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Photosynthetic Proteins in Supported Lipid Bilayers – Towards a Biokleptic Approach for Energy Capture

Lei Wang1,2, Johannes S. Roth1, Xiaojun Han2, and Stephen D. Evans1*

1, School of Physics and Astronomy, University of Leeds, Leeds LS2 9JT, United Kingdom.
2, State Key Laboratory of Urban Water Resource and Environment, School of Chemical Engineering and Technology, Harbin Institute of Technology, Harbin 150001, China

Abstract

In nature, plants and some bacteria have evolved an ability to convert solar energy into chemical energy usable by the organism. This process involves several proteins and the creation of a chemical gradient across the cell membrane. To transfer this process in a laboratory environment several conditions have to be met: i) proteins need to be reconstituted into a lipid membrane, ii) the proteins need to be orientated and functional, and finally iii) the lipid membrane should be capable of maintaining chemical and electrical gradients. Investigating the processes of photosynthesis and energy generation in vivo is a difficult task due to the complexity of the membrane and its associated proteins. Solid supported lipid bilayers provide a good model system for systematic investigation of the different components involved in photosynthetic pathway. In this review, we describe the progress made to date in the development of supported lipid bilayer systems suitable for the investigation of membrane proteins and in particular for the reconstitution of proteins involved in light capture.

1. Introduction

Photosynthesis is the main way of generating and storing useful energy for a variety of organisms, especially in plants and bacteria. The ability to utilize solar energy evolved very early after the first forms of life appeared on earth. Although it is still debated as to how bacteria evolved from the first form of photosynthesis in cyanobacteria to having photosystems I and II and reaction centers in a single organism, the process, which converts solar energy into chemically stored energy, is well understood.

Apart from the proteins required to convert energy from sunlight into a storable form of energy the organisms in which photosynthesis occurs also need membranes, which allow them to create chemical or electrical gradients and host the photosynthetic proteins. These membranes also act as boundaries in cells, protecting the inside of a cell from the environment and allowing the cell to compartmentalize itself. The variety of functions fulfilled by the cell membrane also leads to complex interactions between individual membrane components making the observation of a single process complex in vivo. Therefore, solid supported lipid bilayers (SLBs) have been introduced as a model system for the cell membrane. In addition
to providing the essential properties of membrane fluidity and impermeability their planar geometry allows for more in-depth studies of relevant processes in the membrane. Using SLBs, various membrane processes and associated proteins have been investigated, including electron transfer, lipid-protein interactions, and ion-channels. Since membrane proteins play important parts in all living organisms, they have been the focus of much of this research. Their functions range from receptor proteins, relaying signals to transport proteins, regulating the ions crossing a membrane. In order to investigate these proteins in SLBs, they have to be incorporated into the membrane while retaining their structure and function. This can be achieved in different ways: self-inserting proteins can be introduced into pre-formed SLBs (formed by methods such as Langmuir-Blodgett/Langmuir-Schaefer (LB/LS) deposition, spin coating or vesicle fusion), alternatively protein containing liposomes, proteoliposomes, can be used to form the SLB directly via rupture at the solid-liquid interface to yield SLBs containing membrane proteins. Both approaches have been used to form large area SLBs on different substrates while retaining the membrane stability and fluidity. To enhance the formation of SLBs on substrates on which an SLB normally would not form, anchor molecules that enhance the adsorption and rupture of lipid vesicles on the surface can be used, resulting in tethered lipid bilayer membranes. Despite the versatility of SLBs there are some disadvantages that come with the approach of having a solid support just 1 nm away from the proximal leaflet of the membrane. When membrane proteins with an extramembranous domain larger than the thin cushioning water layer are incorporated into the membrane, these proteins interact with the substrate and can be denatured and thus lose their original functionality. To overcome this problem, lipid membranes can be supported by a soft polymer layer. If the polymer is hydrophilic and biocompatible, the interaction of the proteins with the substrate can be minimized while retaining the fluidity and impermeability of the membrane. Using polymer cushions, where necessary, the SLB is an approach offering the opportunity for different analytical methods to be applied leading to the development of biosensors and enhanced understanding of transmembrane proteins. The primary methods used to characterize the SLBs include: impedance spectroscopy and electrochemistry for the investigation of membrane integrity, surface plasmon resonance (SPR), plasmon-waveguide resonance (PWR) spectroscopy, quartz crystal microbalance with dissipation monitoring (QCM-D), neutron reflectivity (NR) or atomic force microscopy (AFM) for the study of the structural state of the membrane, and fluorescence recovery after photobleaching (FRAP) and fluorescence correlation spectroscopy (FCS) for determination of lipid mobility. Substrates with different morphologies or surface chemistries, such as nanopores or –posts, can be used to further understand the influence of the surface on the SLB. In addition, SLBs offer further advantages: access to both sides of the membrane, localization of proteins within the membrane, and enhanced membrane longevity compared to giant unilamellar vesicles (GUVs) or black lipid membranes (BLMs).
Based on these advantages, SLBs have been utilized for investigations of a broad range of transmembrane proteins. This review provides a brief outline of the different approaches followed for bilayer formation in general before focusing on the systems developed to investigate membrane proteins involved in energy capture and storage from light, especially rhodopsins, light harvesting proteins, reaction centers, cytochromes, and ATPase.

2. Formation of supported lipid bilayers (SLBs):

SLBs can readily be formed on several surfaces using a variety of different methods. These include LB/LS deposition, whereby the individual lipid leaflets that make up the bilayer are deposited sequentially using the Langmuir-Blodgett and Langmuir-Schaeffer methodologies, electrostatically driven vesicle adsorption, rupture and fusion, which is perhaps the easiest and most common route for bilayer formation on silica and mica surfaces or tethered lipid bilayer and hybrid bilayers which use self assembled monolayers (SAMs) to drive vesicle rupture and bilayer formation. In this section we provide an introduction to different substrates used for bilayer formation and then focus on the methods used to obtain SLBs.

2.1 Different substrates for SLBs formation

2.1.1 Solid substrates

SLBs can easily be obtained on a broad range of native surfaces, such as glass, quartz, mica, silica, porous silica nanospheres, ITO, Si₃N₄, photo-oxidized polystyrene surfaces, as well as single crystals of TiO₂ and SrTiO₂. Glass, mica and silica have proven themselves as particularly facile substrates for stable and reproducible SLB fabrication (Figure 1a). These substrates are generally of high-surface free energy and exhibit net surface charge. Dependent on the surface charge, different requirements are necessary for bilayer formation, for example the inclusion of specific ions into a system or the incorporation of charged lipids into the vesicles. For example stable zwitterionic SLBs could only be formed on TiO₂ in the presence Ca²⁺ ions.
2.1.2 SAM systems
When electrical access to the membrane is required, the insulating support can be replaced with a conductive surface, such as gold\cite{40}, silver\cite{41} or platinum\cite{42}, and modified with a SAM, forming a hybrid lipid bilayer (HLB) or a tethered lipid bilayer (TLB) (Figure 1b and 1c).

There are some reviews on the utilization of self-assembly for the modification of electrode surfaces\cite{43} but in short if a hydrophobic SAM layer is formed on the metal surface a single lipid leaflet can be adsorbed or deposited on top of it to produce a HLB (Figure 1b). This method of SAM formation pioneered by Nuzzo and Allara in 1983\cite{44} using methyl-terminated alkanethiols on gold to obtain a well-defined hydrophobic surfaces. However, the alkanethiol layer is more crystalline than a normal leaflet of a lipid bilayer, thus leading to a less biomimetic environment for the insertion of transmembrane proteins\cite{45}. To overcome these limitations the SAM layer can be made to consist of a fraction of lipid linked molecules plus some short hydrophilic “spacer” molecules. This allows for a bilayer being formed in which the lipid like molecules of the SAM insert into the lower leaflet of the adsorbed bilayer leading to an architecture that consists of a complete outer leaflet and a partial inner leaflet and thus making these TLBs more “natural” than the HLBs\cite{46}. The main advantage of these methods is the coupling of the phospholipid bilayer directly to a metallic surface, which makes electrical impedance and cyclic voltammetry measurements possible\cite{47}. Due to the strong interaction between the alkanethiol layer and the underlying substrate HLBs and TLBs are more robust than their solid supported counterparts\cite{48}. For example, their properties can remain unchanged.

Figure 1. Different supporting strategies. a) solid supported bilayers; b) SAM system; c) tethered supported bilayers; and d) polymer cushion solid supported bilayers.
Even after being dried and rehydrated when formed at an air-water interface\(^5\), \(^11\)–\(^50\) Using the TLM approach, it was shown that the space between the bilayer and the solid support can be used as an ion-reservoir and the whole system can be used as a bio-sensor\(^{51}\). Both thiolipids and thiocholesterol derivatives have been used as a lipid membrane tethers. This systems allows for pre-formation of the SAM on the gold substrate and subsequent deposition of vesicles on the substrate and results in a sufficiently small to medium sized reservoir (1–2 nm thick) between the bilayer and the gold to incorporate transmembrane proteins\(^{52}\). A potential advantage of thiol based SAM approach is the ease with which the SAMs can be patterned using either photolithography\(^{53}\) or microcontact printing\(^{54}\).

Recently, a floating supported bilayer was fabricated based on a SAM system, where the previous commonly used silane-grafted phosphatidylcholine on silicon is replaced by a thiol-grafted phosphatidylcholine. In this work, the NR data showed that the coverage of the SAM is much greater (almost 100%) than that typically seen for silane SAMs, which would be better for the fabrication of SLBs with less defects\(^{55}\). Additionally, Jeuken et al. reported that protein-protein interactions regulate the activity of a respiratory-chain enzyme by changing the direction or bias of catalysis, which is based on the reconstitution of proteins in a SAM system\(^{56}\).

2.1.3 Polymer cushioned SLBs

Although solid supported SLBs and TLBs are excellent as sensor platforms for the investigation of many cellular processes, the rigidity of the support and small reservoir (~ 1 nm) between the bilayer and the support is potentially a problem. Using SLBs, the underlying water layer cannot protect peripheral portions of transmembrane proteins from potential immobilization or denaturation when the transmembrane proteins are in contact with the substrate\(^{55}\). Hence, polymer-cushioned membranes were developed in the 1990’s to obtain a reservoir between the membrane and the substrate with a separation of the order of 10 nm\(^{57}\) (Figure 1d). The main advantage of this approach is the increased separation between the membrane and the solid substrate, with the soft polymeric materials acting as a lubricating layer between the membrane and the substrate\(^{55}\). In these systems the frictional coupling between the SLBs and the substrate is reduced, thus decreasing the risk of protein denaturation\(^{59}\). Several investigations of protein / enzyme activity were realized based on polymer-cushioned SLBs\(^{57}\)–\(^{60}\). Although the polymer cushion can promote self-healing of local defects in the membrane over macroscopically large substrates, not all the bilayers formed on polymers were uniform and membrane devoid patches or defects were often present\(^{61}\). This has led to the following suggested requirements for polymer cushions to be used as a bilayer supports: i) high surface uniformity (low rms roughness) to avoid irregularities in the bilayer and ii) hyrophilicity and chemical inertness to avoid reactions with the membrane or proteins within it\(^{59}\). Based on these requirements, some polymers are good candidates for cushions, such as i) carbohydrates: dextran\(^{62}\)–\(^{63}\), cellulose\(^{63}\)–\(^{64}\), chitosan\(^{64}\), agarose\(^{65}\), hyaluronic acid\(^{66}\); ii) polymers: polyacrylamide\(^{67}\), poly(4-vinyl-benzenesulfonic acid)\(^{39}\).
polystyrene-b-poly(4-vinyl-N-methylpyridine iodide), iii) lipopolymer tethers: polyethylene glycol (PEG), poly(2-methyl-2-oxazoline) (PMOXA), polyethyleneimine (PEI), and iv) surface layer proteins.

For example, a thin hydrogel layer of poly(N-(2-hydroxyethyl)acrylamide-co-5-acrylamido-1-carboxypentyl-iminodiacetate-co-4-benzoylphenyl methacrylate) (P(HEAAm-co-NTAAAm-co-MABP)) was used as a soft ‘cushion’ on ITO electrodes, providing a smooth and functional surface to form SLBs onto, which was utilized for the reconstitution of cytochrome c oxidase. Additionally, PEG polymer brushes functionalized with fatty acid moieties (lipo-PEG) were utilized as tethers for vesicles, which, after rupture, formed a continuous membrane. Similarly, a versatile approach for the generation of polymer cushioned SLBs is spin-coating of membrane lipids onto PEG, followed by the incorporation of transmembrane proteins by the fusion of proteoliposomes. However, if the lipo-PEG density were too high, there would be a large immobile fraction of lipids in the lower leaflet. Hence, a new approach using a multistep chemical process for the modification of silicon substrates with polymers of different molecular weights (lengths) was utilized to obtain a highly hydrated surface, which is helpful for the fabrication of SLBs with a uniform density of the polymer layer.

The mobility of lipid bilayers formed on polymer cushions is usually reported to be around 2 µm²/s, which is comparable with that found for lipids in a solid supported membranes on silica supports. However, in cases of very weak coupling of the membrane to the polymer support, the diffusion can reach up to twice the value of that obtained for a glass support. When deposited on a polymeric support, lipid bilayers exhibit similar capacitance values as typically observed on a tethered support, i.e. around 1 µF/cm², notwithstanding this the resistance values determined for such bilayers are relatively low, around 25 kΩ/cm², indicating relatively high defect densities. A further development of this approach has been to introduce lipid tethers into the polymer cushion, to act as anchors for the SLB. A good review of this approach is given by Ribaud et al.

2.2 Methods for bilayer formation

Generally, there are two main methods for the fabrication of SLBs on planar supports, the LB/LS method and bilayer formation via the adsorption, fusion and rupture of vesicles from an aqueous solution. The LB method involves the transfer of a lower leaflet of lipids from the air-liquid interface onto a hydrophilic solid support such that the hydrophobic tails are oriented toward the air interface. The second lipid leaflet is then added by the LS method, which brings the support into contact with the lipid monolayer, compressed at the air/water interface, in a near horizontal configuration. The first report of the application of the LB technique in the fabrication of SLBs comes from the 1980s, where monolayers were successfully transferred sequentially onto several different substrates. The LB/LS method can be utilized for the
fabrication of both symmetric and asymmetric lipid bilayers. Unfortunately, this method is not suitable for
the incorporation of transmembrane proteins, since the approach builds the bilayer in two separate
monolayer steps. In addition, the procedure is time consuming and requires well-controlled conditions for
the deposition process. Despite these limitations, there are some investigations of protein activity using this
approach, such as rhodopsin, bacteriorhodopsin, hydrophobin, and gramicidin. Polarization
modulation infrared reflection absorption spectroscopy or X-ray reflectivity combined with surface
pressure-area isotherms were utilized for the characterization and obtaining the in situ information of the
secondary structure and orientation of the proteins. This basic method offers an opportunity to study the in
plane morphology of SLBs and the functions of proteins.

The second main method for the fabrication of SLBs is the adsorption, rupture and fusion of vesicles to
form SLBs on a substrate. This method was pioneered by McConnell and co-workers and
further developed by others. Vesicle fusion has been utilized to form SLBs from various lipids.
Vesicle fusion is an easy to use method, and, in contrast to LB/LS, can be achieved with lower equipment
costs and generally results in high quality SLBs. Importantly, the method allows for the inclusion of
membrane proteins into the vesicles and thus into the SLBs in a more facile manner. For example, in the
work of McConnell, the H-2Kk protein was reconstituted into egg phosphatidylcholine-cholesterol
vesicles by detergent dialysis, followed by the creation of a planar membrane on glass. The H-2Kk-
containing membrane is useful and significant as a model surface and is capable of eliciting a specific
cytotoxic response when brought into contact with a cell. More recent work is based on the
combination of SAM systems with the fusion of proteoliposomes. For example gold substrates
modified with a mixed thio-cholesteryl / 6-mercaptohexanol SAM, was incubated with vesicles to
produce tethered SLBs and the quality determined using impedance spectroscopy. Based on this approach
cytochrome b0 was studied. Thus demonstrating that the approach can be applied for vesicles containing
membrane proteins and suitable for studying proteins from the respiratory chain. Further, the approach
allows SLBs to be formed from synthetic and/or native membranes though it is noted that vesicles with
high protein content (>20%) are often found difficult to rupture on the surface.

3. Incorporation of proteins into SLBs for light harvesting and energy storage

The absorption of a photon by a light harvesting protein generates an excited electronic state, which is
transferred in several steps through the antenna complexes until it reaches a reaction center. Here the energy
is converted into chemical energy in the form of charge separation across the lipid membrane. During this
process a ubiquinone (UQ), which is diffusing freely in the membrane, is reduced. When the reduced UQ
then arrives at a cytochrome the energy released by oxidizing the UQ is used to establish proton gradient
across the membrane. Such a proton gradient can then be used by other proteins, such as ATP synthase to produce ATP (Figure 2 right). Through this process light energy can be absorbed, converted and stored as chemical energy and is used by many plants, algae, and photosynthetic bacteria.

![Diagram of light energy conversion](image)

**Figure 2.** SLBs with incorporated photosynthetic transmembrane proteins. Left: Proteorhodopsin embedded in an SLBs together with ATPase. Right: Photosynthetic processes as found in bacteria, such as Rhodobacter sphaeroides. (Not to scale).

Perhaps one of the simpler systems for energy conversion is found in bacteriorhodopsin and proteorhodopsin, from marine planktonic bacteria, archaea and eukaryotes in which a single protein acts as a light driven proton pump. There are reports that their properties could be influenced by the acids in the lipid membrane around them. Figure 2 (left) shows a schematic for a SLB with embedded proteins to create systems that would form the simplest biomimetic analogues to the full native systems. The process shown on the right involves more membrane proteins and is an example of the photosynthetic system found in Rhodobacter sphaeroides. Attempts to reconstitute both types of systems into planar bilayers are not only of interest for addressing biological questions but also from a synthetic biology perspective where functional model membranes could be combined with synthetic protein analogues. Here we review the progress made to date in the incorporation of such systems or components of such systems into planar membrane assemblies. We particularly focus on SLBs containing rhodopsin, LHs, cytochromes, and ATPase and combinations of these.

### 3.1 Rhodopsin
Rhodopsin\textsuperscript{101} is one of the best-characterized G-protein coupled receptors and the effects of lipid composition and bilayer structure on its function have been studied since the 1970’s \textsuperscript{19, 102}. Further, there are excellent biochemical and biophysical characterizations of the visual signal cascade, and it is possible to obtain rhodopsin and other components of the cascade in high purity and reasonable quantity, thus making it a model transmembrane protein for studying in supported lipid bilayers \textsuperscript{27b}. Early research focused on the investigation of the structure of rhodopsin incorporated into SLBs, where the LB method was employed \textsuperscript{102a}. The authors utilized freeze-fracture electron microscopy to characterize the location of rhodopsin in the ROS membranes \textsuperscript{86}. Similarly, the LB and vesicle fusion methods have been employed to form SLBs with embedded bacteriorhodopsin on platinum/glass surfaces \textsuperscript{42a}. The photoactivity of bacteriorhodopsin in the reconstituted bilayers was monitored using electrochemistry and compared with that of natural membrane fragments containing bacteriorhodopsin on platinum surfaces. A clear photocurrent was observed from the SLBs with bacteriorhodopsin, while there was no significant signal from the protein-free SLBs. Further investigations focused on the immobilization of G-protein coupled receptors and their interaction with G-proteins. For instance, Vogel and coworkers studied rhodopsin-transducin coupling in patterned membranes \textsuperscript{27a}. In this work, the authors successfully demonstrated light activated release of transducin from the rhodopsin using surface plasmon resonance (SPR) and concluded that the native functionality of rhodopsin was preserved in the SLB. Later studies used time resolved SPR to track ligand binding, G-protein activation, and receptor deactivation of rhodopsin \textsuperscript{103}. From these studies a method of flow-mediated reconstitution of G-protein coupled receptors was reported (see Figure 3) \textsuperscript{104}. The activity of the reconstituted receptor was demonstrated by monitoring the rhodopsin-mediated dissociation of transducin. A clear difference in reflectivity between the flow cell containing light activated proteins and the control sample could be seen under illumination, which was used to show the photoactivity of the reconstituted receptor. The detergent mediated reconstitution method used for protein incorporation into SLBs has advantages in the ease of formation of the SLB. PWR spectroscopy, with enhanced sensitivity and spectral resolution (due to narrower line widths) and the ability to distinguish between mass and conformational changes, was used for the characterization of the kinetics and affinities involved in ligand binding to G-protein coupled receptors (Figure 4) \textsuperscript{105}. Tollin and coworkers reported the effects of lipid composition on conformational changes of rhodopsin induced by light, monitored using PWR, especially the observation of the formation of metarhodopsin II \textsuperscript{27b}. This work also confirmed that; i) lipids that promote a negative spontaneous curvature favor elongation of rhodopsin during the activation process; ii) there is a light activated increase of the G-protein/rhodopsin affinity. Similarly, the photoactivity of rhodopsin and the extent of the conformational transition were measured by the amount of metarhodopsin II using PWR, which was based on the reconstitution of rhodopsin into SLBs with cross-linking of lipid monomers \textsuperscript{19}. Using a similar approach, Saavedra and coworkers investigated the effect of lipid
polymerization on the structure and activity of G-protein coupled receptors. However, the enhancement of the activity of rhodopsin that appeared in the mixture of phosphatidylethanolamine and phosphatidylcholine was eliminated after the polymerization, which is probably a limitation of this method. Furthermore, ultra-high vacuum techniques were used for the structural characterization of SLBs containing rhodopsin, and angle-resolved XPS was employed for the investigation of the location of rhodopsin in SLBs. Another recent work used AFM to visualise the incorporation of UQ in SLBs, as Figure 5. All the work above focused on the photoactivity of rhodopsin in SLBs, but are lacking further use of the photoactivity by other membrane components or investigation of proton pumping or electron transfer. Unfortunately, there is less progress of investigations of rhodopsin in SLBs compared to the investigations in situ or in vesicles, this may be due to the fact that the reconstitution of rhodopsin into an SLB is more difficult than using native membranes fragments or undertaking in situ experiments.
Figure 3. On surface reconstitution of bilayers. (A) Detergent-lipid mixed micelles are injected. (B) When amphiphile-free buffer is running through the flow cell, a detergent monomer concentration is maintained in the mobile phase leading to a very quick extraction of the detergent from the surface. (C) The lipids remain attached to the surface and are able to form a continuous SLB, which hosts functional membrane proteins. The relative proportions of the components in the diagram are not displayed to scale.

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Figure 4. PWR curves (A, p-polarized; B, s-polarized) acquired at each stage of an experiment with a poly(bis-SorbPC/mono-SorbPE) bilayer (1:1 (mol/mol)). The shift in the reflectance minimum and therefore the plasmon resonance from 1 to 5 shows the adsorption of molecules on the PWR prism. - Reproduced with permission. Copyright © 2008 American Chemical Society.

Figure 5. Topographic AFM images of an SLB of DPPC (left) and DPPC: UQ 5:1 (right). Obvious height differences can be seen for the SLB containing a high concentration of UQ. Reproduced with permission. Copyright © 2013 American Chemical Society.
3.2 Light harvesting complex

Light harvesting complexes (LHs) play an important role in the photosynthesis acting as efficient (light) energy absorbers and transferring this energy to the reaction centers (RCs), in the form of excited electronic states, the process ending in the conversion of ADP to ATP. There have been several investigations on the incorporation of LH1 and LH2 complexes into SLBs towards the construction of artificial solar-energy converters. Initial work on reconstituted light harvesting proteins in SLBs used the LB technique. This work showed some examples of artificial photosynthetic systems with not only the incorporation of light harvesting proteins and charge separation but also the fabrication of an artificial light-to-current converter.

In the early 1980s work to reconstitute lipid membranes with LHs into ordered arrays was conducted. In this work, XRD and electron microscopy data proved that planar arrays formed by the LHs incorporated into lipid layers were crystalline at the molecular level. A major draw-back of this work, from the viewpoint of trying to reconstruct the LH systems in an artificial membrane was that the proteins were incorporated into lipid monolayers, which do not have the same properties as natural lipid bilayers. To the best of our knowledge, the first systematic investigations regarding the reconstitution of LH2 in lipid bilayers were conducted in 1994. In this work, near-field fluorescence imaging (NFI) was utilized for the first-time for the characterization of LH2 in SLBs on a mica substrate and the fluorescence lifetime of LH2 complexes was also measured. Despite the advantages these planar systems offer in terms of characterization there has been surprising few functional studies of the LH2 complexes. Negata et al. showed the incorporation of LH1 complexes in SLBs on an ITO substrate, which allowed electrical characterization of the LHs by determination of the photocurrent. The action spectra revealed two peaks corresponding to the absorption bands of the Zn–BChla complex and were used to demonstrate the inclusion of functional LH1 complexes into SLBs. AFM has been commonly used for surface characterization of SLBs containing light harvesting proteins. Cogdell and coworkers studied bilayers containing LH2 using AFM in tapping and contact mode, under ambient and physiological conditions, and were able to show the difference of the endoplasmic and periplasmic sides of LH2 complexes. In addition, the dipole strength for energy transfer between two neighboring LH2 proteins was estimated based on Förster theory. Another AFM investigation on the incorporation of the reaction centre into SLBs has shown high resolution spatial arrangement of the different components of the light harvesting system, LH1 and the associated RCs (Figure 6). Subsequently, Cogdell et al. observed the incorporation of LH2 in SLBs using total internal reflection fluorescence microscopy (TIRF) and developed strategies for the domain selective incorporation of LH2. The process of vesicle fusion and subsequent lateral organization of LH2 is shown in Figure 7. The studies described above provide us with fundamental and important approaches for the construction of SLBs that incorporate LHs. However, to date there have been no reports on the reconstruction of a complete photosynthetic system in SLBs.
Figure 6. Membrane remodeling and high-resolution AFM imaging of core complexes. (a) proteins segregated into close-packing areas. (b) core complexes. (c) LH1-RC. Reproduced with permission. Copyright © 2006 The Biophysical Society.
3.3 Cytochromes

Cytochromes are a class of membrane proteins with a heme group as their central feature. Their main use in organisms is to provide a means for electron transport, which, via some creation of a proton gradient facilitates the generation of ATP. There have been numerous reports on the incorporation of cytochromes into SLBs since the 1990s. Initially, a combination of the LB method and consecutive vesicle fusion was utilized for SLB formation and incorporation of fluorescein labelled cytochrome b₅ by Tamm et al. Using FRAP, it was shown that the mobile fraction of the cytochrome b₅ containing SLBs is as low as 35%. This was attributed to the interaction of the protein with the underlying substrate and a possible dependence of the mobility on the orientation of the C-terminus in the SLB. Subsequent studies utilized different substrates such as silver or gold, or biotinylated lipids in SLBs to which biotinylated cytochrome c was bound via a streptavidin linker. These approaches allowed improved control over the orientation of cytochrome in SLBs. Saavedra and coworkers reported a site-specific immobilization strategy using Cys102 or biotin-streptavidin and used linear dichroism to demonstrate control over the orientation...
distribution of the heme groups in the SLB. Their results indicated that there was no requirement of a close packed monolayer on a streptavidin coated surface for the generation of an oriented protein film. However, this work realized the orientation of cytochrome on top of SLBs rather than embedded in SLBs (Figure 8). Hawkridge et al. incorporated cytochrome c into SLBs under flow conditions and used cyclic voltammetry to show the reaction of cytochrome c at the electrode surface. They were able to show an increase in current at the reduction potential of cytochrome c.

As discussed earlier, transmembrane proteins need a cushioning layer underneath the SLB so that the interaction of the extramembranous domains with the surface is minimized. One approach has been to use poly-ethylene-glycol (PEG) to allow for the incorporation of cytochrome b₅ and Annexin 5 into the SLB while retaining their functionality (see Figure 9). In the work of Wagner and Tamm, PEGylated-lipids were utilized as the cushion and linkers for the SLBs which resulted in two types of diffusion for both, cytochrome b₅ and Annexin 5: fast diffusion with a mobile fraction of around 30% and a slow diffusion with a mobile fraction of around 60%. This approach has been shown to work well for proteins with small extramembranous domains but is limited by the thickness of the PEG spacer (<10 nm).

In 2004, Choi et al. reported that cytochrome c self-inserts into a pre-formed bilayer and resides in the hydrophobic core, with a significant change of the mechanical properties of the bilayers. When cytochrome c was added from solution to a pre-formed bilayer, there was no obvious change of the thickness of the SLBs after the insertion of cytochrome. Mass spectroscopy and visible light absorption spectroscopy were used to confirm the presence of cytochrome c in the lipid bilayer, which indicated that cytochrome c was embedded in the hydrophobic core of the SLB, agreeing with the previous conclusion that cytochrome c is either adsorbed to the surface or inserted into the membrane. A further conclusion which can be obtained from this work, is that the insertion of cytochrome c changed the required force to punch the tip of the AFM through the bilayer by approximately 30%.

Very recently, the interaction between calixarenes (CX) and cytochrome c in SLBs was also investigated using AFM. In this work, single molecule force spectroscopy was used for the analysis of the mechanism of interaction between cytochrome c and CX. The data suggested the existence of both electrostatic and amino group specific interactions between cytochrome c and CX, which opens the field to the design and production of surface based biosensors. Other studies have used different approaches, such as cytochrome sensing based on the reaction between cytochrome c and cytochrome c oxidase, the activity of cytochrome as a function of ubiquinol-10 in SLBs based on cyclic voltammetry (Figure 10), and the effect of Zn ions on the proton release of cytochrome c. Although there are some reports about proton pumping by cytochrome c reconstituted within vesicles, there is still less progress of these experiments carried out on SLBs.
Figure 8.
Schematic of biotinylated cytochrome c attached to an SLB using a biotin streptavidin linker. This system was used to determine the orientation of the heme groups in cytochrome c. Adapted from [116]. Copyright © 1998 American Chemical Society.

Figure 9.
Design of a tethered polymer-supported lipid bilayer. Reproduced with permission. Copyright © 2000 The Biophysical Society. Published by Elsevier Inc.
Figure 10. SLB formation on a gold substrate based on cholesterol tethers. This system allows for the measurement of the activity of cytochrome c depending on the ubiquinol-10 content in the SLB. Reproduced with permission[92]. Copyright © 2010 Elsevier B.V.

3.4 ATP synthase

Adenosine triphosphate (ATP) is an intracellular energy-supplying compound, synthesized from ADP and inorganic phosphate by ATP synthase (ATPase) in an endothermic reaction. As discussed above, a proton gradient in an SLB can be generated by different light harvesting proteins. This gradient then drives ATPase and facilitates the production of ATP. For the measurement of the synthetic activity of F₀F₁-ATP synthases proteoliposome based methods are mainly used. However, many robust and simple surface characterization tools cannot be utilized when ATPase is embedded in a proteoliposome. In contrast SLBs are good model systems, where the activity of ATPase can be easily observed and characterized although there may be challenges in the reconstitution of ATPase in SLBs. To date there has been some fundamental work about functional attachment of ATPase, and direct observation of the rotation of the central rotation surfaces. For example, the rotation of a subunit in F₁-ATPase was demonstrated using single molecule fluorescence imaging[122]. Later, the proton gradient driving the formation of ATP was replaced by a mechanical driving force. This was realized by attaching magnetic beads to the head of ATPase and subsequent application of a rotating magnetic field. In this process, the ATPase rotated under the externally applied torque, followed by the generation of ATP, which proved that a chemical reaction can be induced by a force exerted onto a physically remote site[123]. However, this work was not conducted in SLBs preventing the ATPase to be driven by a proton gradient as it would happen in a natural membrane. Reconstituted ATPase in a lipid
membrane formed via the LB method and characterized using SPR have shown the possibility to detect proton activity in the reaction process.

4. Future prospect for energy conversion based on supported membranes.

SLBs can readily be produced in a laboratory using several methods. However, the reconstitution and the characterization of transmembrane proteins in their natural environment needs sophisticated methods, that requires further work. Despite the photosynthetic process itself being well understood and its individual components being studied in several laboratories, to date a full photosynthetic system has not been reconstituted in a synthetic supported lipid bilayer. Artificial photosynthesis on a chip could then be used for energy generation and storage in a chemical system. Importantly, a fully functioning system would then allow the swapping in and out of components, either natural or synthetic, to generate novel light harvesting systems.

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