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Biomimetic interfaces based on S-layer proteins, lipid membranes and functional biomolecules

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Designing and utilization of biomimetic membrane systems generated by bottom-up processes is a rapidly growing scientific and engineering field. Elucidation of the supramolecular construction principle of archaeal cell envelopes composed of S-layer stabilized lipid membranes led to new strategies for generating highly stable functional lipid membranes at meso- and macroscopic scale. In this review, we provide a state-of-the-art survey of how S-layer proteins, lipids and polymers may be used as basic building blocks for the assembly of S-layer-supported lipid membranes. These biomimetic membrane systems are distinguished by a nanopatterned fluidity, enhanced stability and longevity and, thus, provide a dedicated reconstitution matrix for membrane-active peptides and transmembrane proteins. Exciting areas in the (lab-on-a-) biochip technology are combining composite S-layer membrane systems involving specific membrane functions with the silicon world. Thus, it might become possible to create artificial noses or tongues, where many receptor proteins have to be exposed and read out simultaneously. Moreover, S-layer-coated liposomes and emulsomes copying virus envelopes constitute promising nanoformulations for the production of novel targeting, delivery, encapsulation and imaging systems.

1. Introduction

Supramolecular architectures building up biological systems offer a dazzling array of ideas and inspiration for nanotechnology. A pivotal role concerning the functionality in biological systems is accorded to (membrane) proteins. For micro- and nano-systems, several key factors such as material interface properties, preserving biological viability, as well as self-assembly and bottom-up strategy as a device-fabrication methodology must be considered [1].

Proteins, as natural building units, have attracted a significant amount of attention owing to their unique features, such as conferring elasticity, promoting cross-linking, facilitating material degradation, selectivity, fostering biomineralization, providing specific binding functions and in particular self-assembly [2]. Integration of these units with non-living systems will promise to bridge the worlds of conventional engineering and biology and could dramatically contribute to the development of both. On the one hand, more powerful tools to study, handle and engineer these natural units could be obtained, while, on the other hand, it may be possible to fabricate novel electronic, optic, high-throughput screening, sequencing, drug targeting and delivery, and sensory nano-systems by enhancing current bottom-up techniques [1].

The importance of cell membranes in biological systems has prompted the development of model membrane platforms that recapitulate fundamental aspects of membrane biology, especially the lipid bilayer environment. Supported lipid mono- and bilayers represent one of the most promising classes of model membranes and are based on the immobilization of a planar lipid membrane on a solid support that enables characterization by a wide range of surface-sensitive analytical techniques. Moreover, as the result of molecular engineering inspired by biology, supported lipid membranes are increasingly

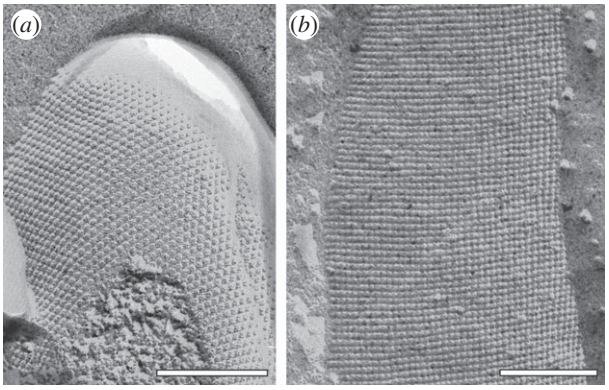

Figure 1. Transmission electron microscopy image of a freeze-etched and metal shadowed preparation of (*a*) an archaeal cell (from *Methanocorpusculum sinense*) and (*b*) a bacterial cell (from *Desulfotomaculum nigrificans*). Bars, 200 nm. (Adapted from [5]. Copyright © 2014 with permission from John Wiley & Sons Ltd.)

able to mimic fundamental properties of natural cell membranes, including fluidity, electrical sealing and hosting integral membrane proteins (MPs). At the same time, new methods have been used to improve the durability of lipid membranes, with shelf-lives now reaching the order of weeks. The capabilities of supported lipid membranes have opened the door to biotechnology applications in medicine, diagnostics, sensor systems, environmental monitoring and energy storage [3–5].

The challenge of integrating non-living systems with biological ones led to problems associated with material interfacing and compatibility, as well as biological issues, such as viability and stability. Furthermore, the integration is also complicated by scaling effects and dynamic interactions between different components. Moreover, water is necessary to maintain biological units, but has adverse effects on engineering components. Although elements used in the biological world are relatively simple, the phenomenon of their assembly is astonishingly diverse. Self-assembly and bottom-up are the most common strategies used for organization of biological units. The extension of this strategy to the creation of hybrid devices is thought to be most promising.

Complex and versatile biological units like cells have extremely elaborate ways to self-assemble functional units like the cell envelope structure. Biomimetic membranes incorporate biological elements or borrow concepts, ideas or inspiration from biological systems [6]. Such membranes can take advantage of the strategies evolved by nature over billions of years. This review is focused on the biomimetic approach of applying the building principle of cell envelope structure for the generation of a versatile biological unit—a protein-supported lipid membrane. The used protein species, termed S-layers (S- is the abbreviation of 'surface'), are defined as 'two-dimensional arrays of proteinaceous subunits forming surface layers on prokaryotic cells' [5,7,8] and are found as the outermost structure in hundreds of different species of almost every taxonomic group of walled bacteria (figure 1) and are an almost universal feature of archaea [4,9–12]. Interestingly, many bacterial and most archaeal S-layer proteins are glycosylated [13–15].

Nonetheless, not only prokaryotic bacteria and archaea (figure 2) have this kind of oriented self-assembly of proteins at their outermost envelope, but also many viruses do

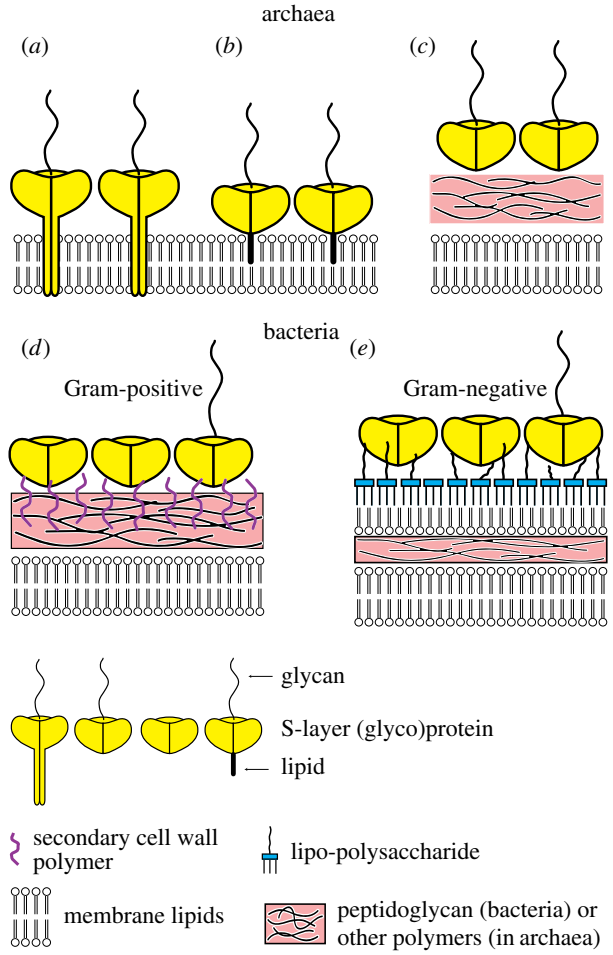

Figure 2. Prokaryotic cell envelopes: schematic of the supramolecular architecture of the major classes of prokaryotic cell envelopes containing surface (S) layers. S-layers in archaea with glycoprotein lattices as an exclusive wall component are composed either of mushroom-like subunits with pillar-like, hydrophobic transmembrane domains (*a*) or lipid-modified glycoprotein subunits (*b*). Individual S-layers can be composed of glycoproteins possessing both types of membrane-anchoring mechanisms. Few archaea possess a rigid wall layer (e.g. pseudomurein in methanogenic organisms) as an intermediate layer between the plasmatic membrane and the S-layer (*c*). In Gram-positive bacteria (*d*) the S-layer (glyco)proteins are bound to the rigid peptidoglycan-containing layer via secondary cell wall polymers (SCWPs). In Gram-negative bacteria (*e*) the S-layer is closely associated with the lipopolysaccharide of the outer membrane. (Adapted from [5]. Copyright © 2014 with permission from John Wiley & Sons Ltd.)

present a regular and well-defined protein layer as the outermost envelope structure.

In a biomimetic approach copying the supramolecular building principle of archaeal cell envelope structures (figure 3*a*), two S-layer-supported lipid membranes (SsLMs) differing in their overall shape and hence function have been generated [4,17,18]. First, the planar SsLM comprising a phospho- or tetraether lipid monolayer (figure 3*b*,*c*,*e*) or bilayer (e.g. phospholipid; figure 3*d*,*f*) and a closely attached S-layer lattice (figure 3*b*–*e*). The S-layer lattice acts as a stabilizing and anchoring scaffold for the lipid membranes. In the case of surface-attached membranes (figure 3*e*,*f*), the S-layer also constitutes a biological cushion covering the inorganic support, provides an ionic reservoir and antifouling surface, allows lateral diffusion of membrane lipids and incorporation of integral MPs with domains protruding from the attached lipid membrane.

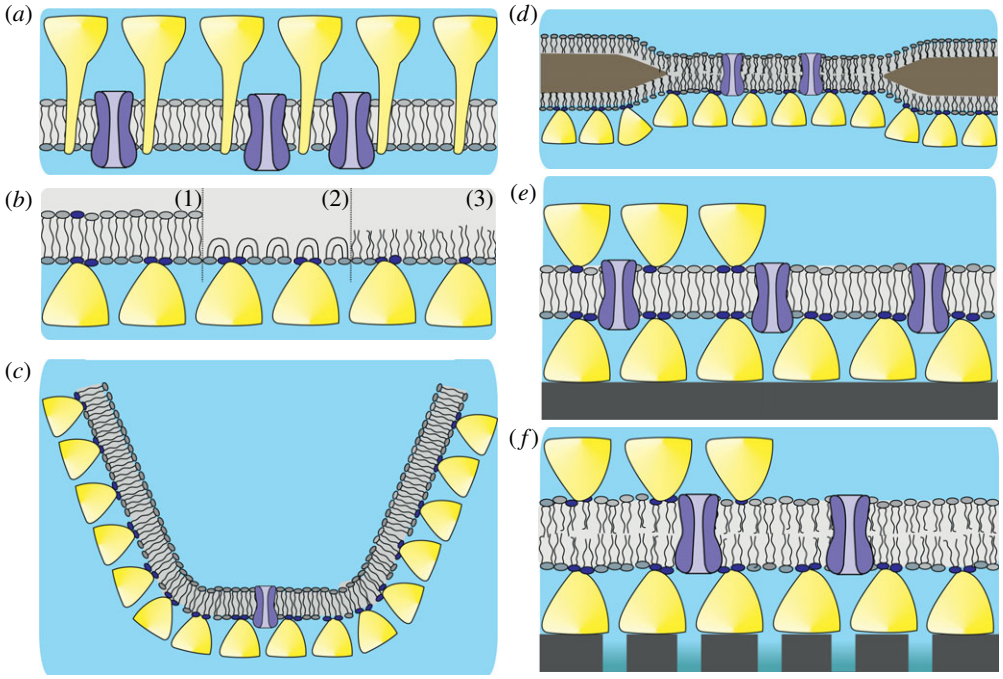

Figure 3. Supramolecular structure of an archaeal cell envelope architecture comprising a cytoplasmic membrane, archaeal S-layer proteins incorporated in the lipidic matrix and integral MPs (*a*). Schematic of various S-layer-supported lipid membranes. (*b*) Lipid monolayer films at the air/water interphase with an underneath recrystallized S-layer lattice. (1) Tetraether lipid monolayer in the upright conformation. (2) Tetraether lipid monolayer in the U-shaped (bent) conformation. (3) Phospholipid monolayer. (*c*) A tetraether lipid monolayer membrane is generated across an orifice of a patch-clamp pipette by the tip-dip method. Subsequently a closely attached S-layer lattice is formed by bacterial S-layer proteins on one side of the lipid membrane. In (*d*), a folded or painted bilayer phospholipid membrane spanning a Teflon aperture is shown. A closed bacterial S-layer lattice can be self-assembled on either one or both (not shown) sides of the lipid membrane. (*e*) Schematic drawing of a solid support where a closed bacterial S-layer lattice has been assembled. On this biomimetic structure, a tetraether lipid membrane was generated by the modified Langmuir–Blodgett method. Optionally as shown on the left-hand side, a bacterial S-layer lattice can be attached on the external side of the solid-supported lipid membrane. (*f*) Schematic drawing of a bilayer lipid membrane generated on an S-layer ultrafiltration membrane (SUM). Optionally as shown on the left-hand side, a bacterial S-layer lattice can be attached on the external side of the SUM-supported lipid membrane. In (*b*–*f*), the head groups of the lipid molecules interacting with the S-layer protein are shown by a darker colour. As indicated in (*c*–*f*), all S-layer-supported model lipid membranes can be functionalized by biomolecules like membrane-active peptides and transmembrane proteins. (Adapted from [16]. Copyright © 2004 with permission from Wiley-VCH.)

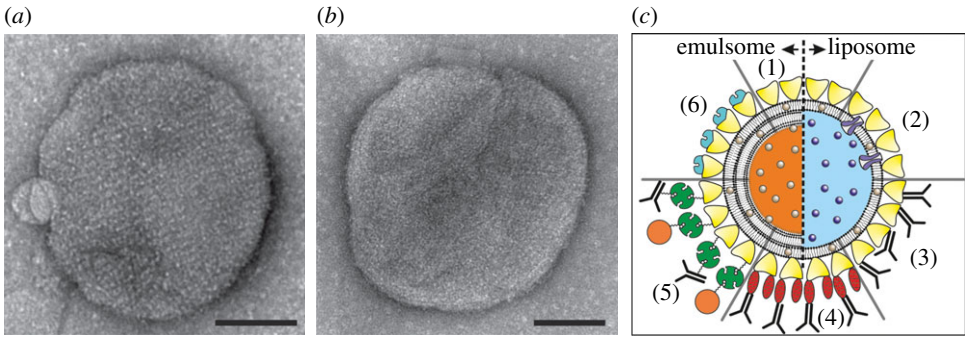

Figure 4. Left: Transmission electron microscopy images of emulsomes coated with the S-layer protein SbsB from *G. stearothermophilus* PV72/p2 (*a*) wild-type SbsB and (*b*) recombinant SbsB. The bars correspond to 100 nm. (Adapted from [19]. Copyright © 2013 with permission from Wiley-VCH.) (*c*) Schematic drawing of (1) an S-layer-coated emulsome (left) and liposome (right) with entrapped functional molecules and (2) functionalized by reconstituted integral proteins. Note, S-layer-coated emulsomes can only transport hydrophobic molecules but with a much higher transport capacity. S-layer-coated emulsomes and liposomes can be used as an immobilization matrix for functional molecules (e.g. human immunoglobulin G) either by direct binding (3) or by immobilization via the Fc-specific ligand protein A (4), or biotinylated ligands can be bound to the S-layer-coated liposome or emulsomes via the biotin–avidin system (5). Alternatively, emulsomes or liposomes can be coated with genetically modified S-layer subunits incorporating functional domains (6). (Adapted from [20]. Copyright © 2002 with permission from Wiley-VCH.)

The second S-layer/lipid assembly structures concern spherically shaped S-layer-coated liposomes and emulsomes (figure 4). In this manner, a biomimetic archaeal cell envelope or viral envelope composed of an S-layer shell, and some kind of lipidic core (liposome or emulsome), is assembled. These biomimetic nanocarrier systems are mainly designed for drug targeting and delivery and for imaging enhancement purposes [19,21–23]. Moreover, the proteinaceous surface of these nanocarriers not only has a stabilizing effect, but also provides the possibility to endow the liposome or emulsomes with a great variety of functionalities such as packed ligands, antigens (Ag), antibodies, enzymes and stealth molecules (figure 4*c*). Both types of SsLM may be functionalized with (antimicrobial) membrane-active peptides and peripheral or integral MPs.

Although both systems are capable of producing an electrical potential, the charge carriers are different, being ions and electrons, for functionalized lipid membranes and inorganic systems, respectively. These differences result in numerous challenges for their integration. These SsLMs, in particular the planar ones on a solid or porous support (figure 3*e*,*f*), are well suited for the development of peptide- or protein-based biosensors. Integrated with inorganic micro- and nano-systems, SsLMs may also constitute a key component for the development of the so-called lipid chips or lab-on-a-chip [24,25].

2. Biological envelope structures

In the following, the envelope structures of bacteria, archaea and viruses will be briefly described to enable the reader to become familiar with the diverse building principles that have evolved in nature. In this context, it is worth mentioning that the so-called Gram stain (the name comes from its inventor, Hans Christian Gram) is an important method of differentiating bacterial species into two large groups: Gram-positive (figure 2*d*) and Gram-negative (figure 2*e*). Differentiation is based on the supramolecular structure and consequently the chemical and physical properties of the cell walls by detecting peptidoglycan, which is present in a thick layer in Gram-positive bacteria. One prokaryote domain, the archaea, have such variability of wall structure that the Gram stain is not a useful differentiating tool [26].

2.1. Cell envelope structures of bacteria

Gram-negative bacteria are composed of a cytoplasmic membrane, a thin peptidoglycan layer, and an outer membrane containing lipopolysaccharide (LPS) in its outer leaflet and phospholipids in the inner leaflet (figure 2*e*). Porins exist in the outer membrane, which act like pores for particular molecules. Gram-positive bacteria, however, are composed of a cytoplasmic membrane and a rigid peptidoglycan-containing layer (murein; teichonic, teichuronic and lipoteichonic acid), which is much thicker than in Gram-negative bacteria (figure 2*d*) [27,28]. In addition, both types of bacteria may have an S-layer lattice as the outermost structure. The S-layer lattice is directly attached to the outer membrane and the peptidoglycan layer in Gram-negative and Gram-positive bacteria, respectively. In the latter, the rigid cell envelope layer is composed of peptidoglycan and accessory (secondary) cell wall polymers (SCWPs; figure 2*d*) [29]. The polymer chains are either covalently linked to the peptidoglycan backbone via phosphodiester bonds or tethered to a lipid anchor moiety [30]. By sequence comparison, S-layer-homologous (SLH) motifs [31] have been identified at the N-terminal part of many S-layer proteins [32–34]. It is now evident that SCWPs serve as anchoring structures for many S-layer proteins. In contrast to the SLH motifs of most S-layer proteins, which reveal a positive net charge, those of *Bacillus sphaericus* strains [34,35] are net negatively charged, which explains why bivalent cations are required for binding of the S-layer subunits to the rigid cell envelope layer [32]. In contrast to most S-layer proteins of Gram-positive bacteria, those of *Geobacillus stearothermophilus* strains [33,36,37] and lactobacillus [38–41] do not possess SLH motifs. Nevertheless, the N-terminal part of the S-layer proteins of *G. stearothermophilus* strains is highly conserved and recognizes a net negatively charged SCWP as the binding site [35,37,42].

2.2. Cell envelope structures of archaea

Many archaea dwell in extreme environments, for example at high pressures, salt concentrations or temperatures. Their cell wall differs in structure from that of bacteria and is thought to be more stable in extreme conditions, helping to explain why some archaea can live in many of the most hostile environments on Earth (figure 2*a*–*c*).

Studies of the archaeal cell envelope resulted in the recognition of a number of archaea-specific features, including a different lipid composition of the cytoplasmic membrane from bacteria and the lack of a general cell wall polymer, resulting in insensitivity to the most common bacterial cell wall-targeting antibiotics. In most archaea (e.g. *Sulfolobus* spp.), S-layer proteins are the exclusive cell envelope constituent (figure 2*a*), whereas in other archaea the cell envelope consists of multiple polymers, including the polysaccharides pseudomurein and methanochondroitin, and can also contain additional S-layer proteins (figure 2*c*) [9,12]. Archaeal S-layers are mostly composed of a single protein or glycoprotein species, which in many cases is associated with the cytoplasmic membrane. In haloarchaea, methanogens, *Staphylothermus* spp. and *Thermoproteus* spp. the main protein constituent of the S-layer is anchored by its carboxy-terminal transmembrane domain to the cytoplasmic membrane (figures 2*a* and 3*a*). The transmembrane domain is often preceded by a stretch of serine/threonine residues that are often glycosylated. In some cases, the S-layer is composed of two S-layer proteins; for example, in *Sulfolobales* spp. the S-layer is composed of the large outer protein which is linked to a small protein called SlaB [12,43]. SlaB, however, is anchored to the cytoplasmic membrane and forms a stalk about 20 nm long [44]. Recently, another type of binding of archaeal S-layer proteins to cytoplasmic membranes has been reported for *Haloferax volcanii*. It could be demonstrated that a subset of secreted euryarchaeal proteins, including the S-layer glycoprotein, is processed and covalently linked to membrane-embedded lipids involving membrane-spanning enzymes referred to as archaeosortases [45,46]. Because the C-terminal structure recognized by archaeosortases of a large number of euryarchaeal S-layer glycoproteins is highly conserved, it is very likely that this proposed lipid-anchoring mechanism is a broadly conserved surface-anchoring mechanism (figure 2*b*) [47–49].

2.3. Envelope structures of viruses

Many types of virus particles exist not as naked nucleocapsids but as nucleocapsids surrounded by lipid membranes. These membranes contain various viral-encoded glycoproteins and perform some subset of the functions that are required for successful viral spread. These structures comprise a lipid bilayer and associated proteins are referred to as viral envelopes. The major component of viral envelopes is one or in rare cases more lipid bilayers. The viral lipid bilayers are generally derived from pre-existing membranes of the host cell; therefore, the lipid components are taken from the cellular membrane. In many cases, the acquisition of an envelope occurs as the nucleocapsid buds out from the cytoplasm to the extracellular milieu [50]. The size of many viruses (e.g. influenza and many animal viruses) is 20–400 nm [51], in the same range as handmade lipid structure like liposomes or emulsomes (figure 4*a*,*b*).

3. Building blocks for biomimetic membranes

Life on Earth is basically composed of the four building blocks: amino acids, nucleic acids, carbohydrates and lipids. The self-assembly and interaction of different polymers of these basic molecules make up the majority of life's structure and function. From a very general point of view, nucleic acids store and transfer genetic information; carbohydrates store energy, reconstitute recognition systems and provide building materials; lipids make membranes and store energy; and proteins are structure elements and perform the chemistry of the cell [52]. Lipids are a loosely defined group of molecules with one main characteristic: they are insoluble in water. Phospholipids are, among lipids, the most important molecules of bacterial and mammalian cells, as they form, with the exception of archaea, the core of all biological membranes. Archaea, in contrast, are composed of ether- or tetraether lipids that show a higher resistance against chemical hydrolysis [53]. Proteins are the most abundant ones of the organic molecules, constituting about 50% of a cell's dry weight. A particular protein's overall conformation can be considered on four levels: primary, secondary, tertiary and quaternary structure. These levels of structure combine to create a complete protein that may serve many different functions within a cell. The building blocks lipids, proteins and carbohydrates (polymers) will be discussed later on the basis of their importance in creating biomimetic surfaces, in particular functional SsLMs.

3.1. Lipids

Natural bacterial cell membranes are complex structures composed of a variety of polymers, lipids and proteins. The weight ratio of protein to lipid varies from 20% to 70%; however, it is the lipid component that gives the membrane the gross morphology of a closed structure [54]. Lipids may not only be regarded as building materials or structural elements, they also have important functional tasks. Phospholipids, in many instances, constitute an important barrier function and are necessary for the stabilization and function of native membrane-bound proteins [55]. The lipidic components of natural cell membranes consist, among other minor components such as sphingolipids, glycol(sphingo)lipids and sterols, mainly of glycerolphospholipids, each differing in charge, acyl chain composition and physical properties [54,56].

However, the most frequently used lipid molecules for membrane formation are synthetic zwitterionic phospholipids carrying isoprene side chains like 1,2-diphytanoyl-*sn*-glycero-3-phosphatidylcholine (DPhPC; CAS number 207131-40-6) and, thus, forming fluid lamellar structures at biologically relevant ambient temperatures [54,57–59]. Other frequently used phospholipids are lecithin (PC) extracted from egg yolk or soya bean or unsaturated phosphatidylcholine like the zwitterionic palmitoyl-oleoyl-phosphatidylcholine (POPC; CAS number 26853-31-6) doped with small amounts of positively or negatively charged phospholipids such as phosphatidylethanolamine (PE) or phosphatidylglycerol (PG) and -serine (PS), respectively [56,60]. Membrane lipids of archaea, however, are unique and distinct from those found in eukarya and bacteria. The polar lipids consist of isoprenoid chains, 20–40 carbons in length and usually saturated, which are attached via stable ether bonds to the glycerol carbons at the *sn*-2,3 positions [61]. Polar head groups differ at the genus level of diversity and consist of mixtures of glyco groups (mainly disaccharides), and/or phospho groups, primarily phosphoglycerol, phosphoserine, phosphoethanolamine or phosphoinositol. In this context, it is interesting to note that archaeal lipids comprise isoprene side chains which are bound via an ether linkage to the glycerol moiety of phospholipids. Furthermore, the so-called tetraether lipids, consisting of two different hydrophilic head groups, which are ether-linked by two C_{40} isoprenoidic chains having up to five cyclopentane rings, have been used to generate a monomolecular membrane (figure 3*a–c*,*e*) with the same overall structural features and barrier function as common phospholipid bilayers (figure 3*d*,*f*) [62,63]. Glycerol dialkyl nonitol tetraether lipid, extracted and purified from *Sulfolobus* and *Metallosphaera* strains and the main phospholipid isolated from *Thermoplasma acidophilum*, has also frequently been used for membrane formation. The advantage of etherlipids is their pronounced stability towards oxidative degradation, resistance against hydrolysis, even under extreme environmental conditions, and their fluid characteristics over a broad range of temperatures [64–66]. However, a disadvantage is that tetraether lipids may adopt instead an upright conformation (figure 3*b*(1)), an unstable so-called U-shaped (bent) conformation at the air/water interphase where both hydrophilic head groups interact with the aqueous subphase (figure 3*b*(2)) [64,67,68]. Thus, films comprising tetraether lipids may not be stable in terms of maintaining the adjusted surface pressure over time as permanent rearrangement of the lipid molecules occurs [62,69,70]. Owing to their amphiphilic nature, ether- and phospholipids can nicely be self-assembled at the air/water interphase with the hydrocarbon chains pointing towards the air and the polar head groups facing the aqueous phase (figure 3*b*(3)) [71,72].

3.2. S-layer proteins

Despite the diversity of cell envelope structures observed in prokaryotic organisms [28,73], among the most commonly observed cell surface structures are mono- or bimolecular arrays composed of identical species of protein or glycoprotein subunits (figure 1). Because S-layers account for approximately 10% of cellular proteins in bacteria and archaea and because the biomass of prokaryotic organisms surpasses the biomass of eukaryotic organisms [74], S-layers can be considered as one of the most abundant biopolymers on our planet [5,9]. S-layers also represent the simplest biological protein or glycoprotein membranes developed during evolution [75–77].

High-resolution electron and atomic force microscopy studies on the mass distribution of S-layer lattices revealed that the S-layer covers the entire cell surface as a coherent layer [78–81]. Most S-layers are monomolecular assemblies of single subunit species with a molecular weight ranging between 40 and 200 kDa. S-layer lattices generally exhibit oblique (p1, p2), square (p4) or hexagonal (p3, p6) space group symmetry with a centre-to-centre spacing of the morphological units of 3.5–35 nm [12,28,82]. Hexagonal lattice symmetry is predominant among archaea [9,83]. Depending on the lattice type the morphological units consist of one, two, three, four or six protein or glycoprotein monomers, respectively. Bacterial S-layers are generally 5–10 nm thick, whereas archaeal S-layers frequently exhibit a much thicker 'mushroom-like structure' with pillar-like domains [12]. Bacterial S-layers reveal a rather smooth outer and a more corrugated inner surface (figure 2*d*,*e*). Moreover,

S-layers represent highly porous protein lattices (30–70% porosity) with pores of uniform size and morphology in the 2–8 nm range [35,84,85]. Many S-layers possess two or even more distinct classes of pores [12,20,28,82,86].

Biological functions of S-layers vary depending on the different S-layer lattices and organism and may include (i) protection against bacteriophages, bdellovibrios and phagocytosis, (ii) resistance against low pH, (iii) a barrier or protective coat against high- and low-molecular-weight substances (e.g. lytic enzymes), (iv) stabilization of the membrane, (v) provision of adhesion sites for exoproteins, and (vi) provision of a periplasmic compartment in Gram-positive prokaryotes together with the peptidoglycan and the cytoplasmic membranes [5,28,87–90]. Interestingly, in the S-layer of *Deinococcus radiodurans* ion-gating properties of microbial S-layer protein arrays have also been determined [91]. Ion transport appears to be mainly due to an electrical gradient inside the pores, presumably originating from the negative charges found on this S-layer lattice. The gating characteristics of the nanoporous membranes towards various ionic species were evaluated and revealed that the immobilized S-layers undergo a strong interaction with cations, in particular Ca^{2+} ions.

Recently, an outstanding antifouling characteristic of the S-layer protein of *Lysinibacillus sphaericus* CCM 2177 (SbpA) in the presence of high-protein solutions (e.g. 70 mg ml^{-1} human serum albumin), plasma and whole blood samples was observed. This finding is explained by the inherently (zwitterionic) neutral surface charge of the S-layer protein SbpA [92].

One important feature of S-layer proteins is the capability of isolated native or recombinantly produced subunits to self-assemble into crystalline arrays on many surfaces such as glass, silicon oxide and nitride, mica, noble metals like gold, titanium and platinum, but also on stainless steel or many polymers such as polystyrene, polyester and cellulose, and on technically relevant surfaces like highly oriented pyrolytic graphite or indium tin oxide [92,93]. Transmission electron microscopy (TEM) [94–96] and atomic force microscopy (AFM) [97–101] are the most appropriate techniques to elucidate the recrystallization process of S-layer proteins. Crystal growth at interfaces (e.g. solid supports, air–water interface or lipid membranes) is initiated simultaneously at many randomly distributed nucleation points, and proceeds in plane until the crystalline domains meet, thus leading to a closed, coherent mosaic, of individual, several micrometres large, S-layer domains [97,102–104]. The growth of extended S-layers domains is favoured at low monomer concentrations owing to the corresponding low number of nucleation sites. The individual domains are monocrystalline and separated by grain boundaries.

The formation of coherent crystalline domains depends on the S-layer protein species used, the environmental conditions of the subphase, such as ionic content and strength, the pH value and the surface properties of the interface. Interestingly, it was shown that a self-assembled layer, depending on whether the inner or outer side is exposed to the aqueous environment, can exhibit against cells in tissue cultures either cell adhesive (cytophilic) or cell repulsive (cytophobic) surface properties, respectively. The different orientation and function of the S-layer protein can simply be achieved by altering the recrystallization protocol from a basic (pH 9; resulting in an outer smooth cytophobic side) to an acidic (pH 4; resulting in an inner rough cytophilic surface pattern) condition [105].

While the reassembly of S-layer proteins at the air–water interface and at planar lipid films is well defined [95,96, 106–108], the deliberate modification of the surface properties of a solid support allows the reassembly process to be specifically controlled [86,97,103,109]. For example, the S-layer protein SbpA, which is currently one of the most studied S-layer proteins for functionalizing solid supports, forms monolayers with a height of 9 nm on hydrophobic and double layers on hydrophilic silicon supports [97]. The height of the double layer structure is 15 nm and not twice the height of a monolayer, indicating that the two layers are resting on each other like two interdigitated toothed racks. Furthermore, in comparison with hydrophilic surfaces, the layer formation is much faster on hydrophobic supports, starting from many different nucleation sites and thus leading to a mosaic of small crystalline domains (two-dimensional powder) [75]. Along this line, the importance of the interplay between hydrophobic and hydrophilic regions was studied in detail by reassembling the S-layer protein SbpA on self-assembled monolayers (SAMs) on gold composed of dialkyl disulfide derivatives with different end groups (hydroxyl versus methyl) and lengths of the individual methylene bridges [110]. The formation of monolayers was observed when the hydrophobic methyl end groups surmounted the hydrophilic hydroxyl groups. On the contrary, double S-layers were formed when hydrophilic hydroxyl groups superseded the hydrophobic methyl end groups. The threshold for the transition between native and non-native S-layer parameters was four methylene bridges. Finally, it must be noted that different lattice constants were observed.

Self-assembled monolayers were also used to study the influence of the introduced surface chemistry [111]. The SAMs carried methyl, hydroxyl, carboxylic acid or mannose, respectively, as terminating functional groups. It was confirmed that electrostatic interaction (carboxylic acid functional groups) induces a faster adsorption than hydrophobic (methyl groups) or hydrophilic (hydroxyl groups) interaction—as already shown for the reattachment on the bacterial cell [75,112] and at liposomes and polyelectrolyte nanocapsules [113–116].

Although native S-layer proteins have already demonstrated their great potential as patterning elements and nanoscale building blocks, genetic approaches opened up the possibility of modifying and changing the natural properties of S-layer proteins [117,118]. However, although S-layer proteins incorporate insertions or fusions of foreign proteins or domains, the capability to assemble into geometrically well-defined layers must be retained. To date, two different strategies for the production of nanobiotechnologically relevant S-layer fusion proteins are pursued, namely homologous expression and secretion by the cells or production inside a host, mostly *Escherichia coli* [117]. By these means bio-inspired materials with designed functional properties can be produced. Moreover, the possibility to modify the natural properties of S-layer proteins by genetic manipulations and to incorporate single or multi-functional domains of other proteins has led to a broad spectrum of applications ranging from fluorescent biomarkers, immobilized biocatalysts, vaccine development, diagnostics and sensor development, to biosorption of heavy metals and nanoparticle arrays [5,21,79,119]. In this context, S-layer proteins may also be genetically engineered in order to introduce domains for the covalent binding of lipid molecules and, thus, enhancing the stability of the whole composite SsLM [17,18,120,121]. Table 1 summarizes the

Table 1. Summary of the strategies investigated to date to bind lipid molecules on S-layer protein lattices. SLP, S-layer protein; SMCC, succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate; SPDP, *N*-succinimidyl 3-(2-pyridyldithio)-propionate; TCEP, Tris (2-carboxyethyl) phosphine hydrochloride; NTA, nitrilotriacetic acid.

type	reactive group	cross-linker	targeted group	reference
natural SLP				
electrostatic interaction	negatively charges on SLP		zwitterionic or positively charged lipids	[106,107,122]
lectin-type like binding	S-layer-homologous domain on SLP		secondary cell wall polymer coupled to lipids	[123,124]
chemical modification of SLPs				
covalent bond	carboxyl groups on SLP	carbodiimide analogues	primary amine group from lipids	[125–129]
covalent bond	primary amino groups on SLP	SMCC analogues	thiol group from lipids	[130]
covalent bond	primary amino groups on SLP	SPDP/TCEP; introduction of thiol group in SLP	maleimide group from lipids	personal communication[a]
chemical binding of linker on SLPs				
strong ligation	streptavidin chemically coupled to SLP		biotinylated lipids	[115,131]
genetically engineered SLPs				
covalent bond	thiol group from introduced cysteine		maleimide group from lipids	[98,132,133]
multiple chelation	multiple histidines (His_6-tag) on SLP		nickel(II)-NTA from lipids	[134]
strong ligation	streptavidin fused to SLP		biotinylated lipids	[118]
strong ligation	strep-tag fused to SLP	streptavidin	biotinylated lipids	[99–101]

[a]B Schuster *et al.* 2014, personal communication.

possibilities of how S-layer proteins and phospholipid molecules can be interconnected.

3.3. Cell wall polymers

Studies on a great variety of S-layer proteins from *Bacillaceae* revealed the existence of specific binding domains on the N-terminal part for sugar polymers, the so-called SCWPs, which are covalently linked to the peptidoglycan of the cell wall [135]. These SCWPs mediate the oriented binding of S-layer proteins to the underlying cell wall.

For Gram-positive bacteria, at least two major types of binding mechanism between S-layer proteins and SCWPs have been described (figure 2*d*). This specific molecular interaction is often mediated by a recurring structural motif of approximately 55 amino acids, which is mostly found in triplicate at the N-terminus of S-layer proteins. These so-called SLH motifs are involved in cell wall anchoring of S-layer proteins by recognizing a distinct type of SCWP which carries pyruvic acid residues [32,112,136–140]. Moreover, the coexistence of two N-terminally located binding domains for SCWPs and peptidoglycans was also described for the SLH domain carrying S-layer protein of *G. stearothermophilus* PV72/p2 (SbsB) [29,141].

By contrast, S-layer proteins devoid of SLH motifs are anchored to different types of SCWP via their N- or C-terminal regions. By using affinity studies and surface plasmon resonance spectroscopy, a further main type of binding mechanism was described for *G. stearothermophilus* which involves a non-pyruvylated SCWP containing 2,3-diacetamido-2,3-dideoxymannuronic acid as the negatively charged component and a highly conserved N-terminal region lacking an SLH domain [36,37,42,142].

In Gram-negative bacteria, no general S-layer anchoring motif has been identified and the S-layer is attached with its N- or C-terminus to the LPS component of the outer membrane (figure 2*e*) [143–145]. For the *Caulobacter crescentus* S-layer protein (RsaA), recrystallization on lipid vesicles was obtained only when the vesicles contained the specific species of *Caulobacter* smooth LPS that previous studies implicated as a requirement for attaching the S-layer to the cell surface [146]. The specific type of phospholipid did not appear critical; phospholipids rather different from those present in *Caulobacter* membranes or archaeal ether lipids worked equally well. However, the source of LPS was critical. Furthermore, efficient recrystallization and long-range order could not be obtained with pure protein, though it was apparent that calcium was required for crystallization [146].

Few methanogens possess pseudomurein, a polymer with a thickness of approximately 15–20 nm that is similar to bacterial peptidoglycan. In these species (e.g. *Methanosphera* and *Methanothermus*), the S-layer proteins interact with the

pseudomurein (figure 2*c*) [12]. However, most archaea, in particular the *Sulfolobales* and *Thermoplasmatales* spp., lack a polymer and the S-layer protein is anchored to the cytoplasmic membrane via a protein domain (figure 2*a*) or a lipid anchor bound to the S-layer protein (figure 2*b*) [49]. Interestingly, although the members of, for example, *Thermoplasmatales* do not possess a cell wall, these organisms dwell in a harsh environment such as under extremely low pH values (pH 1–2) and at temperatures of around 60° C [12].

3.4. Membrane-active peptides and integral membrane proteins

MPs as amphiphiles possess complex refolding processes that allow them to attain the three-dimensional configurations only in lipid membranes which are necessary for restoration of functionality. Moreover, these proteins are of major interest in medicine, diagnostics and the pharmaceutical industry. Approximately 60% of the over 430 drug targets that are presently known are MPs [147,148]. The latter are involved with cell sensing, signal transduction, immune recognition, transport of ions and nutrients, and a host of other vital process and, thus, in health and disease [149]. Among this 60%, G-protein-coupled receptors (GPCRs) are the most prevalent (19%), followed by ion channels (17%) and receptors (13%). Membrane-associated enzymes (6%), solute carriers and transporters (together approx. 5%) are also important drug targets [150]. Moreover, the results from the human proteome project suggest that more than 30% of proteins are membrane or membrane-associated proteins like pores, ion channels, membrane-anchored enzymes and most important (G-protein-coupled) receptors [151–154]. However, investigations on these proteins pose significant technical challenges, primarily because of their physico-chemical structure and sensitivity outside their native environment. They may contain large hydrophobic moieties that anchor them in the membrane, but which consequently limit their solubility in most other media, particularly if their structure has to be preserved. For this reason, biomimetic model lipid membranes have attracted lively interest because of their unique feature to provide an amphiphilic matrix for reconstitution of integral MPs [150,155,156]. Hence, MPs as preferred targets for pharmaceuticals received widespread recognition in drug discovery and protein-ligand screening and are of high interest for the development of biosensor platforms [150,155].

Very early after developing techniques for planar lipid bilayer generation membrane-active peptides incorporated in lipid membranes were studied [157]. One very prominent reason is that peptides can more easily be reconstituted in contrast to MPs [156,158,159]. Later on, supported model lipid membrane systems that complemented the existing ones (e.g. Langmuir monolayers, vesicular liposomal dispersions and bimolecular ('black') lipid membranes) were introduced [18,160–162]. These planar systems are thought to be promising platforms to study the mode of action of antimicrobial peptides (AMPs) but also for biophysical studies of and with artificial membranes or for sensor development employing, for example, antimicrobial membrane-active peptides and membrane integral proteins. Moreover, it offers the additional advantage of allowing for studies of the influence of membrane structure and order on the function of integral proteins; for example, on how the composition and organization of lipids in a mixed membrane influence the ion translocation activity of integral channel proteins [159,163–165].

By far the most literature can be found for the membrane-active peptides alamethicin, valinomycin and gramicidin [166–172]. The last was also used to determine the fluidity of the lipid membrane and as a molecular force probe [173,174]. In recent years, AMPs gained interest as it turned out that global antibacterial resistance is becoming an increasing public health problem [175]. Indeed, certain naturally derived peptides have been successfully used as antibiotics for many years. Despite the potential obstacles that remain, peptide therapeutics is poised to play a significant role in the treatment of diseases ranging from Alzheimer's disease to cancer [176].

4. The biomimetic approach

It is interesting to note that, in archaea possessing S-layers as the sole cell wall component (figures 2*a* and 3*a*), the protein lattices have been identified to be involved in the generation and maintenance of cell shape and in the cell division process [80,177,178]. Moreover, it was postulated by theoretical considerations that S-layers in archaea contribute to osmoprotection [88]. Hence, S-layers must therefore integrate the basic functions of mechanical and osmotic cell stabilization as these organisms dwell under extreme environmental conditions such as temperatures up to 120°C, pH down to zero, high hydrostatic pressure or high salt concentrations [64,179,180]. Since suitable methods for disintegration of archaeal S-layer protein lattices (figure 3*a*) and their reassembly into monomolecular arrays on lipid films are not yet available, S-layer proteins from Gram-positive bacteria are used to copy the supramolecular building principle of archaeal envelopes for the generation of S-layer-stabilized lipid membranes (figure 3*b–f*). S-layer proteins can be used as biofunctional surfaces [181] and constitute a fascinating structure for hosting and stabilizing functionalized lipid membranes [4,5,16,18–20,22,76,79,182,183]. These model lipid membranes consist either of a tetraether lipid monolayer (figure 3*b*,*c*,*e*) or of an artificial phospholipid bilayer (figure 3*d*,*f*) that replaces the cytoplasmic membrane and isolated bacterial S-layer proteins are assembled as monomolecular lattices onto the lipid membrane (figure 3*b–f*).

Moreover, a second S-layer acting as a protective molecular sieve and further stabilizing the scaffold can be recrystallized on the top of the previously generated SsLM (figure 3*e*,*f*). Hence, S-layer lattices constitute unique supporting architectures resulting in lipid membranes with a highly retained fluidity of the lipid molecules and a considerably extended longevity [17,184–187] (see also §6.1).

Besides archaeal cell envelope structures, many animal and human viruses with viral envelopes covering their protective protein capsids provide the building principle in particular for S-layer-coated liposomes or emulsomes (figure 4). The virus envelopes typically are derived from portions of the host cell membranes (phospholipids and proteins), but include some generally densely packed viral glycoproteins [188,189]. Interestingly, the size of a virus is 20–400 nm [51], i.e. in the same range as the artificial virus envelopes generated by coating liposomes or emulsomes with a monomolecular S-layer lattice (figure 4*a*,*b*) [19,22].

The following section intends to give a survey on biomimetic planar and spherical lipid membranes comprising the lipid matrix and a closely associated proteinaceous

S-layer lattice. The prerequisite for creating such supramolecular structures is given by the unique non-covalent interaction of S-layer proteins with lipid head groups within planar and spherical lipid mono- and bilayers [17,18,186,187,190].

5. Generation of S-layer-supported lipid membranes

SsLMs have attracted lively interest because of three main reasons. (i) They constitute a versatile biomimetic model to study the characteristics of the archaeal cell envelope by a broad arsenal of surface-sensitive techniques and sophisticated microscopical methods. (ii) Surfaces with new properties such as elevated antifouling characteristics for application in material science and nanomedicine can be generated using SsLMs. And finally, (iii) SsLMs provide an amphiphilic matrix for reconstitution of MPs. This is an important prerequisite to characterize integral MPs in a native environment, to apply the latter in basic research, but also to generate bio-inspired materials like MP-based biosensors and lab-on-a-chip architectures.

Besides the intrinsic interaction of phospholipids with certain domains or amino acid residues of the S-layer protein (which will be discussed in §§5.1 and 5.2), the S-layer protein and lipid molecules can be interconnected by means of (i) chemical modification of the S-layer protein for the subsequent binding of lipid molecules; (ii) chemical binding of molecules (cross-linker) for the indirect coupling of the S-layer protein with lipid molecules; and (iii) introduction of a binding site exposed on the S-layer protein by genetic engineering (table 1). Interestingly, it recently became evident that in nature archaeal proteins are targeted for post-translational modifications such as the addition of a lipid [46]. Hence, our approach to link lipids covalently to S-layer proteins is a biomimetic one occurring in archaea where a portion of membrane-bound proteins are anchored through a covalent association of their carboxy-termini with the lipid bilayer [47].

5.1. Planar lipid membranes

There are two basic methods for the generation of a free-standing bilayer lipid membrane (BLM) across an aperture which links two fluid-filled chambers (figure 3*d*) [191–194]: first, 'painted' bilayers are formed from a dispersion of either one or a mixture of purified phospholipids in a non-polar solvent such as *n*-decane. Prior to bilayer formation, the hole on which the bilayer is to be formed is 'primed' with a small quantity of the phospholipid dispersion. The priming dispersion is allowed to dry, the cup is positioned in the block and both *cis* and *trans* chambers are filled with the desired experimental solution. Additional phospholipid dispersion is then drawn across the hole using a 'stick'. This implement varies considerably from laboratory to laboratory and may be a small brush, a plastic rod or in some cases an air bubble at the end of a glass capillary.

An alternative procedure for the formation of a planar phospholipid bilayer involves the apposition of two phospholipid monolayers formed at the interface of an aqueous solution and the air [192,195]. This so-called 'folded' BLM has been adopted by some workers because the resultant bilayer contains somewhat less solvent than painted lipid membranes. However, again prior to bilayer formation, the hole on which the bilayer is to be formed is pretreated with a small quantity of hexadecane dissolved in pentane (1 : 10) [192,196].

Monolayers can be formed by applying phospholipids in a volatile solvent such as pentane, chloroform or hexane to the surface of an aqueous solution; the solvent evaporates in minutes leaving a phospholipid monolayer at the air–solution interface [195,197]. Alternatively, monolayers can be formed by allowing phospholipid liposomes or mixtures of liposomes and native membrane vesicles to equilibrate with a monolayer at an air–water interface [198,199]. The final structure of painted and folded BLMs is schematically depicted in figure 3*d*.

As implied by its name, this method involves the folding of two monolayers to form a planar bilayer. The apparatus used for bilayer formation via monolayer folding is in many respects similar to that used in the production of painted bilayers, which it involves two chambers separated by a septum containing a hole. A major difference exists in that the septum used for monolayer folding is not of rigid plastic but is composed of a very thin (10–25 µm) polytetrafluorethylene (PTFE; Teflon) membrane. Interestingly, no practical difference in terms of vesicle fusion was observed for painted and folded BLMs, respectively [200].

BLMs may also be formed at the end of conventional patch-clamp pipettes [201–203] with tip diameters in the range 0.5–5 µm either with or without fire polishing [197,204–206]. With this so-called tip-dip technique, the tip of the pipette is immersed in the desired experimental solution in a compartment of a multi-well disposable tray, and a phospholipid monolayer is formed at the air–water interface. Subsequently, a portion of the monolayer is transferred to the pipette tip by raising the pipette into the air. The polar head groups of the phospholipids orientate so that they interact with the aqueous pipette-filling solution and the glass wall of the pipette. The hydrocarbon chains of the molecules face the air. A bilayer is constructed by re-immersion of the pipette in the bath solution. As the tip of the pipette crosses the monolayer at the air–solution interface, a second region of monolayer interacts with the monolayer in the pipette to form a bilayer. The tip-dip technique, however, is also appropriate to generate an electrically tight tetraether lipid monolayer spanning the orifice of the pipette (figure 3*c*) [207].

Consequently, free-standing SsLMs are generated by two steps. First the lipid membrane is produced by either the painted, folded or tip-dip technique and subsequently an S-layer protein is recrystallized on it (figure 3*c*,*d*) [18,120,190]. Although the impact of the attached S-layer lattice on the membrane capacitance and resistance, and the boundary potential on free-standing BLMs, is negligible, the mechanical properties of SsLMs are considerably altered. Hydrostatic pressure applied across painted BLMs caused them to bulge resulting in an area of expansion measured by an increase in membrane capacitance [208]. A significantly higher area of expansion is observed for BLMs than for SsLMs whenever pressure is applied from the S-layer faced side. This experimental result supports the 'osmoprotecting effect' of the S-layer lattice, which was proposed 5 years later based on theoretical considerations to be one of the biological functions of S-layer lattices [88].

The membrane tension of BLMs after attachment of S-layer proteins is determined by dynamic light scattering [209]. For plain BLMs, the collective motions of the lipid molecules are dominated by membrane tension rather than by membrane curvature energy. S-layer lattices recrystallized

on both sides of the BLM have resulted in a considerable reduction of the membrane tension by a factor of approximately five. However, the membrane bending energy increases by three orders of magnitude, indicating that the attached S-layer lattice facilitates transverse shear motions of lipid molecules [209]. In accordance with voltage pulse experiments [210], a significant increase in the previously negligible surface viscosity of the membrane is observed during S-layer protein attachment [209].

However, although free-standing SsLMs revealed a higher mechanical stability (e.g. against hydrostatic pressure) and longevity, in particular with reconstituted peptides or proteins in comparison with BLMs without an attached S-layer lattice, these membranes are up to now not stable enough for many practical applications [17,18,120]. Hence, a promising strategy is to attach BLMs to porous or solid supports to enhance their practical applicability [25,123,211–214].

Solid-supported lipid membranes involving S-layers as key constituents to provide a stabilizing and defined tethering layer to decouple the BLM from the (inorganic) support, but also to generate an ionic reservoir if desired, have been fabricated in several ways. S-layer proteins have been recrystallized on glass and modified silicon surfaces before generating a tetraether lipid monolayer (figure 3*e*) or a BLM (figure 3*f*) by the Langmuir–Blodgett (vertical transfer of a lipid monolayer)/Langmuir–Schaefer (horizontal transfer of a lipid monolayer) (LB/LS) technique [215,216].

SsLMs comprising a tetraether lipid monolayer or a phospholipid bilayer are generated by a modified LB technique on a polymer aperture covering either a porous or solid support [184,217]. SsLMs without the need of an aperture are generated by a combined LB and LS technique and, as recently described, either by the rapid solvent exchange (RSE) technique [125] or by a newly developed vesicle fusion technique [126]. In brief, the latter novel technique is based on the finding that β-diketone-carrying molecules form complexes when europium (Eu^{3+}) ions (Eu) are present. When an amphiphilic β-diketone ligand [218] is incorporated in giant unilamellar vesicles (GUVs) consisting of phospholipids, the addition of Eu induced inter-liposomal complex formation and finally fusion of the GUVs [219]. For this versatile method, small unilamellar vesicles (SUVs) were fabricated comprising, beside, the main membrane forming phospholipid PC, a small amount of β-diketone ligand and linker lipids, capable of binding to chemically activated S-layer proteins. The addition of Eu to SUVs bound on the S-layer lattice resulted in rupture and fusion of the SUVs. By this procedure, a closed planar lipid bilayer is spontaneously formed on the S-layer lattice. This highly reproducible process can exactly be followed by quartz crystal microbalance with dissipation monitoring (QCM-D), but also by combined QCM-D with electrochemical impedance spectroscopy (EIS) measurements [126]. Another approach was to bind disc-shaped micellar lipid structures, called bicelles on the S-layer lattice [127,128,134]. The advantage is that no opening and rearrangement of this structure is necessary as bicelles are bilayered architectures, but, unfortunately, scarce fusion events caused in most cases an incomplete coverage of the S-layer lattice by the lipid membrane.

A further option is to bind thiolated SCWPs on gold surfaces before the S-layer protein is recrystallized on the support. This SCPW layer provides a defined orientation of the S-layer protein on the support and, as determined by AFM measurements, the crystallites of the S-layer lattice are larger and the S-layer lattice appears to be smoother than an S-layer lattice directly recrystallized on a solid support. Moreover, the soft thiolated SCWP layer with a thickness of approximately 6.2 nm [181] decouples the S-layer protein from the gold surface, which might cause denaturation of the protein structure at the area of contact when, for example, a certain electrical potential is applied [220].

5.2. Spherical lipid membranes (liposomes and emulsomes)

Unilamellar liposomes are artificially prepared spherical containers comprising a phospholipid bilayer shell and an aqueous core (figure 4*c*, right-hand side) [221–223]. Hydrophilic drugs can be stored and transported in the core, whereas the lipidic shell can be loaded with hydrophobic drugs. Emulsomes, however, are spherical systems with an internal solid fat core surrounded by phospholipid mono- and bilayer(s) (figure 4*c*, left-hand side) [224,225]. Hence, emulsomes show a much higher capacity to transport lipophilic drug molecules. Both nanocarriers can be used for targeted drug delivery for cancer and other diseases [23,225,226]. Furthermore, S-layer lattices as the outermost envelope structure covering the spherical containers constitute biomimetic 'artificial cell envelopes' or 'artificial virus-like particles', both enabling stabilization of the nanocarriers and presenting addressor molecules in a well-defined orientation and special distribution (figure 4).

Isolated S-layer subunits were recrystallized on positively charged, unilamellar liposomes comprising 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), cholesterol and hexadecylamine [22,114,115]. The S-layer attached to positively charged liposomes by their inner, negatively charged face. This is the orientation identical to the S-layer lattice on intact cells. Coating of the positively charged liposomes with the S-layer protein SbsB from *G. stearothermophilus* PV72/p2 resulted in inversion of the zeta-potential from an initially positive value to a negative one [114]. A similar behaviour was observed for liposomes coated with S-layer proteins from lactobacilli [227,228].

Emulsomes composed of a solid tripalmitin core and a phospholipid shell were reproducibly generated with an average diameter of approximately 300 nm using temperature-controlled extrusion steps [19]. The advantage of emulsomes is their high capacity to transport lipophilic substances such as curcumin [23]. Both wild-type (wt) and recombinant (r) S-layer protein SbsB formed an S-layer lattice covering the entire surface of emulsomes, as demonstrated by TEM (figure 4*a*,*b*). Upon coating with wtSbsB, the positive charge of emulsomes shifts to a highly negative zeta-potential, whereas those coated with rSbsB become charge neutral. This observation is attributed to the presence of the negatively charged SCWP, which is associated only with wtSbsB.

6. Performance of composite S-layer lipid membranes

The most challenging property of model lipid membranes is the feasibility to incorporate membrane-active (antimicrobial) peptides (AMPs) [229,230] and, more important, (complex) integral MPs in a functional state [150].

Up to now, the following membrane-active and/or AMPs have been incorporated in SsLMs: the ion carrier

Table 2. Summary of membrane-active peptides incorporated in S-layer-supported lipid membranes.

membrane-active peptide	source	remarks	references
gramicidin A (gA)	*Bacillus brevis*	linear pentadeca peptide	[217]
alamethicin (Ala)	*Trichoderma viride*	linear, 20 amino acids	[184]
valinomycin (Val)	several *Streptomyces* strains, e.g. *S. tsusimaensis* and *S. fulvissimus*	cyclic dodecadepsipeptide, 12 amino acids and esters	[184,207]
peptidyl-glycine-leucine-carboxyamide (PGLa) analogue	synthesized via protein chemistry	20 amino acid; PGLa(−)	[129]

Table 3. Summary of transmembrane proteins reconstituted in S-layer-supported lipid membranes.

transmembrane protein	source	remarks	references
α-HL	exotoxin from *Staphylococcus aureus*	pore-forming; homo-heptamer	[196,239,240]
ryanodine receptor 1 (RyR1)	skeletal muscle cells	Ca^{2+}-release channel; homo-tetramer	[241]
nicotinic acetylcholine receptor (nAChR)	plasma membranes of neurons; on postsynaptic side of the neuromuscular junction	ligand-gated ion channel; five subunits	[130,134]
VDAC	located on the outer mitochondrial membrane; also produced by cell-free expression	porin, voltage gated; ion channel monomeric but can cluster	personal communication[a]
M2 segment from nAChR	segment, forms ion-conducting channel (see nAChR)	ion-conducting channel	[242,243]

[a]S Damiati & B Schuster 2013, personal communication.

valinomycin, channel-forming alamethicin [184], the pore-forming gramicidin [217] and the AMP analogue peptidyl-glycylleucine-carboxyamide, where all lysine residues were replaced by glutamic acid, termed negatively charged analogue of peptidyl-glycylleucine-carboxyamide (PGLa(−); table 2) [129]. Furthermore, reconstitution of the staphylococcal pore-forming protein α-haemolysin (αHL) [24,231], the M2 segment that forms the ion-conducting channel of the nicotinic acetylcholine receptor (nAChR) [232], the ryanodine receptor/Ca^{2+} release channel (RyR1) [233] and the voltage-dependent anion channel (VDAC) [234–238] into plain and SsLMs has successfully been performed (table 3).

6.1. Free-standing membranes

SsLMs were first characterized by dual label fluorescence, surface pressure, transmission electron microscopy and AFM, Fourier transform infrared spectroscopy, and X-ray and neutron reflectivity measurements [96,106–108,216,244,245]. Formation of S-layer lattices covering the entire area of lipid films has been observed on zwitterionic phospholipids like PCs and in particular PEs [122]. S-layer proteins did not form crystalline lattices on negatively charged phospholipids. Membranes comprising a small portion of positively charged surfactants [22,210] or lipid derivatives [209] facilitated the crystallization process particularly on PCs. Electrostatic interaction is thought to exist between exposed carboxyl groups on the S-layer lattice and zwitterionic lipid head groups. At least two or three contact points between the S-layer protein and the attached lipid film have been identified [122]. Hence, less than 5% of the lipid molecules of the adjacent monolayer are anchored to these contact points (protein domains) on the S-layer protein whereas the remaining 95% or more of lipid molecules may diffuse freely within the membrane between the pillars consisting of anchored lipid molecules [120,187]. This calculation is based on the lattice constants of SbpA having a square unit cell with a spacing of 13.1 nm [97,116] and an area per lipid molecule of 0.65 nm^2 [246]. These nanopatterned lipid membranes are also referred to as 'semifluid lipid membranes' [95] because of their widely retained fluid behaviour [209,215]. Most important, although peptide side groups of the S-layer protein interpenetrate the phospholipid head group regions almost to their entire depth, no impact on the hydrophobic lipid alkyl chains has been observed [106–108,196,207].

To prove whether a phospholipase is able to hydrolyse a lipid monolayer shielded by an S-layer lattice, porcine pancreatic phospholipase A_2 (PLA_2) has been injected underneath an S-layer/lipid monolayer membrane. Interestingly, the S-layer lattice has neither constituted a significant barrier for the PLA_2 nor induced lipid packing defects, which would have resulted in shorter enzymatic lag periods [247]. Hence, there is significant evidence that the recrystallized S-layer lattice did not modulate a large proportion of the head group region of the phospholipid monolayer to an extent that could seriously impede the recognition of phospholipids by the biological interplay with PLA_2 [247].

Free-standing S-layer-supported tetraether lipid monolayers generated on the tip of a glass micropipette (tip-dip technique) and functionalized with valinomycin (figure 3*c*)

revealed a 10-fold higher life time than a membrane without an attached S-layer lattice (table 2) [207]. No reconstitution of αHL could be achieved with tetraether lipid membranes. In membranes mainly composed of the branched phospholipid DPhPC, αHL formed lytic pores when added to the lipid-exposed side of the SsLM (figure 3*d*). No pore formation was detected upon addition of αHL monomers to the S-layer face of the SsLM. Therefore, one can conclude that the intrinsic molecular sieving properties of the S-layer lattice do not allow passage of αHL monomers through the S-layer pores to the lipid membrane [196]. In addition, these data represent a quality control for the existence of a closed S-layer lattice without any defects and a tight attachment to the BLM. Compared with plain BLMs, SsLMs have a decreased tendency to rupture in the presence of αHL, again indicating an enhanced stability owing to the attached S-layer lattice [196]. Nevertheless, even single pore recordings have been performed with αHL reconstituted in free-standing SsLMs (table 3) [239].

SsLMs formed by the tip-dip technique were functionalized with M2 ion channels [242]. The M2 ion channel characteristics were studied and it turned out that the attached S-layer lattices were non-intrusive to the channel functionality and characteristics. The ability to stabilize BLMs and their non-intrusive character on ion channel activity make S-layer proteins attractive for biosensor applications, especially those that enhance the stability of BLMs beyond the use of tethers or polymer supports [3,242,243]. nAChR is a ligand-triggered ion channel consisting of five channel-forming subunits, which are arranged symmetrically around a central pore. For reconstitution studies in SsLMs, nAChR was isolated from the electric organ of the Pacific electric ray *Torpedo californica*. Reconstitution of nAChR was followed in liposomes, free-standing membranes with and without an attached S-layer lattice. When activated with carbamoylcholine, the nAChR channels reconstituted in all model lipid membranes were found to be functional and revealed a single channel conductance very close to those reported by others [127,130,206].

6.2. Surface-attached membranes

SsLMs prepared by the LB/LS technique without the need for an aperture have been compared with a silane- and dextran-supported phospholipid bilayer [215]. Most probably owing to the repetitive local interaction of the S-layer lattice with the lipid head groups, the nanopatterned fluidity of lipids was highest in SsLMs compared with the other supported bilayers, as determined by the fluorescence recovery after photobleaching technique. Phospholipid bilayers and tetraether lipid monolayers have also been generated on S-layer-covered gold electrodes. The tetraether lipid monolayer sandwiched by an S-layer lattice on each side (figure 3*e*) revealed an exceptional long-term robustness of approximately one week [17,18,120,187]. This finding also reflects the optimization of the archaeal cell envelope structure by nature over billions of years of evolution.

Lipid membranes generated on a porous support combine the advantage of easy manual handling, individual access to both membrane surfaces, and an essentially unlimited ionic reservoir on each side of the BLM (figure 3*f*). This is seen as a basic requirement of experiments copying the *in vivo* situation (e.g. plasmatic/exoplasmatic side). However, the surface properties of porous supports, such as roughness or great differences in pore size, have significantly impaired the stability of attached BLMs [248]. Hence, a straightforward approach is the use of S-layer ultrafiltration membranes (SUMs) with the S-layer as a stabilizing and smoothening biomimetic layer between the lipid membrane and the porous support [184,217,240].

SUMs were produced by depositing S-layer fragments as a coherent layer on microfiltration membranes [22,249–251]. The mechanical and chemical stability of their composite structure was subsequently obtained by inter- and intramolecular cross-linking [250,252–254]. The uniformity of functional groups on both the surface and within the pore area of the S-layer lattice could be used for very accurate chemical modifications in the sub-nanometre range, allowing the molecular sieving as well as antifouling characteristics of SUMs to be tuned [250,254,255]. Moreover, SUMs can be prepared with different net charges, hydrophilic or hydrophobic surface properties. That is why SUMs have been used as supporting and stabilizing structures for functional lipid membranes [5,18,121,182].

Whereas composite SUM-supported DPhPC bilayers were found to be highly isolating structures with a life time of up to 17 h [184,217,240], BLMs on plain microfiltration membranes revealed a life time of only approximately 3 h. The life time increased significantly to about 1 day by formation of an S-layer–lipid membrane–S-layer sandwich-like structure, i.e. an additional monomolecular S-layer protein lattice recrystallized on the lipid-faced side (figure 3*f*) [184,240]. An even further increase in the stability of this composite supramolecular structure can be expected upon cross-linking those lipid head groups involved in direct contact with the S-layer proteins. Hence, the nanopatterned anchoring of the membrane is a romising strategy for generating stable *and* fluid lipid membranes.

The functionality of SsLMs resting on solid supports has been investigated by the incorporation of the membrane-active peptides valinomycin, alamethicin, gramicidin D and the AMP analogue PGLa(−) (table 2) [184,256,257]. SsLMs with incorporated valinomycin, a potassium-selective ion carrier, revealed a remarkable high resistance bathed in sodium buffer. However, bathed in potassium buffer a decrease in resistance by a factor of 500 was observed for the same membrane owing to valinomycin-mediated ion transport [184].

SsLMs generated by the RSE technique are used to perform combined surface-sensitive QCM-D and EIS measurements. This study evidenced not only the attachment and/or insertion of PGLa(−) in the supported lipid membrane but also indicated toroidal pore formation in a concentration-dependent fashion [129]. Hence, SsLMs constitute a promising platform for studying the interaction and insertion of membrane-active (antimicrobial) peptides [229].

Incorporation of the membrane-active peptide gramicidin D could be demonstrated by measurements on single gramicidin D pores in all the above-mentioned SsLMs [217].

Finally, alamethicin channels could not only be incorporated in SsLMs on solid supports, the channels could even be specifically blocked as increasing amounts of inhibitor (amiloride) gave rise to a significant increase in membrane resistance (table 2) [184]. Thus, proof of concept for the applicability of these composite S-layer/lipid structures for biosensing purposes has been demonstrated. In future, the ability to reconstitute integral MPs in defined structures on, for example, sensor surfaces is one of the most important concerns in designing biomimetic sensing devices [3,18,258].

MPs have also been successfully reconstituted in SsLMs (table 3). Reconstitution of αHL, moreover even single pore

recordings, could be achieved with SUM-supported DPhPC bilayers but no pore formation was observed with BLMs generated on the pure micro-filtration membranes [240].

RyR1, isolated from rabbit muscle cells, was successfully reconstituted in SsLMs [241]. For this purpose, the supported membranes were formed by the β-diketone ligand-triggered vesicle fusion technique either on glass for fluorescence experiments or on gold for QCM-D measurements. Preliminary measurements clearly indicated that incorporation of RyR1 occurred, which was verified by control experiments to exclude misinterpretation due to unspecific adsorption to the bilayer or the S-layer lattice [241]. Nevertheless, further experiments, for example combined QCM-D with EIS studies or patch-clamp measurements on a chip, have to be performed. Finally, SsLMs may constitute a versatile and stabilizing scaffold allowing the detailed investigation of different drugs on isolated RyR1 in high-throughput screening-like devices.

SsLMs made by the newly developed europium-induced vesicle fusion technique were incubated with VDAC (table 3). A significant decrease in membrane resistance could be observed but the membrane capacitance did not vary significantly. Moreover, increasing VDAC concentration decreased membrane resistance, which indicates an increasing number of channels reconstituted spontaneously into the SsLM (S Damiati & B Schuster 2013, personal communication). It is well known that VDAC reconstituted in artificial membranes forms a voltage-gated channel. At low membrane potentials (less than 10 mV), VDAC is in the open state and switches to the closed state at high membrane potentials [235,236]. Indeed, this behaviour could also be clearly observed for the SsLMs with incorporated VDAC channels. Furthermore, the membrane resistance decreased again after reducing the voltage from 10 mV back to zero but the resistance was higher than the first measurement. This may be explained by the re-opening of some channels while others remain closed. In addition, it is conceivable that keeping the channels in the closed state for a long period of time during the measurements may reduce the rate of re-opening of VDAC and cause some structural rearrangements in order to achieve a more stable closed conformation [259]. Moreover, it has been shown that the presence of the nucleotides nicotinamide adenine dinucleotide hydride (NADH) or nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) induce channel closure and, thus, the conductance of the VDAC channels is significantly reduced [260–262]. Indeed, addition of NADH to the SsLM with reconstituted VDAC caused a significant increase in the membrane resistance; this is strong evidence for the blocking of VDAC channels by NADH molecules (S Damiati & B Schuster 2013, personal communication).

6.3. Interphases in solution (liposomes and emulsomes)

The influence of an S-layer coating on the stability of liposomes was studied by the release of the encapsulated hydrophilic marker carboxyfluorescein (CF) during the application of mechanical and thermal challenges. S-layer-coated liposomes (figure 4*c*(1)) released only half the amount of enclosed CF compared with plain liposomes upon exposure to shear forces or ultrasonication as mechanical stress [114]. Furthermore, temperature shifts from 25°C to 55°C and vice versa induced considerably less CF release from S-layer-coated than from plain liposomes. Moreover, the S-layer protein on the liposome can be cross-linked with glutaraldehyde or bis(sulfosuccinimidyl)suberate [257,263,264]. In addition, cross-linking can also be used for covalent attachment of macromolecules (figure 4*c*(3,4)). In turn, a layer of intact liposomes can also be reversibly tethered via the specific nickel–His-tag linkage on an S-layer lattice [130]. Moreover, liposomes functionalized with reconstituted integral proteins may also be stabilized by a coat comprising an S-layer lattice (figure 4*c*(2)).

The thermotropic phase behaviour of liposomes with (figure 4*c*(1)) or without an S-layer coating was characterized by differential scanning microcalorimetry. The data indicated for both preparations a broad phase transition around 50°C due to the chain melting from a liquid-ordered gel-like to a liquid-ordered fluid phase, similar to that described for DPPC/cholesterol mixtures. The slightly higher phase transition temperature for the S-layer-coated liposomes was explained by increased intermolecular order [265].

S-layer-coated liposomes have been investigated for their ability to act as a versatile system for entrapping and binding target molecules (figure 4). Indeed, S-layer-coated liposomes constitute a proper matrix for the covalent attachment of macromolecules such as ferritin [22]. Another approach was the biotinylation of S-layer-coated liposomes which resulted in two accessible biotin residues for subsequent avidin binding per S-layer subunit (figure 4*c*(5)) [115]. An ordered monomolecular layer of streptavidin was formed on the surface of the S-layer-coated liposomes as visualized by labelling with biotinylated ferritin. Furthermore, biotinylated anti-human immunoglobulin G (IgG) was attached via streptavidin to the biotinylated S-layer-coated liposomes. The biological activity of the bound anti-human IgG was confirmed by enzyme-linked immunosorbent assay [115].

S-layer proteins recrystallized on liposomes were exploited as the immobilization matrix for anti-human IgG (figure 4*c*(3)). The interaction of rabbit or swine anti-human IgG as Ag was studied by measuring changes in ultrasound velocity [131]. The ultrasound velocity decreased linearly following an increase in Ag concentration, presumably caused by changes in hydration of the membrane due to the binding process. Finally, no substantial differences in the behaviour of ultrasound velocity were observed for interaction of human IgG with rabbit or swine anti-human IgG [131].

Moreover, S-layer/streptavidin fusion proteins have been constructed (figure 4*c*(6)), and hence the biotinylated binding partner can be bound in a much more defined orientation and position. By this method, three biotin residues accessible for subsequent avidin binding were introduced per S-layer subunit [118].

An interesting approach is the recrystallization of functional chimeric S-layer fusion proteins carrying the sequence of the enhanced green fluorescent protein (EGFP) on liposomes (figure 4*c*(6)) [21]. The uptake of S-layer/EGFP fusion protein-coated liposomes into eukaryotic cells, for example a cell type in an immortal human cell line (HeLa cell) could be nicely visualized by the intrinsic EGFP fluorescence. The major part of the coated liposomes was internalized by HeLa cells within 2 h of incubation by endocytosis [21]. With regard to further experiments, the most interesting advantage can be seen in co-recrystallization of, for example, S-layer/EGFP fusion protein and the S-layer/streptavidin fusion protein [118] on the same liposomal surface. The uptake of these specially coated liposomes by target cells and the functionality of transported drugs could be investigated simultaneously without the need for any additional labels.

In vitro cell culture studies revealed that S-layer-coated emulsomes (figure 4) can be taken up by human liver carcinoma cells (HepG2) without showing any significant cytotoxicity [19]. Moreover, in a very recent study the low-water-soluble polyphenolic compound curcumin was encapsulated inside the solid lipid core of emulsomes. These emulsomes were termed CurcuEmulsomes [23]. Not only the particular characteristics of CurcuEmulsomes were investigated but also the delivery of curcumin via CurcuEmulsomes into HepG2 cells as the first cellular model system. Hence, this type of nanocarrier provides an interesting platform for the delivery of hydrophobic bioactive agents whose medical use is otherwise limited. The utilization of S-layer fusion proteins equipped in a nanopatterned fashion by identical or diverse functional domains or biomolecules may lead to attractive nanobiotechnological applications, particularly as carrier and/or drug targeting and delivery systems, as artificial virus envelopes in, for example, medicinal applications, as diagnostic tools, for molecular imaging and in gene therapy [18,115,121,263,266].

7. Conclusion and perspectives

Considerable knowledge has accumulated over past years concerning the isolation and purification of S-layer proteins [257], the experimental conditions required for obtaining coherent extended S-layer lattices on solid or porous supports, but also on the generation and intrinsic properties of S-layer-supported lipid films, and application of various microscopic and biophysical techniques. The study and use of biomimetic membrane systems generated by bottom-up processes is a rapidly growing scientific and engineering field. Elucidation of the supramolecular construction principle of archaeal cell envelopes composed of S-layer-stabilized lipid membranes (figure 2*a–c*), as well as envelopes of a great variety of animal and human viruses, led to new strategies for generating more stable functional lipid membranes with nanopatterned fluidity at the meso- and macroscopic scale. In this biomimetic architecture, artificial or isolated lipids replace the cytoplasmic membrane and isolated native or recombinant S-layer proteins derived from *Bacillaceae* are attached on either one or both sides of the lipid membrane (figure 3).

An important strategy to design stable membranes on surfaces and interfaces concerns the application of SCWPs as constituents of the molecular construction kit. The use of SCWPs enables reassembly of S-layer lattices on surfaces and lipid films in a predetermined orientation (figure 5*a*) and is, thus, essential for many nanobiotechnological applications. Moreover, SCWPs [29,267], chemically modified SCWPs (e.g. pyridyl disulfide-activated ones) [112] or SCWPs linked to lipid molecules [123] provide useful anchors or spacers between S-layer lattices and inorganic supports (figure 5 and table 1) [124]. The distance between the lipid membrane and the electrode, and, thus, the ionic reservoir, may be modulated in the nanometre range by this intermediate polymer. SCWP-linked phospholipids incorporated in the outer leaflet of the lipid membrane will allow the recrystallization of a second S-layer lattice on the top of the composite architecture at a certain distance so that previously incorporated integral MPs are shielded from the external environment (figure 5*a*). Moreover, the crystalline top layer composed of glycoproteins serves as a nanoporous filter with antifouling characteristics and as a protecting layer for, for example,

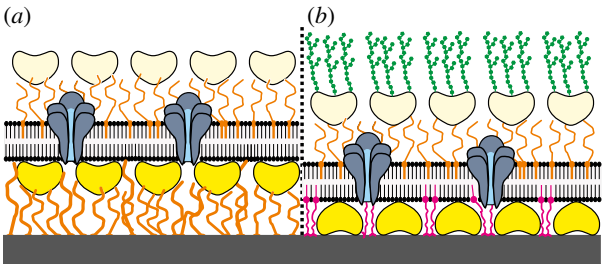

Figure 5. Schematic drawing of a lipid membrane on an S-layer-covered porous or solid support. (*a*) A thiolated SCWP is chemisorbed on the surface of a gold sensor and an S-layer lattice is recrystallized on it. The orientation of the S-layer lattice is determined by the SCWP layer. Subsequently, the lipid membrane is generated on the S-layer lattice and may be functionalized by integral MPs. Finally, a further proteinaceous lattice composed of S-layer proteins can be recrystallized on the top via SCWP-linked phospholipids which are anchored in the outer leaflet of the lipid membrane. (*b*) The lipid membrane is bound to the surface by membrane-anchored tethered lipid molecules reaching from the support through the pores of the S-layer lattice. Again, in this lipid membrane, transmembrane proteins can be reconstituted. Finally, a further proteinaceous lattice composed of S-layer glycoproteins (with carbohydrate moieties pointing to the external side) can be recrystallized on the top via SCWP-linked phospholipids which again are anchored in the outer leaflet of the lipid membrane. (Adapted from [18]. Copyright © 2009 with permission from Elsevier Inc.)

mechanical challenges (figure 5*b*). This approach mimics the functional aspects of many bacterial and archaeal S-layer lattices [5,28].

Chemical binding of the head groups of lipids on the S-layer protein was revealed to be an appropriate approach to generate stable and functional SsLMs. In addition, chimeric S-layer fusion proteins containing streptavidin can be recrystallized on their corresponding SCWPs or directly on solid supports [118]. As a result biotinylated lipids mixed with synthetic phospho- or isolated tetraether lipids of the fluid lipid membrane can be linked to this S-layer fusion protein via the biotin/streptavidin bridge to obtain an enhanced stability (table 1).

Another opportunity is the application of biotinylated integral MPs which may also be linked to the streptavidin S-layer fusion protein. The concept of these functional SsLMs is new and might have considerable impact on the development of solid-supported nanopatterned biomimetic membranes with functional proteins distributed in regular arrays following the lattice constant of the S-layer.

Finally, as most of the native S-layer proteins do not contain cysteine residues, it is possible to introduce cysteine residues on surface-exposed positions [132,133] that will be used for binding lipid molecules carrying a maleimide residue on their head group.

The very accurate nanopatterned fluidity of the lipid molecules and maintenance of lateral mobility of membrane-active peptides and MPs [18,120,217,240] in these biomimetic membranes provides significant advantage compared with other supported membrane systems [268]. Moreover, the combination of S-layer proteins and the specific interacting SCWPs enables a defined variation of the distance between the S-layer lattice and the solid support [76,77,124], which is seen as a basic requirement for the incorporation of MPs with domains exposed on the inner and/or outer membrane surface and for the assembly of multi-component membrane functions such as GPCRs, kinases, etc.

Copying nature's solution for the assembly of lipid membranes dwelling under most extreme environmental conditions is seen as one of the most promising strategies for various applications. Exciting areas for application of composite S-layer membrane systems concern mainly sensor systems involving specific membrane functions. Up to date, the use of such devices is primarily hampered by the instability and short longevity of membranes and, in particular, problems emerging upon drying biomimetic membranes. Native S-layer glycoproteins or S-layer proteins with carbohydrate moieties attached by chemical procedures should significantly help to overcome this problem (figure 5*b*). Moreover, previous studies have demonstrated that chemical cross-linking of S-layer glycoproteins through their glycan chains is feasible [269].

This approach is inspired by nature both in the nanobiotechnological design of supported lipid membranes and in the use of MPs [270] as they have attracted interest in their potential with respect to possible applications such as MP-based biosensors for detecting small hydrophobic molecules, ligand fishing, DNA sequencing, high-throughput screening and in (lab-on-a-) biochip technology. The same development is needed for biosensors such as artificial noses or tongues, in which many receptor proteins have to be exposed and read out simultaneously to create the response.

Another area of future development concerns copying virus envelopes by reassembly of S-layer (fusion) proteins on liposomes and emulsomes (figure 4) for the production of new targeting, delivery, encapsulation, and imaging systems, and vaccines.

The understanding of the mechanisms involved in the interaction of biological systems with inorganic materials is of interest in both fundamental and applied disciplines. A deep knowledge of the surface/biological fluid interface processes is needed for the design of new biocompatible materials for implants, drug targeting and delivery and nanodevices for diagnosis and therapy.

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