipid bilayer and the capacity of the membrane protein to be reinserted; the composition of the bilayer in terms of length and/or unsaturation of the fatty acyl chains on one hand, and the nature of the phospholipid polar head on the other hand, being essential for ensuring successful functional reincorporation of the membrane proteins.

In summary, tBLMs were originally developed for reinsertion of transmembrane proteins in view of their use as a recognition element for biosensing applications [41]. As seen above, a number of membrane proteins has been reinserted in tBLMs. Most of them are small pore-forming peptides such as *gramicidin*, *mellitin*, *alamethicin*, *valinomycin*, *cholesterol-dependent cytolysins* (CDCs), Aβ oligomers, which spontaneously insert into the preformed membrane from an aqueous solution, but larger transmembrane proteins are rarer; they concern *porins* or large oligomeric bacterial toxins such as *α-hemolysin*, receptors e.g., *integrins*, *nicotinic acetylcholine receptor*), *H^+-ATPases* or redox proteins such as *cytochrome b_5*, *cytochrome bo3*, *cytochrome c oxidase* or *CymA*, a menaquinol-7 dehydrogenase, which, except *α-hemolysin*, *H^+-ATPases* and *CymA*, do not possess bulky extra-membrane domains.

4.5. Vesicle Fusion Method to Form tBLMs: A "Top-Down" Approach

In order to circumvent the drawbacks of the "bottom-up" procedure to prepare tBLMs, another concept for forming tethered membranes, based on the fusion of lipid vesicles containing both the anchor and the spacer molecules on a functionalized surface has been proposed as an easier and versatile approach. Subsequently, the two leaflets of the tethered bilayers originate from the membranes of the vesicle, and are never built independently. This approach combines functionalized/spacer lipids and their use for receptor/ligand binding. This approach is expected to overcome some limitations of the different constructions developed so far, as the tether density, for instance [126]. This "top-down" approach can be performed with PEG [35,39,211–213], specific recognition/ligand receptors [81,118,214] (Figure 3) or peptides [43,200] as anchoring spacers (Figure 2).

4.5.1. Using PEG as Anchoring Spacer

In this polymer-coated liposome spreading procedure, the bilayer is achieved by deposition of vesicles containing a mixture of PC and either DSPE-PEG-NHS (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-poly-(ethyleneglycol)-N-hydroxysuccinimide) [35] or DSPE-PEG-PDP (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-poly(ethyleneglycol)-2000-N-[3-(2-pyridyldithio)propionate]) [39], which act as both spacer and anchor molecules. These spacer lipids are terminated by an activated group, which reacts with any amine-coated surface (cysteamine-coated gold or amino-silanized glass) or gold surface, respectively (Figure 3a). Once the vesicles are bound to the surface, their rupture, possibly triggered by a fusogenic agent [213], leads to the formation of the bilayer. Formation of

these *polymer-tBLMs* has been followed using SPR spectroscopy [35,36,130], QCM-D [39] and characterized by AFM [36,83]. They can be used to reconstitute raft lipid domains in the tBLMs in a microarray format [83]. Studies of the diffusion coefficient of individual lipids within the bilayer by FRAP measurements have indicated that the bilayer is fluid and continuous to preserve the high lateral mobility of membrane constituents despite the proximity of the solid support [35,36,130].

This type of bilayer has been successfully used to investigate the protein/membrane interactions of different kinds of integral proteins such as the voltage-dependent anion channel (VDAC) of the outer mitochondrial membrane [36] or the *aquaporin Z* (AqpZ) [39]. By using a sophisticated model mimicking cellular compartmentalization obtained thanks to polymer-coated liposomes spreading associated with distinct protein markers of intra- and extra-cellular milieu [130], Rossi and her group have succeeded in studying for the first time, the translocation of the adenylate cyclase, a bacterial toxin produced by *Bordetella pertussis* (CyaA) through a lipid bilayer [129]. It should be noted that this last contribution is a remarkable example of the usefulness of tBLM models to understand molecular mechanisms that occur at the level of plasma membranes, since it allows a real breakthrough in the understanding of the protein translocation and transport across biological membranes under precisely defined conditions [215,216].

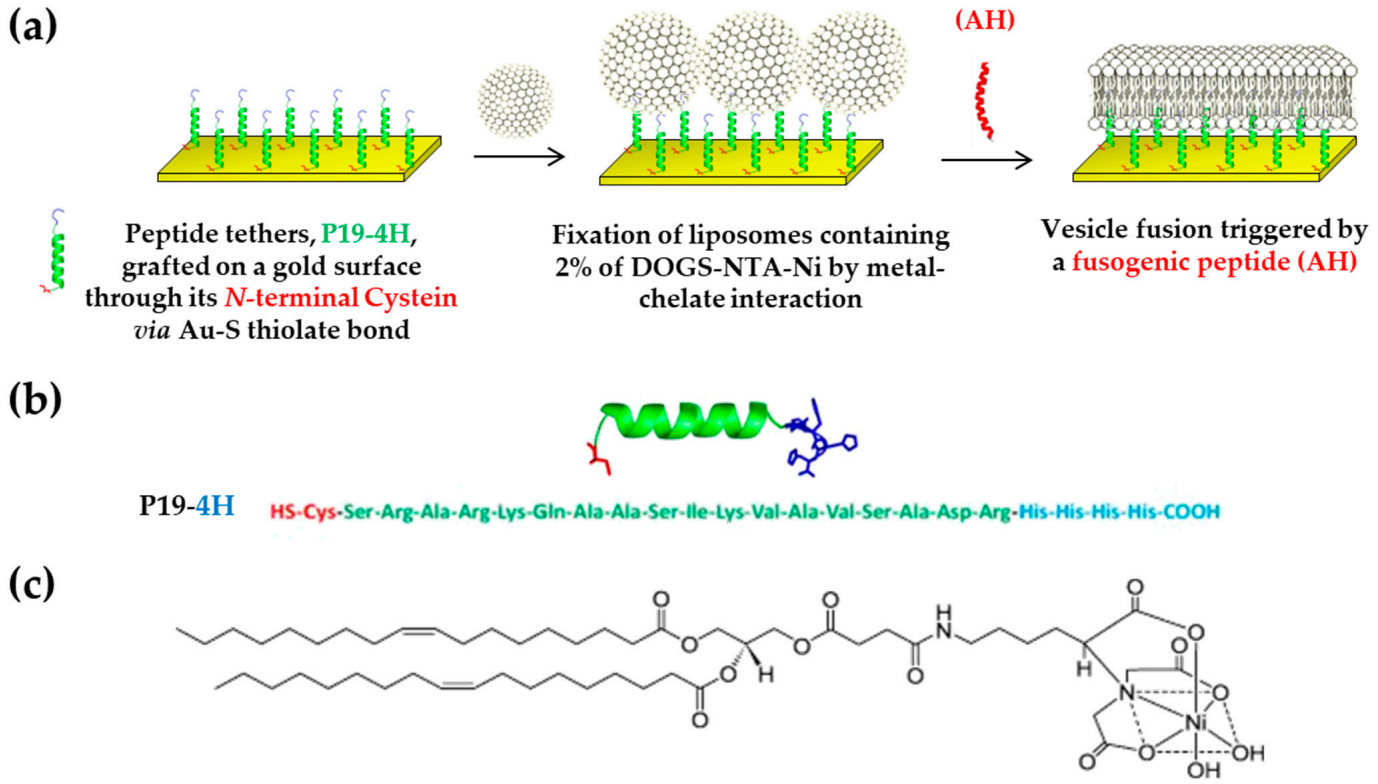

Figure 2. (**a**) Principle of formation of pep-tBLM by "top-down" approach onto peptide tethers. The pep-tBLM is obtained after vesicle fusion of liposomes containing 2% of a chelating lipid DOGS-NTA-Ni, tethered onto P19-4H peptides, grafted themselves on gold surface via stable Au-thiolate bond; the vesicle fusion is triggered by an amphipathic α-helix (AH) peptide derived from the N-terminus of the hepatitis C virus NS5A protein; (**b**) 3D modelling of P19-4H; the three characteristic parts of the peptide are represented with the cysteine residue in red for the grafting on the gold surface, the central α-helix in green for the spacing and the 4 Histidine residues in blue for the attachment of liposomes containing DOGS-NTA by chelation in the presence of nickel salt. The sequence of P19-4H and P19 peptide from which P19-4H has been derived are indicated below; (**c**) Structure of the DOGS-NTA-Ni able to interact specifically with P19-4H (adapted with permission from [200]. Copyright 2017, American Chemical Society).

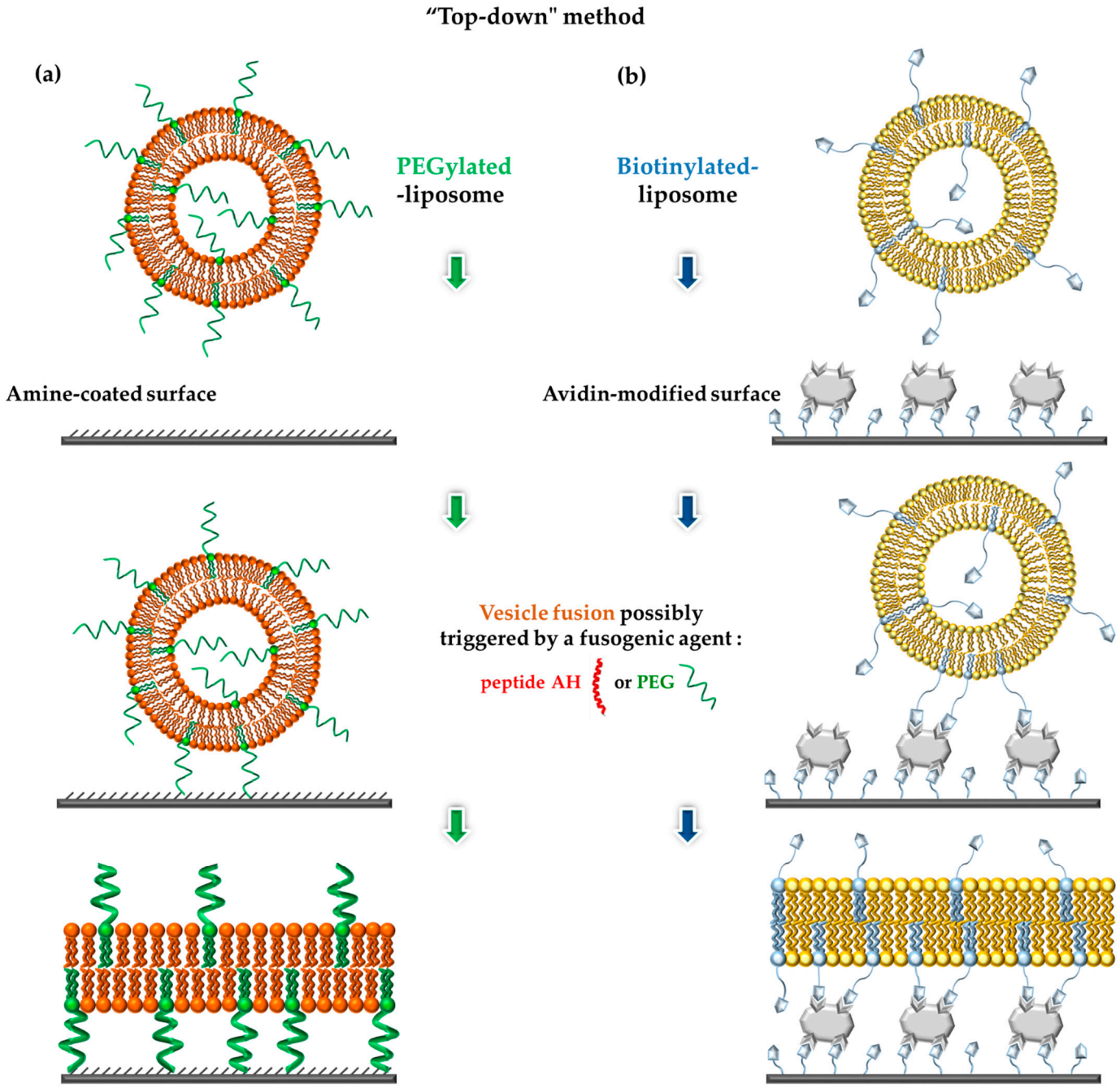

Figure 3. tBLMs formed by fusion of lipid vesicles containing both the anchor and the spacer molecules on a functionalized surface via a "top-down" approach. (**a**) using PEG as spacers, by fusion of liposomes containing either DSPE-PEG-NHS or DSPE-PEG-PDP on an amine-coated surface; (**b**) using biotin/avidine as spacers, by fusion of biotinylated vesicles on a sublayer of immobilized streptavidin. In both cases, the liposome fusion can be triggered by a fusogenic agent (i.e., PEG-8000 solution [81,134,214,217] or an amphipathic α-helix (AH) peptide [39]). DSPE-PEG-NHS: 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-poly-(ethyleneglycol)-N-hydroxysuccinimide; DSPE-PEG-PDP: 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-poly(ethyleneglycol)-2000-N-[3-(2-pyridyldithio)propionate].

An important advantage of the direct incorporation of the spacers in vesicles is the possibility to control their percentage. This provides a spacer layer with a density lower than the fully tethered proximal leaflet formed by self-assembly of the *telechelics* [30,104] or even lipopolymer [91]. As seen above, a high tether density leads to a poorly hydrated sub-membrane compartment which adversely affects the membrane fluidity and constitutes a

steric hindrance for reincorporation of membrane proteins having large extra-membrane domains [51].

Another important point when studying integral protein concerns the lipid composition of the bilayer. Indeed, it is now well-known that the lipid environment has an impact on the proper functioning of membrane proteins [8–10,218,219]. Ideally, MPs should be investigated in their native environment. Thus, the lipid composition of tBLMs must be as close as possible of the one of the natural lipid membrane in which the membrane protein is embedded. A particular attention should also be paid to the importance of non-bilayer lipids (Non-bilayer lipids can be defined as cone-shaped lipids with a preference for non-bilayer structures with a negative curvature, such as the hexagonal (HII) phase) [220,221] in the functional activity of intrinsic MPs. These lipids are present in large amounts in all membranes, and especially in energy-converting membranes where they constitute about half of their total lipid content [222]. Due to their conical shape, they alter the lateral pressure profile in the lipid bilayer—called *lateral pressure model* (LPM)—and generate elastic energy of curvature—called *flexible surface model* (FSM). Hence, they contribute to the structural flexibility and plasticity of membranes by stabilizing regions of high curvature or packing around the hydrophobic core of MPs [223]. Their role in membrane functioning and their impact as regulators of functional MP activities are now widely described [15,16]; they should be much more taken into account in the lipid composition of membrane models than they are currently. Until now, the compositional simplicity of biomimetic membrane models in general, often limits the level of knowledge that can be gained about the structure and the function of transmembrane proteins. Recently, Pace et al. [224] have explored a generic methodology wherein "*native cell-membrane vesicles*" (NMVs) are merged with a synthetic vesicle containing POPC and PEGylated lipids that promote vesicle fusion. These hybrid vesicles readily spontaneously rupture into a continuous polymer-supported membrane, which contains essentially all the natural components of the plasma membrane of the cell line from which the NMVs are generated. By using the *β-secretase 1* (BACE1), a transmembrane protease—overexpressed in an insect cell line and exhibiting asymmetric ectoplasmic and cytoplasmic extra-membrane protruding regions which can be probed independently—the authors demonstrated that the mobility and activity of transmembrane proteins are preserved. This "top-down" approach corresponds to the first report demonstrating the lateral mobility of a transmembrane protein in a *polymer*-tBLM derived from the cell membrane, with a large (>1 nm) protruding region facing the substrate. It provides new insight into how biomimetic surfaces can be fabricated from native membranes, for fundamental studies of membrane biophysics and production of surface-based analytical sensor platforms [224].

For the sake of completeness, we can cite the works of Roder et al. [93], who propose an intermediate solution between the "bottom-up" and "top-down" approaches, with a pre-functionalization of the surface with a lipopolymer tether and a subsequent fusion of (proteo-)liposomes to form continuous tethered polymer-supported membranes. The latter offer the possibility to reproduce liquid-disordered (l_d) and liquid-ordered (l_o) phase segregation within both leaflets [225]. In this approach, highly monodisperse very small unilamellar vesicles (VSUVs) generated by extraction detergent with cyclodextrin, including fluorescent lipids and transmembrane protein models, were first captured onto a dense PEG polymer brush functionalized with fatty acid moieties (i.e., palmitic acid); their fusion and the consecutive self-assembly of the membrane were induced by PEG solution. This liposome spreading on pre-functionalized surface gives the opportunity to mix vesicles containing different components on demand. Lipid and protein diffusion were characterized in details by FRAP, fluorescence correlation spectroscopy (FCS) and single molecule imaging (SMT). Full mobility of lipids and a high degree of protein mobility as well as homogeneous diffusion of both were observed. Additionally, quantitative ligand binding studies confirmed functional integrity of a transmembrane receptor reconstituted into these polymer-tBLMs [93]. It could be noticed that the lipopolymer used in this approach is constituted of only one fatty acid chain. It is possible that the smaller volume of

the hydrophobic anchor interferes less with the diffusivity of the membrane compounds, unlike what was observed previously with the former polymer-tBLMs (see Section 4.1). The advantage of this method lies in the formation of VSUV by detergent extraction with cyclodextrin which is compatible with a broad spectrum of detergents used for the solubilization of membrane proteins and thus, allows rapid and automatized reconstitution of the latter [93].

Finally, a similar combined approach between the "bottom-up" and "top-down" method, has been recently proposed by Daniel's group to form polymer-supported mammalian cell membranes directly created from plasma cell membrane vesicles [226]. These vesicles, also called cell blebs, that bud from the plasma membrane, are readily generated either by mechanical cell lysis or by chemically induced methods, and they correspond to small biopsies of the intact plasma membrane. Cell blebs, expressing either GPI (glycosylphosphatidylinositol)-linked yellow fluorescent proteins or neon-green fused with a 6-pass α helix transmembrane P2X2 receptor, were induced to fuse and rupture on glass surfaces using PEGylated lipid vesicles which both trigger the bleb rupture and provide some space between the glass surface and the lower leaflet of the planar bilayer to accommodate the cytosolic extramembranous domains. Single molecule tracking and moment scaling spectrum (MSS) analysis have revealed mobility of over 50% for multipass transmembrane proteins and over 90% for GPI-bound proteins, and enzymatic assays, that both proteins are oriented in the planar bilayer with extracellular domains facing toward the bulk, indicating that the dominant mode of bleb rupture is via the "parachute" mechanism [226]. In order to enhance the mobility of the transmembrane proteins over 75%, such membranes have been prepared on a surface-tethered tunable-length polyelectrolyte cushion (i.e., PMETAC: poly([(2-methacryloyloxy)ethyl]trimethylammonium chloride)), involving direct electrostatic interaction between the naturally negatively charged cell plasma membrane vesicles and the positive charges polyelectrolyte brushes to induce vesicle rupture [92]. More recently, they have been formed on optically transparent and electrically conducting polymer poly(3,4-ethylenedioxythiophene):poly(styrene sulfonate) (i.e., PEDOT:PSS) in order to monitor ATP (adenosine 5-triphosphate)-gated P2X2 ion channel activity through EIS [227]. In all cases, enzyme accessibility assays have confirmed that the protein orientation is preserved in these types of polymer-tBLMs, and results in the extracellular domain facing toward the bulk phase and the cytosolic side facing the support. The main advantage of this approach is to promote the direct incorporation of membrane proteins into the planar bilayer without using detergents or reconstitution, and to preserve its natural environment. But on the contrary, the protein of interest must be overexpressed in cell membranes to be sure to not bias the functional studies since in situ, the analyzed compound is not pure. Indeed, if the membrane protein activity is analyzed inside the cell membrane, the result is highly challenging since many artefacts can modify the accuracy of the measurement: cell membranes can also contain homologous proteins with identical activity, or agonists and antagonists. That being said, the platform presented here retains the complexity of the plasma membrane and preserves the natural orientation and mobility of MPs. It corresponds so far to the best representative mimic of native cell membranes, which may find many applications in biological assays aimed at understanding cell membrane phenomena.

4.5.2. Using Biotin/Avidin as Spacers

Another strategy for forming tBLMs by liposome spreading uses the affine avidin/biotin system as spacer for tethering the membrane (Figure 3b). In a first step, intact biotinylated vesicles are accumulated by affinity on a sublayer of immobilized streptavidin. As liposomes do not fuse spontaneously on soft matter, the formation of the bilayer is triggered by addition of soluble poly(ethylene glycol) (PEG), a fusogenic agent of lipid vesicles [118,214] or by replacing the medium by a hypotonic solution [228]. One of the crucial steps in the formation of tBLMs is the achievement of vesicle fusion on the template, in such a way that a continuous fluid bilayer without defects can be produced in a reasonable time, compatible

with the preservation of the native membrane proteins. The formation of a tethered bilayer on a streptavidin sublayer in this "top-down" assembly makes it possible to overcome the constraints imposed by uncontrolled kinetics of spontaneous vesicle fusion since this latter requires triggering [81,217]. Additionally, since no particular anchorlipid is needed in this strategy, this allows the lipid composition to be modified as desired. Thus, by using this system, Elie-Caille et al. [134] have successfully tethered and fused phospholipid-enriched proteoliposomes prepared from the inner mitochondrial membrane and containing the transmembrane proteins of the respiratory chain at a protein area fraction of about 15%. The overall process and the triggered fusion of the immobilized (proteo-)liposomes have been monitored by SPR and FRAP, respectively. A homogenous flat surface of soft material corresponding to a biomimetic (proteo-)lipid bilayer anchored to the solid substrate was obtained [81,134]. Sumino et al. [228] used the avidin–biotin interaction to tether lipid bilayer containing photosynthetic antenna proteins (light-harvesting complex 2, LH2- and light-harvesting core complex, LH1-RC) for functional analysis. The energy transfer—obtained from LH2 to LH1-RC within the tethered membrane and analyzed by fluorescence spectroscopy—has been ascribed to the diffusivity of the proteins inside the bilayer. Such a result indicates that biotinylated planar membranes tethered by pillars of avidin molecules and formed by spreading of proteoliposomes in a "top-down" approach, offer the possibility of reconstituting in vitro large photosynthetic complexes and mimic a natural situation. As demonstrated through these examples, the main advantage of forming tBLMs by vesicle fusion incorporating only 0.5–1 mol% of biotinylated-phospholipids (e.g., DMPE, DPPE: 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine, or DOPC) lies in the possibility to complexify the membrane lipid composition [217] or even to be able to use extracted membrane lipids [134]. Finally, the affine avidin/biotin system is commonly used in the design of microarrays. Avidin/biotin-tBLM arrays have been built in a parallel microfluidic system (i.e., in parallel microchannels) using the "top-down" approach, and used for multiplexed label-free analysis of lipid-protein interactions by SPRi. Multiplexed analysis of protein interactions has been demonstrated with several systems, including monosialoganglioside (GM1) receptor for cholera toxin (CT) and phosphatidylinositol phosphate (PIP) lipid for PIP-binding proteins, for which a calibration curve was obtained. The tethered membrane array technology, in combination with *surface plasmon resonance imaging* (SPRi), offers an attractive platform for studies of membrane proteins, and can also find a range of applications for rapid screening of drug candidates interacting with MPs embedded in the near-native environment [217].

4.5.3. With Peptide as Tethers

An alternative approach to take advantage of pep-tBLMs benefits which truly mimics the intra- (i.e., cytoskeleton) or the extra- (i.e., extracellular matrix) protein nature of the membrane environment, and to form the bilayer in a single step, but avoiding any chemistry, has been proposed. In this approach, liposomes including or not reconstituted membrane proteins are attached by a metal-chelate interaction to peptide spacers grafted on gold surface, before triggering their fusion by a fusogenic agent to form pep-tBLMs (Figure 2a) [200,229]. The metal-chelate interaction has been already used to graft MPs onto chemically modified gold surface via the affinity of their histidine-tag for a nickel-chelating nitrilotriacetic acid (NTA) surface before reconstituting them into the lipid environment (see below Section 4.6) [107], but never for attaching the lipid membrane onto grafted peptide spacers. For this purpose, a new peptide spacer derived from the natural thiopeptide (P19) and modified in its *C*-terminal extremity by four histidine residues (P19-4H, Table 2 and Figure 2b) to bind liposomes containing 2% of a chelating lipid (DOGS-NTA-Ni: 1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (nickel salt)) (Figure 2c) has been recently designed [229]. Among all the peptides spacers already used to form pep-tBLMs, P19 was chosen since its N-terminal cysteine residue allows spontaneous grafting on gold surface via stable Au-thiolate bond formation, making it one of the most widely used for that purpose. As it is a water-soluble peptide derived

from the subunit α of laminin, an extracellular matrix glycoprotein involved in the cell–cell interaction and cell differentiation, its natural composition offers an adequate hydrophilic sub-membrane environment for membrane protein reinsertion [37,203,205,206,230]. DOGS-NTA presents a chelating headgroup that forms a coordination complex with histidine residues in the presence of nickel with a high binding affinity ($K_d = 10^{-13}$ M for a six-residue polyhistidine tag at pH 8.0) [231]. Suitable to coat materials, chelating lipids have been used to immobilize proteins and functionalize different systems, like lipid surfaces for bioreceptor immobilization [232,233]. After tethering, liposome fusion is triggered by a fusogenic agent to obtain a planar membrane, as previously proposed for other systems (see above). In the present approach, an amphipathic α-helix (AH) peptide derived from the N-terminus of the hepatitis C virus NS5A protein [234] was used to induce vesicle rupture, as already demonstrated for formation of tBLMs on polymer (PEG) cushion [39] or on mesoporous silica [213]. The main reason of this choice is because after promoting vesicle swelling and rupture, this peptide desorbs from planar bilayer, leading to the formation of a pure lipid bilayer exempt of any "extra" molecules [235–237].

The mode of attachment by chelation between P19-4H and the headgroup of DOGS-NTA proposed in this "top-down" method has conferred a great stability during handling compared to other pep-tBLMs described in the literature, in which the lipid bilayer is only anchored by hydrophobic interaction (i.e., hydrophobic chains of DMPE attached to the *C*-terminal end of peptide spacer inserted inside the bilayer, see Section 4.4), possibly leading to a delamination and/or partial destruction of the membrane architecture with formation of defects in the bilayer structure. FRAP and AFM studies have confirmed that using this "top-down" approach, the bilayer obtained with or without membrane proteins is homogenous, fluid, continuous and the lateral mobility of membrane compound constituents are preserved. By developing an original test involving the peculiar adsorption property of a cytoplasmic protein, the *nucleoside diphosphate kinase-B* (or NDPK-B) to associate in a discriminatory manner to nude gold surface or anionic lipid membranes, it was shown that the pep-tBLMs is formed without defects [200].

The main original feature of this methodology, compared with the classical way to form pep-tBLMs, is that it is based on the use of preformed entire vesicles before fusion, and as we saw before, the lipid composition of the bilayer can be thus easily tuned and made more complex (Table 3), or even formed from natural membrane lipid extracts (unpublished data). For instance, some non-bilayer lipids, like PE, can be added in the lipid composition (Table 3). Due to its conical shape, this fusogenic lipid preferentially forms non-bilayer phases and promotes inverted hexagonal (HII) phase intermediates that can favor membrane fusion [223,238,239]. Hence, the lipid composition can be readily adapted for (trans)membrane protein reinsertion since it offers the possibility to reincorporate them in a native-like lipid environment, which is essential to keep their natural conformation and their biological activity on a gold surface for performing ligand binding assays.

Therefore, the fusion of a proteoliposome of complex lipid composition, produced by cell-free expression and inserting a functional C-X-C motif chemokine receptor 4 (CXCR4), a seven-transmembrane protein belonging to the large superfamily of G-protein-coupled receptors (GPCRs) of high pharmacological interest, led to an efficient reinsertion of this bulky membrane protein exhibiting extra-membrane subunits in the planar pep-tBLMs (Figure 4), while retaining its lateral mobility [200]. (*GPCRs are an important class of membrane receptors implied in numerous diseases and cellular dysfunction with a great relevance in pharmaceutical research and drug screening applications. CXCR4 represents an important therapeutic target involved in cancer cell migration and in HIV-1 entry*). The functionality of the reincorporated receptor was assessed by ligand binding assays using SPRi, and revealed that the receptor retains both its native folding and its proper orientation in the planar pep-tBLMs, suggesting that the opening of the proteoliposomes induced by the AH peptide followed a "parachute" mechanism. This successful result could probably also be ascribed to the fact that proteoliposomes were produced by cell-free expression that allows a vectorial insertion of proteins in the membrane directly in a functional orientation (see

Section 5). The possibility to create pep-tBLMs inserting the functional GPCR in a microarray format by spotting P19-4H on micropatterned support for multiplexed drug screening has been also demonstrated [43]. Due to being based on peptide tethers, this versatile approach can be considered for the reconstitution of other membrane proteins and even, to study the influence of the peptide composition of the sub-membrane compartment on the functionality of the reconstituted integral MPs, as one can see it in natural cell membranes.

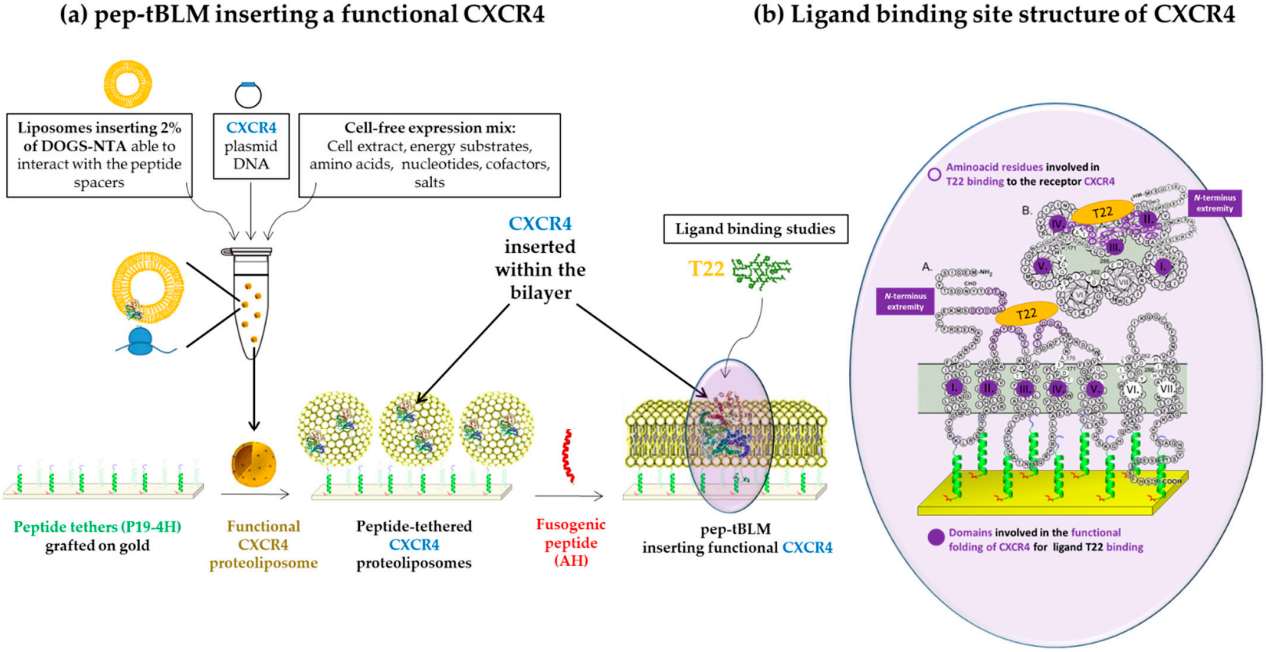

Figure 4. (**a**) pep-tBLM obtained by "top-down" approach, integrating a functional C-X-C motif chemokine receptor 4 (CXCR4), a seven-transmembrane protein belonging to the large superfamily of G-protein-coupled receptors (GPCRs) of high pharmacology interest. The functional proteoliposome has been previously obtained by a cell-free expression system in presence of pure liposomes; (**b**) side (A) and top (B) views of the binding site structure of functional CXCR4 inserted in the pep-tBLM with domains and aminoacid residues (highlighted in purple) involved in the specific binding of T22, an antagonist ligand which specifically inhibits human immunodeficiency virus type 1 (HIV-1) infection mediated by CXCR4. This structure reveals that the binding site of T22 is localized outside the membrane and that 5 domains need to be correctly folded for a functional activity. Hence, characterizing T22 binding is not only a good indicator of the functional folding of the receptor, but also gives information about the orientation of the receptor inside the membrane (adapted with permission from [200]. Copyright 2017, American Chemical Society).

Table 3. Non-exhaustive lipid compositions leading to the formation of a fluid and continuous pep-tBLMs (Reprinted with permission from [200]. Copyright (2017), American Chemical Society).

Lipid Composition	Molar Percentage (Mol%) [1]
POPC	100
DOPC	100
DOPC/DOPS [2]	75:25
DOPC/DOPS doped with fluorophores	75:25
Egg PC/brain PS	68:32
Egg PC/brain PS/brain PIP_2 [3]	68:30:2
DOPC/DOPE/DMPA [4]/Chol	31:17:20:32
POPC/SM [5]/POPE [6]/Chol [7]	44:35:10:11

[1] All doped with 2% of DOGS-NTA-Ni; [2] 1,2-dioleoyl-sn-glycero-3-phospho-L-serine; [3] phosphatidylinositol-4,5-bisphosphate; [4] 1,2-dimyristoyl-sn-glycero-3-phosphatidic acid; [5] sphingomyelin; [6] 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine; [7] cholesterol.

4.6. Protein-tBLMs or ptBLMs

In most tBLMs previously described, and allowing the incorporation of MPs, the protein orientation in the membrane is rather casual; the addition of either solubilized channel proteins or the fusion of proteo-liposomes results in the spontaneous incorporation of proteins into the tethered membranes, but does not allow for the desired control of their orientation [72]. Indeed, if one of the two extramembraneous domains of the protein is much bulkier than the other, incorporation in a tBLM occurs preferentially with the bulkier domain turned towards the aqueous phase, in view of the limited space of the hydrophilic sub-membrane compartment of the tBLMs. Thus, it is extremely difficult to control the insertion and this random orientation may prevent the functional analysis of the protein. Moreover, the surface concentration of the reconstituted protein in the lipid bilayer is not well controlled.

To overcome these problems, another strategy has been proposed to develop tBLMs: it concerns the tethering of membrane proteins, instead of the membrane lipids [175] (Figure 5). First developed by Knoll's group [112], this tBLMs are called *Protein-tBLMs* or *ptBLMs*. For this purpose, a recombinant membrane protein presenting a '*His-tag*', e.g., a sequence of typically 6 Histidine genetically added in C- or N-terminus, and solubilized in detergent micelles is first attached by affinity to nitrilotriacetic acid (NTA) moiety grafted to a metal surface via a sulfhydryl group, in the presence of Cu^{2+} or Ni^{2+} cations. In a second step, the detergent molecules are substituted by lipid molecules by in situ dialysis, or in the presence of microporous biobeads and detergent-solubilized lipids, thus forming a lipid bilayer that is tethered by the protein itself. By using *cytochrome c oxidase* (an ubiquitous membrane protein of the respiratory chain in eukaryotes) (*CcO*) as model, the successful reconstitution of the ptBLMs was assessed by SPR, QCM [112] and SEIRAS [107]. The water layer remaining interposed between the lipid bilayer and the NTA moiety can act as an ionic reservoir as demonstrated by EIS with the active protons transport across the lipid bilayer during catalytic redox cycle of the reconstituted *CcO* in the presence of reduced cytochrome c [112]. Catalytic activity and electron transfer through functional coupling of the redox protein to the electrode (referred to as "electronic wiring") have been also assessed by *cyclic voltammetry* (CV) in combination with *surface-enhanced resonance Raman spectroscopy* (SERRS) and provides mechanistic and structural insights into the proton translocation coupled to electron transfer across the redox center, i.e., the heme center, during the catalytic redox cycle in the presence of cytochrome c under aerobic conditions [120,240,241] (for more details, see the review by Naumann & Knoll (2008) [132]).

ptBLMs can be seen as universal systems suitable for immobilization and surface-focused reconstitution of all His-tagged membrane proteins. Purification of the proteins could even be avoided, if the preparation of ptBLMs was performed using the crude cell lysate [112]. This strategy allows controlling the orientation [122,242] and the packing of the reconstituted membrane protein in the bilayer [243]. In contrast, it is only relevant for studies of membrane properties or protein functions in which the lateral mobility of the protein is irrelevant since all the proteins are immobilized on the surface [71]. Given examples are redox proteins, like *cytochrome c oxidase*, that one might want to connect electronically to the base electrode because they need to be "wired" to the support for an efficient heterogeneous electron transfer between the external circuit and the redox center of the protein [76]. The ptBLMs methodology has been applied (i) to study the voltage-dependent structural changes of a seven-helical transmembrane protein sensory *rhodopsin II* belonging to the family of microbial rhodopsins that act as light-driven pumps [244], (ii) to investigate photoexcitation of bacterial reaction centers (RCs) from *Rhodobacter sphaeroides*, a purple bacterium that can obtain energy through photosynthesis [245], or (iii) to functionally probe the energy-converting *NADH* (reduced nicotinamide adenine dinucleotide)-*ubiquinone oxidoreductase*, the respiratory complex I of *Escherichia coli*, against various substrates and inhibitors [246] via SEIRAS, SERRS or CV. It has been also transposed to functionalize hydrogel deposited as cushions on indium-tin oxide (ITO) used as transparent electrode. In this new approach of polymer-supported lipid membrane, the hydrogel was functionalized

with NTA group; then, the His-tagged enzyme (i.e., *CcO*) was first immobilized and the lipid bilayer was then formed around the immobilized protein. Since the mesh size of the hydrogel was smaller than the protein size, ptBLMs were formed only on the top of the hydrogel cushions. EIS showed good electrical sealing properties and in the presence of reduced cytochrome c, CV demonstrated that CcO was incorporated into the gel-supported ptBLM in a functionally active form [247].

Recently, ptBLMs have been transposed from flat surfaces to spherical particles from µm sized agarose gels beads [248,249] to nm sized silica nanoparticles [250], to develop proteo-lipobeads (PLBs) applicable to fluorescence and UV/Vis spectroscopies, and laser-scanning confocal fluorescence microscopy (LSM) [133]. Multi-redox center proteins (MRPs), such as *cytochrome c oxidases* (CcO) [249,250] or photosynthetic reactions centers (RCs) [248,251] were used as models to demonstrate that these enzymes keep their electron and proton transfer activities on PLBs, which opens a new way for investigation of reconstituted integral membrane proteins in bilayer lipid membranes.

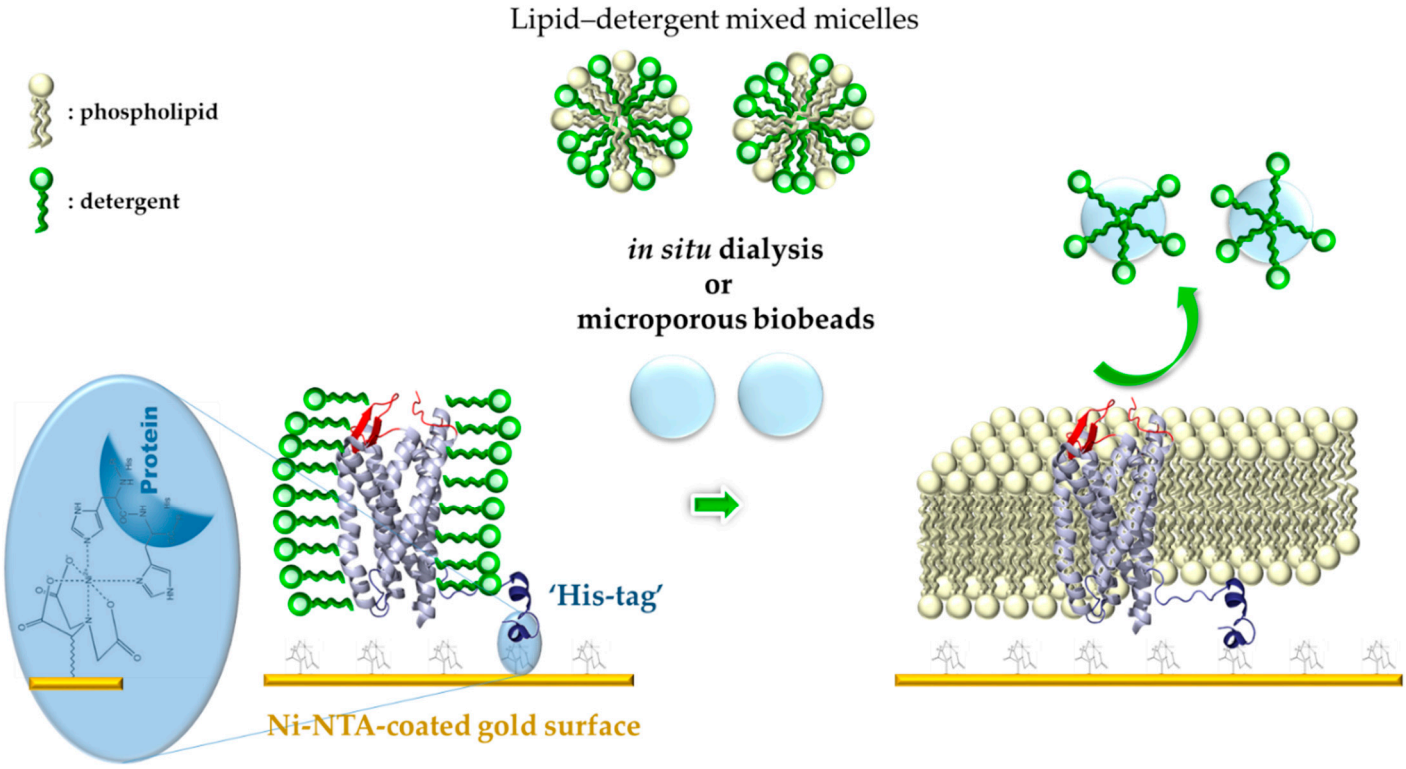

Figure 5. Principle of formation of *protein-tBLMs* or ptBLMs. A recombinant membrane protein with a *'His-tag'* in C- or N-terminus solubilized in detergent micelles is first attached by affinity to nitrilotriacetic acid (NTA) moiety grafted to a metal surface in the presence of Ni^{2+} (or possibly Cu^{2+}) cations. The lipid bilayer surrounding the attached protein is obtained in a second step by in situ dialysis or using microporous biobeads in the presence of lipid–detergent mixed micelles (adapted with permission from [107]. Copyright 2004, American Chemical Society, and with permission from [132]. Copyright 2008, American Vacuum Society).

5. Cell-Free Expression for Reinsertion of Membrane Proteins before or after Formation of tBLMs

One of the major difficulties with regard to the reconstitution of MPs in tBLMs is the possibility of preparing proteoliposomes which retain their functional activity for their use during the vesicle fusion step. In addition, the material losses and/or the possible protein denaturation that could be encountered during this latter step, when using classical "leaflet-by-leaflet" formation of tBLMs in the "bottom-up" approach, leads more often to a very low amount of proteins which is finally reinserted into the tBLMs, and at the end of the day, does not allow to measure any protein activity.

To address this issue, the reconstitution of integral MPs produced in vitro by cell-free expression synthesis directly into preformed tBLMs has been proposed, whatever the tethering molecules used e.g., polyethylene glycol (PEG) [39], *telechelics* [38], peptides [37,203,205,230,252] or proteins [253]. The basic idea is to bypass the difficult expression, purification and reconstitution procedures inherent to dealing with integral MPs. The production of recombinant membrane proteins by classic overexpression techniques presents some limiting features (i.e., low expression rate and/or cytotoxicity for the host cells). Another common problem is the detergent-based purification and refolding of MPs, possibly aggregated, in phospholipid environment like liposomal membranes into their native functional conformation. Cell-free transcription/translation systems are based on gene expression in the presence of cell extracts of various species [254–261] like bacteria (e.g., *E. coli*), insects (e.g., *Spodoptera frugiperda*), plants (e.g., *wheat germ*) or eukaryotes (e.g., rabbit reticulocytes). The relevant gene, provided in the form of cDNA (complementary deoxyribonucleic acid), is mixed with the cell lysate and the translated protein directly inserts in the membrane systems provided, generally liposomes, nanodisks or amphipols (a class of amphiphilic polymers designed to keep membrane proteins soluble in water without the need for detergents). One of the advantages of these in vitro systems over classic in vivo systems, is their ability to synthesize cytotoxic MPs, regulator or unstable proteins. Moreover, they are completely open systems, allowing each parameter of the reaction (such as pH, redox potential, ionic strength etc.) to be modified depending on the target protein. They offer the possibility of the integral proteins to spontaneously reincorporate in lipid membranes in a functional orientation and sufficient amount. Currently, many research workers have opted for these synthesis systems as tools for structural and functional studies [261,262].

Therefore, a number of membrane proteins have been successfully reincorporated in tBLMs. They mainly concern integral proteins like (i) GCPR, e.g., *olfactory receptor 5* (i.e., *OR5*) [230,252], *cytochrome* bo_3 *ubiquinol oxidase* (i.e., *Cyt-*bo_3) [37,203] or *hERG* (*human ether-a-go-go-related gene)* potassium channel [205] in pep-tBLMs formed with the natural P19 peptide spacer, for which the orientation has been assessed by immunolabeling and/or surface plasmon enhanced fluorescence spectroscopy (SPFS) [203,230]; (ii) the human voltage-dependent anion channel *(VDAC*) of the outer mitochondrial membrane in S-layer supported bilayer [253]; (iii) Aquaporin Z [39] in PEG-tBLMs; (iv) a plant membrane transport protein, designated *Bot1* [38] or *OprF* porin of *Pseudomonas aeruginosa* [195] into an anchorlipid-tBLM. Surprisingly, the functionality of the inserted protein was only reported for GPCRs [230], *Bot1* [38] and *OprF* [195].

In order to investigate the conformation of 7TM model membrane protein *bacteriorhodopsin* (BR), as a GPCR model, in a defined orientation by AFM-based force spectroscopy, Bronder et al. have proposed a combined approach between (i) cell free expression of BR that contains a His-tag, (ii) the formation of sparsely PEG-NTA group grafted on quartz glass substrate and diluted with a PEG- anchor lipid (PE), and (iii) the completion of the bilayer by applying a mixture of the protein with free lipid solubilized by detergent to form the ptBLMs. The authors succeeded in performing the study of MPs in a native conformation and in a defined orientation by single-molecule force spectroscopy, which is challenging to carry out with a random protein orientation [86].

Therefore, cell-free expression appears to be a promising approach to prepare functional proteoliposomes that can be used for the preparation of tBLMs [200] or for the direct insertion of membrane proteins into tBLMs. By cell-free expression, membrane proteins can be incorporated into the model membranes in statu nascendi and assume to correctly fold during the synthesis from cellular extracts. This approach addresses the challenge of considering the incorporation of a broad spectrum of MPs that could not be reinserted using conventional membrane protein purification methods.

6. Perspectives: Are tBLMs Placed in the 3R System?

Since the mid 1990s, the design of tBLMs has currently evolved towards more and more sophisticated models with the aim to reproduce as close as possible the complexity of the lipid composition which surrounds MPs. Now, the design of tBLMs starts to resemble to a molecular toolkit giving the opportunity to expand the range of applications of this type of artificial membranes. Several examples have demonstrated the utility of these lipid platforms for studying fundamental research on MPs and their potential for practical applications in biosensing and drug screening. The big plus for the further boom of this technology is the increasing number of techniques available for their characterization. Additionally, the cell-free protein synthesis method represents a promising alternative to solve the problems associated with the cell-based purification methods and opens new perspectives for reconstitution of a large panel of MPs difficult to extract. Therefore, a very reasonable strategy is to pursue the development of artificial membrane platforms, like tBLMs, to analyze drug candidates, as an alternative method. Currently, animal models are used for drug screening, which is not only time consuming, expensive and unethical, but also has variable results. For this reason, preclinical testing using artificial membranes systems integrating MPs has attracted increasing attention and is an actively developing field [263]. Regulatory authorities have endorsed the principle of the 3Rs: replacement, refinement and reduction, a concept developed at the beginning of the 1960s by Russell and Burch [264] and now largely accepted as criteria for limiting the animal use in research and testing. Under this prospective, tBLMs appear as a serious potential candidate for the reduction of animals involved in structural or functional studies of MPs [14]. Indeed, tBLMs can be viewed as a powerful complementary tool for a better understanding of the interactions and effects of lipid membrane-targeting compounds.

Author Contributions: Data curation—writing original draft—review and editing (A.P.G.-E.); writing—review and editing (O.M.). All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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