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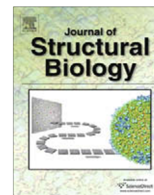

Review

Composite S-layer lipid structures

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ABSTRACT

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 how S-layer proteins, lipids, and polysaccharides 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 for application of composite S-layer membrane systems concern sensor systems involving specific membrane functions.

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1. Introduction

A remarkable number of processes in or between cells such as cell–cell interaction, information exchange, and transport processes are membrane mediated (Gennis, 1989; Hille, 1992; Lipowsky and Sackmann, 1995; Derby and Gleeson, 2007; Sudhahar et al., 2008). Consequently, the potential of membranes reconstituted at meso- or macroscopic dimensions, incorporating specific functional attributes (e.g. ion channels, carriers, pores, receptors, proton pumps) has attracted much attention in both basic and applied research (Hille, 1992; Galdiero et al., 2007; Viviani et al., 2007; Kiessling et al., 2009). Since lipid membranes are fluid, highly dynamic structures in their functional state, long-term investigations of their structural/functional principles and application potential are frequently limited by their short longevity and low mechanical stability (Singer and Nicolson, 1972; Cevc and Marsh, 1987; Hanke and Schlue, 1993; Marsh, 1996a; Edidin, 2003; Engelman, 2005; Jacobson et al., 2007).

The observation that the cell envelope of most archaea (formerly archaebacteria) dwelling under extreme environmental conditions like temperatures up to 120 °C, pH down to zero, high hydrostatic pressure, or high salt concentrations (De Rosa, 1996; Stetter 1999; Hanford and Peeples, 2002) is exclusively composed of a cytoplasmic membrane and a closely associated or even integrated monomolecular crystalline (glyco)protein lattice (termed surface (S)-layer) (Figs. 1 and 2a,b) led to the concept of exploiting this supramolecular principle for stabilizing planar or vesicular membrane systems (Fig. 2c–g) (Pum et al., 1993; Pum and Sleytr, 1996; Sleytr et al., 2004; Schuster and Sleytr, 2006, 2008; Schuster et al., 2008). Since suitable methods for disintegration of archaeal S-layer protein lattices and their reassembly into monomolecular arrays on lipid films are not yet available, S-layer proteins from Gram-positive bacteria (Fig. 2b) were used for copying the archaeal building principle of S-layer stabilized lipid membranes. Single or oligomeric S-layer subunits either isolation from S-layer carrying bacterial cells or recombinantly produced in host cells like, e.g. *Escherichia coli* (Sleytr et al., 2009) can be recrystallized into up to 20 μm large crystalline patches which form closed lattices on a great variety of solid supports (Fig. 1a and b) like silicon, glass, metals, polymers, semiconductors, and lipid structures (Schuster et al., 2004, 2005). Moreover, in Gram-positive bacteria assembly of S-layer proteins in defined orientation on the outer surface of the rigid peptidoglycan containing wall layer is determined by a specific (lectin-type) interaction with short glycans (termed: secondary cell wall polymers "SCWPs") covalently linked to the peptidoglycan matrix of the cell wall (Fig. 2b) (Sára, 2001; Egelseer et al., 2008). SCWPs are now used as important construction elements for the assembly of S-layer proteins on surfaces and interfaces (Sleytr et al., 2000; Egelseer et al., 2009).

In this review, we provide a state of the art survey how S-layer proteins, lipids, and SCWPs may be used as basic building blocks for assembly of supramolecular structures incorporating functional lipid membranes with increased stability and life time.

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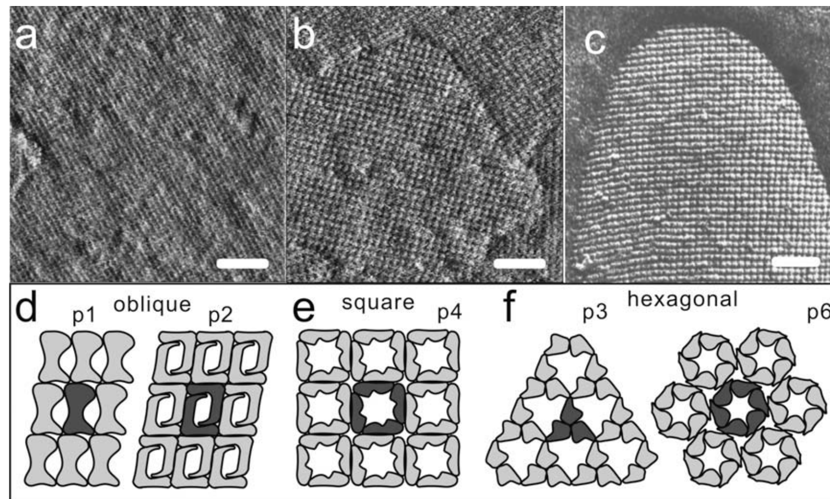

Fig. 1. Atomic force microscopical images the S-layer protein from *Geobacillus stearothermophilus* PV72/p2 exhibiting an oblique S-layer lattice (a) and the S-layer protein from *Lysinibacillus sphaericus* CCM 2177 exhibiting a square S-layer lattice (b). In the latter image crystalline patches forming the closed S-layer lattice are visible. The bars correspond to 50 nm. (c) Electron micrograph of a freeze-etched and Pt/C-shadowed preparation of a Gram-positive organism exhibiting a square S-layer lattice. The bar corresponds to 100 nm. Schematic drawing illustrating the various S-layer lattice types. In the oblique lattice (d), one morphological unit (dark grey) consists of one (p1) or two (p2) identical subunits. Four subunits constitute one morphological unit in the square (p4) lattice type (e), whereas the hexagonal lattice type is either composed of three (p3) or six (p6) subunits (f). Modified from Sleytr et al. (1999) with permission from Wiley–VCH.

2. The molecular construction kit

2.1. S-layer proteins

Despite the diversity of cell envelope structures observed in prokaryotic organisms (Beveridge and Graham 1991; Sleytr and Beveridge, 1999) one of the most commonly observed cell surface structures are monomolecular arrays composed of identical species of protein or glycoprotein subunits (Fig. 1) with a molecular mass ranging from 40 to 200 kDa. Such surface layers (termed S-layers) (Sleytr, 1978; Sleytr et al., 1988) have been identified on organisms of nearly every taxonomical group of walled bacteria (Fig. 1c) and are an almost universal feature of archaea (Sleytr et al., 1996, 2002; Sleytr and Beveridge, 1999; Sára and Sleytr, 2000). S-layers represent the only wall component external to the plasma membrane in archaea lacking a rigid cell wall layer (Fig. 2a) (Kandler, 1982; König, 1988; Baumeister and Lembcke, 1992; König et al., 2007; Engelhardt, 2007a). Being composed of a single species of constituent (glyco)protein subunits, S-layers can be considered the simplest type of biological membranes developed during evolution (Sleytr, 1975; Sleytr and Glauert 1975; Sleytr and Plohberger, 1980; Sleytr and Beveridge, 1999).

High resolution electron microscopical and atomic force microscopical studies revealed, that S-layer lattices can have oblique (p1, p2; (Fig. 1d), square (p4; (Fig. 1e) or hexagonal (p3, p6; Fig. 1f) symmetry with a center-to-center spacing of the morphological unit of approximately 5–35 nm. S-layers are generally 5–25 nm thick, whereas S-layer lattices of archaea reveal a thickness of up to approximately 70 nm. Most S-layers reveal a rather smooth outer surface and a more corrugated inner surface. Frequently, archaeal S-layers possess pillar-like domains which integrate into the hydrophobic domains of the plasma membrane (Fig. 2a) (Baumeister and Lembcke, 1992; Peters et al., 1995). S-layers are highly porous protein lattices with a surface porosity of 30–70%. Since S-layers are composed of identical species of subunits, they exhibit pores of identical size and morphology. In many S-layers two or even more distinct classes of pores with diameters up to 8 nm were identified providing them with the function of a very accurate molecular sieve in the ultrafiltration range (Sára and Sleytr, 1987; Sára et al., 1996a). Although considerable knowledge has been experimentally accumulated on the structure of S-layer proteins (Baumeister et al., 1990; Hovmöller et al., 1993), as to date, only part of an S-layer protein from a *Bacillus* species has been resolved at atomic resolution (Pavkov et al., 2008). Recently, structural predictions have also been attempted by the mean force method (Horejs et al., 2008).

Since prokaryotes carrying S-layers are ubiquitously found in the biosphere and because S-layers represent one of the most abundant cellular proteins, it is now evident, that the porous protein lattices reflect specific adaptions to a broad spectrum of ecological conditions and selection criteria. Up to now, many functions have been identified (Sára et al., 1996b; Sleytr, 1997; Sleytr and Beveridge, 1999; Sleytr et al., 1999, 2000). In context of this review it is interesting to note, that in archaea possessing S-layers as sole cell wall component the protein lattices have been identified to be involved in the generation and maintenance of cell shape and in the cell division process (Messner et al. 1986; Wildhaber and Baumeister, 1987; Sleytr and Messner, 1989; Pum et al., 1991). More recently, it was postulated that S-layers in archaea contribute to osmoprotection (Engelhardt, 2007b).

2.2. Heteropolysaccharide (SCWPs)

With the exploiting of S-layers as basic building block in a molecular construction kit for nanobiotechnological applications it became evident that recrystallization of S-layer proteins in defined orientation with respect to solid supports, the air/water interface or lipid films is most relevant (Sleytr et al., 2000, 2007a; Györvary et al., 2003, 2004; Egelseer et al., 2009). For S-layer proteins of Gram-positive bacteria which are used for the assembly of supramolecular structures, common structural organization principles have been identified (Sára et al., 1998, 2005, 2006; Egelseer et al., 2008, 2009; Sleytr et al., 2009). Most relevant, a cell wall targeting domain was found either at the N-terminal or C-terminal region of S-layer proteins, which mediates specific binding to a specific heteropolysaccharide, the so-called secondary cell wall polymer (SCWP). For Gram-positive bacteria at least two major types of binding mechanisms between the N-terminal region of S-layer proteins and SCWPs have been described (Sára, 2001; Mader et al., 2004; Huber et al., 2005; Schäffer and Messner,

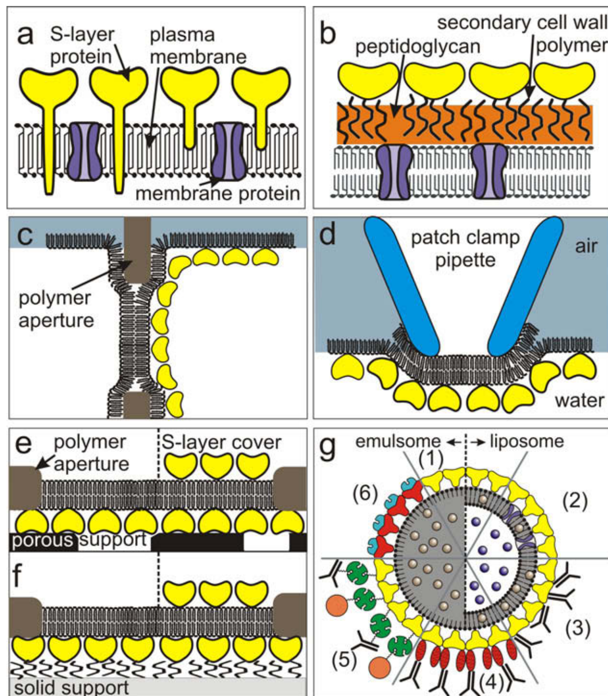

Fig. 2. Supramolecular structure of an archaeal (a) and Gram-positive bacterial cell envelope (b). Schematic illustrations of various S-layer-supported lipid membranes. In (c), a folded or painted membrane spanning a Teflon aperture is shown. A closed S-layer lattice can be self-assembled on either one or both (not shown) sides of the lipid membranes. (d) A bilayer lipid 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 on one side of the lipid membrane. (e) Schematic drawing of a lipid membrane generated on an S-layer ultrafiltration membrane (SUM). Optionally, an S-layer lattice can be attached on the external side of the SUM-supported lipid membrane (right part). (f) Schematic drawing of a solid support covered by a layer of modified secondary cell wall polymer (SCWP). Subsequently a closed S-layer lattice is assembled and bound via the specific interaction between S-layer protein and SCWP. On this biomimetic structure a lipid membranes is generated. As shown in (e), a closed S-layer lattice can be recrystallized on the external side of the solid supported lipid membrane (right part). (g) Schematic drawing of (1) an S-layer-coated emulsome (left part) and S-liposome (right part) with entrapped water-soluble (blue) or lipid-soluble (brown) functional molecules and (2) functionalized by reconstituted integral membrane proteins. S-layer-coated emulsomes and S-liposomes can be used as immobilization matrix for functional molecules (e.g. IgG) either by direct binding (3), by immobilization via the Fc-specific ligand protein A (4), or biotinylated ligands can be bound to S-layer-coated emulsome and S-liposome via the biotin–streptavidin system (5). Alternatively, emulsomes and liposomes can be coated with S layer fusion proteins incorporating functional domains (6). Modified after Sleytr et al., 2002, Copyright (2002) and Sleytr et al., 2004, Copyright (2004), with permission from Wiley–VCH.

2005; Sleytr et al., 2007a). With respect to the first binding mechanism, so-called S-layer homology (SLH) motives (Lupas et al., 1994) each comprising about 50–60 amino acids, recognize a distinct type of pyruvylated SCWP as the correct anchoring structure. A further type of binding mechanism has been characterized by the interaction of a non-pyruvylated SCWP containing the negatively charged 2,3-dideoxy-diacetamido mannosamine uronic acid with a highly conserved N-terminal region lacking an SLH domain (Egelseer et al., 2009). In the case of *Lactobacillus* S-layer proteins, SLH-motifs have not been found either, yet the attachment of the S-layer protein to the cell wall seems to involve also SCWPs in several lactobacilli (Åvall-Jääskeläinen and Palva, 2005).

2.3. Lipids

Natural bacterial cell membranes are complex bilayer structures composed of a variety of lipids and proteins. The weight ratio of protein to lipid varies from 20% to 70% (Jain, 1972), however; it is the phospholipid component that gives the membrane the crass morphology of a closed bilayer. Lipids may not be regarded only as building materials or structural elements they also have important functional tasks. Phospholipids, in many instances, are necessary for the stabilization and function of native membrane-bound proteins (Sandermann, 1978). The lipidic components of natural cell membranes consist amongst others of phospholipids and glycolipids, each differing in charge, acyl chain composition, and physical properties.

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; Fig. 3a) and thus, forming fluid lamellar structures at biologically relevant ambient temperatures (Cevc and Marsh, 1987; Hanke and Schlue, 1993; Marsh, 1996a). 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, so-called tetraetherlipids, 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 with the same overall structural features and barrier function as common phospholipid bilayers (Strobl et al., 1984; Bakowsky et al., 2000). In our group, glycerol dialkyl nonitol tetraetherlipid (GDNT; Fig. 3b), extracted and purified from *Sulfolobus* and *Metallosphaera* strains and the main phospholipid isolated from *Thermoplasma acidophilum* (MPL; Fig. 3c) has most frequently used for membrane formation. The advantage of etherlipids is their pronounced stability towards oxidative degradation (Seltmann and Holst, 2002), resistance against hydrolysis even under extreme environmental conditions (De Rosa, 1996; Gliozzi et al., 2002), and their fluid characteristics over a broad range of temperature (Nicolini, 1995).

2.4. Integral peptides and proteins

Membranes provide a surface on which membrane-active peptides and peripheral proteins can be concentrated and oriented, but also a proper matrix for the functional embedding of integral peptides and proteins (Hille, 1992). Typically, transmembrane proteins have one or more membrane-spanning domain(s) composed of either α-helices, each composed of approximately 22 hydrophobic amino acids (Marsh, 1996b), or a β-barrel structure, composed of up to seven subunits, which functions as a conductive channel (Song et al., 1996; Arora and Tamm, 2001). In addition to the hydrophobic interaction of the membrane-spanning part, positively charged residues are typically found flanking the hydrophobic domains (von Heijne, 1986; Hartmann et al., 1989).

The function of membrane proteins is influenced by the physical characteristics and dynamic properties of the membrane, such as fluidity, charge, and chemical composition. For instance, fluid bilayers allow diffusion and recognition in protein systems, for example, formation of enzyme–substrate complexes and multiprotein aggregates (Hille, 1992). Thus, for exploitation and utilization of natural, chemically modified, or genetically engineered membrane proteins, the chemical composition and physical properties of the artificial lipid bilayer must be considered (Gennis, 1989).

The exploitation of transmembrane proteins reconstituted in lipid model membranes constitutes a particular challenging task for the following reasons: (1) Membrane proteins constitute a prominent class of biomolecules as one-third of all proteins are membrane proteins such as pore-forming proteins, ion channels, receptors or enzymes (Gerstein and Hegyi, 1998; Galdiero et al., 2007). (2) Membrane proteins are directly affected in many diseases and therefore preferred sites for drugs. This can be seen from the fact, that more than 60% of all consumed drugs act on mem-

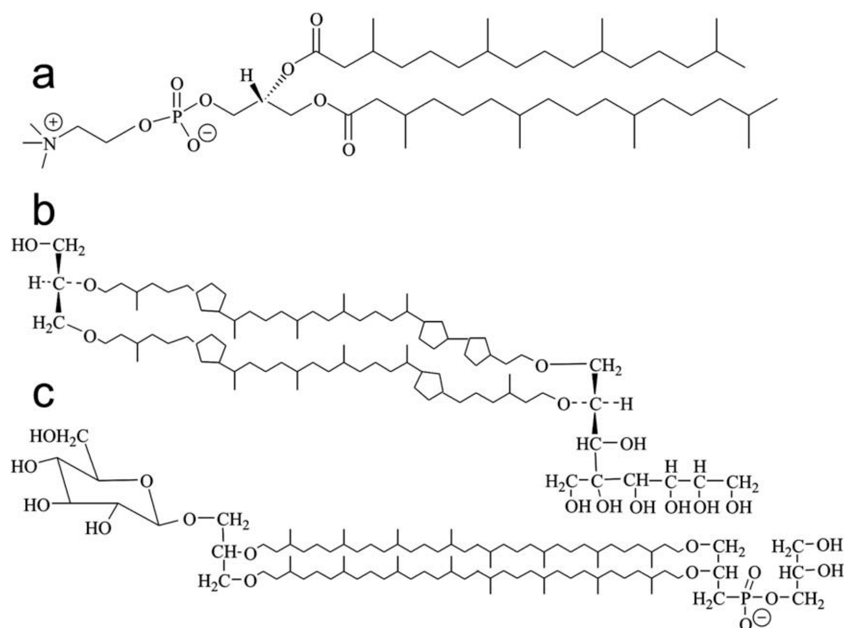

Fig. 3. Chemical structures of (a) the phospholipid 1,2-diphytanoyl-*sn*-glycero-3-phosphatidylcholine, (b) the membrane-spanning tetraetherlipids glycerol dialkyl nonitol tetraetherlipid (GDNT) extracted and purified from *Sulfolobus* and *Metallosphaera* archaea, and (c) the Main Phospholipid (MPL) isolated from *Thermoplasma acidophilum*.

brane proteins (Ellis and Smith, 2004; Viviani et al., 2007). (3) The physiological methods of perception are based on sensory receptors (Lindemann, 1996; Frings and Bradley, 2004). From this role in nature it has been deduced that membrane-active peptides, but more important transmembrane proteins can be utilized as highly specific and sensitive sensor elements at the single molecular level (Martinac, 2007). Possible applications range from diagnostic devices and lab-on-a-chip designs, pharmaceutical drug screening, proteomic analyses, over biosensing devices for environmental monitoring and for the detection of pollutants and biological warfare agents, to artificial noses or tongues (Bayley and Cremer, 2001; Anrather et al., 2004; Schuster et al., 2004; Chambers et al., 2008).

3. Fabrication of S-layer lipid structures

3.1. Planar lipid membranes

There are two main techniques for the generation of S-layer/lipid structures. At the first technique the planar lipid membrane spanning a Teflon aperture or a tip of a glass pipette is generated either by the painting or the folding method (Fig. 2c), or by the tip–dip method (Fig. 2d), respectively.

In the painting method (Mueller et al., 1962; Fettiplace et al., 1975), bilayer membranes are formed by placing a small amount of a lipid solution onto an aperture in a septum separating two aqueous solutions. After spreading the lipid solution across the aperture, most of the solvent drains away and a bilayer is formed spontaneously. The folded method (Takagi et al., 1965; Montal and Mueller, 1972) on the other hand, employs monolayers formed at the air–water interface of aqueous compartments. By raising the water levels in both compartments, the two monolayers are apposed within the aperture (Fig. 2c). Although both methods efficiently produce bilayer membranes, some differences are noted. Painted membranes contain a certain amount of organic solvent used in the membrane-forming lipid solution, but bilayers formed from monolayers are virtually free of solvent (Benz et al., 1975). The presence of organic solvent in painted membranes may affect the function of membrane-active peptides and reconstituted membrane protein. Solvent-free micro lipid membranes can be prepared on the tip of a glass pipette (tip–dip method) either from phospholipid bilayers (Coronado and Latorre, 1983; Sugawara and Hirano, 2005) or tetraetherlipid monolayers (Schuster et al., 1998a). In brief, the tip of a filled pipette is vertically immersed in a vessel solution using a micromanipulator. Keeping the tip of the pipette immersed, a lipid solution is spread on the surface. After allowing solvent to evaporate, the pipette is slowly moved out and again moved into the solution that sustains a lipid layer at the air/water interface resulting in a lipid membrane formation across the tip of the glass pipette (Fig. 2d).

In a next step, S-layer protein monomers or oligomeric precursors dissolved in an appropriate buffer are added to one or to both sides of the preformed lipid membrane. Depending on the type of S-layer protein and lipid composition, the recrystallization takes approximately one to twelve hours to obtain a lipid structure entirely covered by a closed S-layer lattice (Schuster et al., 2005).

In the second technique, a closed S-layer lattice is provided onto which a lipid membrane is generated (Schuster et al., 2001, 2003; Gufler et al., 2004). One option is to use the so-called S-layer ultrafiltration membrane (SUM) as support for the lipid membrane (Fig. 2e) (Sára and Sleytr, 1987; Weigert and Sára, 1995). SUMs are isoporous structures with very sharp molecular exclusion limits and are manufactured by depositing S-layer-carrying cell wall fragments on commercial microfiltration membranes (MFMs) with an average pore size of approximately 0.4 μm (Sára and Sleytr, 1987; Sára et al., 1988; Weigert and Sára, 1995). Finally, chemical cross-linking of the S-layer fragments can be performed to increase the stability of the whole filter. Scanning force microscopy revealed a ~3 times lower roughness for SUMs compared to untreated MFMs (Schuster et al., 2003). Furthermore, the S-layer protein can be recrystallized on a solid support like various sensor surfaces, gold, silver or platinum electrodes, or indium tin oxide coated glass (Fig. 2f). A layer of chemically modified SCWP can be bound on the solid support to provide a biomimetic surface for the S-layer proteins resulting in a defined orientation of the recrystallized S-layer lattice (Schuster and Sleytr, 2009). The solid or porous substrate is subsequently covered by a polymer foil with an aperture.

The lipid membrane resting on the S-layer lattice is fabricated across the aperture as described for folded membrane generation (Fig. 2e and f).

3.2. Liposomes

Liposomes (lipid vesicles) are formed when thin lipid films or lipid cakes are hydrated and stacks of liquid crystalline bilayers become fluid and swell (Ryman and Tyrrell, 1979; Szoka and Papahadjopoulos, 1980; Rigaud and Pitard, 1995). The hydrated lipid sheets detach during agitation and self-close to form multilamellar vesicles. Another possibility for liposome fabrication is to dissolve the tetraether- or phospholipids in ethanol and inject a small amount of this solution by a syringe into a buffer (Ryman and Tyrrell, 1979; Szoka and Papahadjopoulos, 1980). Once multilamellar vesicles have formed disruption to produce unilamellar vesicles and size reduction can be achieved by sonication or extrusion. Finally, a solution of S-layer proteins is added to the liposomes and recrystallized over night under gently shaking (Küpcü et al., 1995; Mader et al., 1999, 2000). S-layer-coated liposomes (S-liposomes) represent simple model systems resembling features of archaeal cell or virus envelopes. S-layer proteins, once crystallized on liposomes, can be cross-linked and exploited as a matrix for the covalent attachment of functional molecules as required for drug-targeting or immunodiagnostic assays (Fig. 2g) (Ilk et al., 2004; Sleytr et al., 2005). Alternatively, S-layer fusion proteins incorporating specific functions (e.g. ligands, antibodies, enzymes) can be used for liposome functionalization (Schuster et al., 2006; Sleytr et al., 2007a,b; Ilk et al., 2008).

4. Performance of S-layer lipid structures

A fascinating feature of S-layer proteins is their unique noncovalent interaction with lipid molecules within lipid monolayers, planar lipid membranes, or liposomes (Schuster and Sleytr, 2000, 2005, 2006, 2008). S-layer supported lipid membranes (SsLMs) mimic the supramolecular assembly of archaeal cell envelope structures (Fig. 2a) as either an artificial phospholipid bilayer or a tetraetherlipid monolayer replaces the cytoplasmic membrane and isolated bacterial S-layer proteins are attached either on one (Fig. 2) or both sides of the lipid membrane (Figs. 2e, f and 4).

SsLMs have been characterized by dual label fluorescence microscopy, FTIR spectroscopy, transmission electron microscopy and by X-ray and neutron reflectivity measurements (Diederich et al., 1996; Weygand et al., 1999, 2000, 2002; Smit et al., 2001). Formation of S-layer lattices that cover the entire lipid film area has been observed with zwitterionic phospholipids. The addition of a small portion of positively charged surfactants (Küpcü et al., 1995; Schuster et al., 1999) or lipid derivatives (Hirn et al., 1999) facilitated the crystallization process. From this observation it has been concluded that electrostatic interaction exists between exposed carboxyl groups on the S-layer lattice and zwitterionic lipid head groups. For such an alignment, it has been suggested that there are at least two to three contact points between the lipid film and the attached S-layer protein (Wetzer et al., 1998). Thus, less than 5% of the lipid molecules of the adjacent monolayer are anchored to these contact points on the S-layer protein whereas the remaining ⩾95% lipid molecules diffuse freely in the membrane between the pillars consisting of anchored lipid molecules (Schuster, 2005; Schuster and Sleytr, 2006). The calculation for this nanopatterned tethered structure is based on the S-layer lattice of SbpA from *Lysinibacillus sphaericus* CCM 2177 having a unit cell with a spacing of 13.1 nm (Györvary et al., 2003; Toca-Herrera et al., 2004) and an area per lipid molecule of 0.65 nm^2 (Lee et al., 2004). This nanopatterned lipid membrane is also referred to as 'semifluid membrane' (Pum and Sleytr, 1994) because of its widely retained fluid characteristic (Györvary et al., 1999; Hirn et al., 1999). But most important, although peptide side groups of the S-layer protein have been shown to interpenetrate the phospholipid head group regions almost in its entire depth, no impact on the hydrophobic lipid alkyl chains has been observed (Schuster et al., 1998a,b; Weygand et al., 1999, 2000, 2002). A second S-layer acting as protective molecular sieve and as a further stabilizing scaffolding has been recrystallized on the top of the SsLM (Fig. 4). In general, the S-layer lattices may show a congruent coverage resulting in opposing immobilized phospholipids (Fig. 4a, left). In the case of the tetraetherlipids, the molecules are immobilized on both polar lipid head groups (Fig. 4a, right). More conceivably, the S-layer lattices are displaced to some extent as it has been observed for crystalline SbpA double layers (Györvary et al., 2003). In such an arrangement (Fig. 4b), the immobilized phospholipids do not oppose each other (Fig. 4b, left) and the tetraetherlipids are anchored to the S-layer just by one lipid head group (Fig. 4b, right). With two S-layers, nanopatterned fluidity determined by S-layer/lipid head group interactions from both sides are introduced (Fig. 4). This is particularly the case if S-layer lattices differing in lattice constants and symmetry are used. Thus, S-layer lattices constitute unique supporting scaffolding resulting in lipid membranes with nanopatterned fluidity and considerably extended life times (Schuster and Sleytr, 2000, 2005, 2006, 2008; Schuster et al., 2004; Schuster, 2005).

4.1. Free-standing membranes

Schematic illustrations of SsLMs on a septum at a bilayer lipid membrane (BLM) chamber and on a tip of a micropipette are shown in Fig. 2c and d, respectively. The effect of an attached S-layer lattice on the capacitance, resistance, and the boundary potential on free-standing BLMs has been investigated and found to be negligible. In contrast, the mechanical properties of the SsLMs are considerably altered. Relaxation experiments revealed a considerably longer delay time between the voltage pulse and the

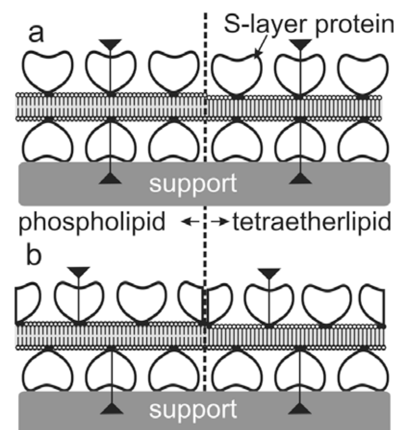

Fig. 4. Schematic illustration of the sandwich structure of an S-layer supported lipid membrane with an additional S-layer cover (not drawn to scale). (a) The S-layer lattices may show a congruent coverage resulting in opposing immobilized phospholipids (left side). In the case of the tetraetherlipids, the molecules are immobilized on both polar lipid head groups (right side). The immobilized lipid head groups are drawn in black, whereas the free-lipid head groups are drawn in white. In (b), the two S-layer lattices sandwiching the membrane are displaced. In such an arrangement, the immobilized phospholipids do not oppose each other (left side) and the tetraetherlipids are now anchored to the S-layer just by one lipid head group (right side). Please consider this is only a two-dimensional schematic drawing showing a cut where the lipid head groups interact with binding domains on the S-layer proteins. Modified after Schuster and Sleytr, 2006, Copyright (2006), with permission from Bentham Science Publishers Ltd.

appearance of the initial defect at SsLMs (Schuster et al., 1999). Hydrostatic pressure (11 N/m^2) applied across BLMs caused them to bulge resulting in an increase of capacitance due to area expansion. At a hydrostatic pressure of 11 N/m^2, a significantly higher area expansion was observed for BLMs (~3.7 nF) compared to SsLMs forced from the S-layer faced side (~1.9 nF), demonstrating an osmoprotecting effect of the S-layer lattice (Schuster et al., 1999; Schuster and Sleytr, 2002a). The membrane tension of BLMs upon the attachment of S-layer proteins has also been determined by dynamic light scattering (Hirn et al., 1999). For BLMs, the collective motions of the lipid molecules are dominated by membrane tension rather than by membrane curvature energy. Crystalline S-layer lattices at both faces of the BLM resulted in a considerable reduction of the membrane tension up to a factor of approximately five (from 0.9 mN/m to 0.2 mN/m), whereas the membrane bending energy increased by three orders of magnitude to 5×10^{-17} J. These data demonstrate that the attached S-layer lattice facilitate the transverse shear motions of the lipid molecules (Hirn et al., 1999). In accordance with voltage pulse experiments (Schuster et al., 1999), a significant increase of the previously negligible surface viscosity of the membrane has been observed as a consequence of the S-layer protein attachment (Hirn et al., 1999).

In reconstitution experiments, the incorporation of valinomycin and the self-assembly of the staphylococcal pore-forming protein α-hemolysin (αHL) (Bhakdi and Tranum-Jensen, 1991; Bayley and Cremer, 2001) has been examined at plain and SsLMs (Schuster et al., 1998b; Schuster and Sleytr, 2002b). S-layer supported GDNT monolayers functionalized with valinomycin revealed a tenfold increase in life time compared to a GDNT membrane without an attached S-layer lattice (Schuster et al., 1998a). Lytic pores were formed in a membrane mainly composed of DPhPC when αHL was added to the lipid-exposed side of the SsLM. No pore formation was detected upon adding 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 surface (Schuster et al., 1998b). In addition, these data represent a quality control for the existence of a closed S-layer lattice without any defects tightly attached to the BLM. In comparison to plain BLMs, SsLMs have a decreased tendency to rupture in the presence of αHL, indicating an enhanced stability due to the attached S-layer lattice (Schuster et al., 1998b). Even single pore recordings have been performed with αHL reconstituted in SsLMs and also with BLMs resting on an SUM (Schuster et al., 2001; Schuster and Sleytr, 2002b). However, although SsLMs revealed a higher mechanical stability (e.g. against hydrostatic pressure) and in particular with reconstituted peptides or proteins a significantly enhanced life-time compared to BLMs lacking an S-layer support (Schuster, 2005; Schuster and Sleytr, 2006), these membranes are not stable enough for many practical applications.

4.2. Filter supported membranes

In general, lipid membranes generated on a porous support combine the advantage of possessing an essentially unlimited ionic reservoir on each side of the BLM and of easy manual handling (Fig. 2e). Furthermore, the possibility for generating functional lipid membranes on S-layer ultrafiltration membranes open a great variety of possibilities for individual excess to both membrane surfaces witch is seen as basic requirement of experiments copying the *in vivo* situation (e.g. plasmatic/exoplasmatic side) is desired. However, the surface properties of porous supports, like roughness or great differences in pore size have significantly impaired the stability of attached BLMs. Thus, the strategy to use an SUM with the S-layer as the stabilizing and smoothening biomimetic layer between the lipid membrane and the porous support was initiated (Schuster et al., 2001).

Composite SUM-supported DPhPC bilayers have been shown to constitute highly isolating structures during their whole life-time of up to 17 h (Table 1) (Schuster et al., 2001; Gufler et al., 2004). For a comparison, lipid membranes on a plain MFM revealed a life-time of approximately 3 h. Interestingly, an additional monomolecular S-layer protein lattice recrystallized on the lipid-faced side, forming an S-layer–lipid membrane–S-layer sandwich-like structure, increased the life time significantly to about one day (Schuster et al., 2001; Gufler et al., 2004). Stable lipid membranes on SUMs have also been generated with MPL, mixtures of MPL with DPhPC, and pure DPhPC (Schuster et al., 2003). Interestingly, the capacitance of these electrically tight SUM-supported membranes increased continuously with increasing MPL to DPhPC ratio (Table 1). This result nicely demonstrated that the pure DPhPC membrane was thicker than membranes with a certain amount of MPL and finally, the pure MPL monolayer constituted the thinnest membrane (Schuster et al., 2003).

In a further study, the membrane-active peptide gramicidin D was incorporated (Schuster et al., 2003). The most striking result was that high-resolution conductance measurements on single gramicidin D pores could be performed in all SsLMs above mentioned. The reconstitution of αHL to form lytic pores could be achieved with SUM-supported DPhPC bilayers but no pore formation was observed with BLMs generated on the pure MFMs (Schuster et al., 2001).

Table 1
Electrophysical parameters of S-layer stabilized lipid membranes.

Lipid	Solvent	Support	Capacitance (μF/cm^2)	Resistance (MΩ cm^2)	Stability (h)	Method
DPhPC	*n*-Decane	No	0.40	33.3	–	VC
DPhPC	Hex/Eth	No	0.84 ± 0.05	1.15 ± 0.07	6 – 7	VC
DPhPC	Hex/Eth	Gold/SbpA	0.53 ± 0.14	5 – 80	5 – 46	EIS
DPhPC	Hex/Eth	SUM	0.69 ± 0.04	25 – 50	⩽17	EIS
		SUM	0.62 ± 0.03	>10	7.9 ± 2.4	VC
DPhPC/MPL 1/1	Hex/Eth	SUM	0.66 ± 0.04	>10	–	VC
DPhPC/MPL 1/5	Hex/Eth	SUM	0.75 ± 0.02	>10	–	VC
MPL	Hex/Eth	SUM	0.77 ± 0.08	5 – 55	⩽18	EIS
		SUM	0.76 ± 0.01	>10	8.3 ± 2.9	VC
MPL	Hex/Eth	Gold/SbpA	0.75 ± 0.07	0.9 – 60	4 – 44	EIS
MPL	Hex/Eth	Gold/SbpASbpA cover	0.76 ± 0.01	>10	21.2 ± 3.1	VC
GDNT	Hex/Eth	Gold/SbpA	0.68 ± 0.03	20 – 125	30 – 46	VC
GDNT	Hex/Eth	Gold/SbpA SbpA cover	0.68 ± 0.03	20 – 125	41 – 106	VC

DPhPC, diphytanoyl phosphatidylcholine (main lipid for bilayer formation); MPL, Main Phospholipid isolated from *Thermoplasma acidophilum*; GDNT, glycerol dialkyl nonitol tetraetherlipid; SbpA, S-layer protein from *Lysinibacillus sphaericus* CCM 2177; SUM, S-layer ultrafiltration membrane; Hex/Eth, *n*-hexane/ethanol = 9/1; $CHCl_3$, chloroform; VC, voltage clamp; EIS, electrochemical impedance spectroscopy. Modified after Schuster and Sleytr (2006), Copyright (2006), with permission from Bentham Science Publishers Ltd.

4.3. Solid supported membranes

Solid-supported lipid membranes involving S-layers as constituents have been fabricated by several ways. In a first approach, S-layer proteins have been self-assembled on silanized silicon surfaces before generating a BLM by the Langmuir–Blodgett-technique. This composite structure has been compared with a silane- and a dextran-supported phospholipid bilayer (Györvary et al., 1999). Most probably due to the repetitive local interactions of the S-layer lattice with the lipid head groups, the nanopatterned fluidity of lipids was highest in the SsLMs compared to silane- or dextran-supported bilayers. Phospholipid bilayers and tetraetherlipid monolayers have also been generated on S-layer covered gold electrodes (Fig. 2f). To enhance the long-range order and the smoothness of the S-layer lattice, a layer of thiolated SCWP has been chemisorbed on the gold surface prior recrystallization of the S-layer protein (Schuster and Sleytr, 2008, 2009). A sandwich-like structure of a GDNT monolayer in between the S-layer covering the gold electrode and a second S-layer on the top revealed an exceptional long-term robustness of more than four days (Table 1) (Schuster, 2005; Schuster and Sleytr, 2006). This clearly demonstrates that nanopatterning of the membrane fluidity by the attached S-layer lattice is a promising strategy for generating stable lipid membranes.

The functionality of this type of lipid membranes has been demonstrated by the reconstitution of the membrane-active peptides alamethicin, gramicidin, and valinomycin (Gufler et al., 2004). For example, a membrane with incorporated valinomycin, a potassium-selective ion carrier, revealed a remarkable high resistance when bathed in a sodium buffer. In contrast, due to the valinomycin-mediated ion transport a pronounced decrease in resistance by a factor of almost 500 was observed for the same membrane bathed in a potassium buffer (Gufler et al., 2004). Furthermore, it has been demonstrated that conductive alamethicin channels can be blocked as increasing amounts of an inhibitor gave rise to an increased membrane resistance (Gufler et al., 2004). Thus, proof-of-principle for the applicability of these composite structures for biosensing purposes has been demonstrated. The ability to reconstitute integral membrane proteins in defined structures is one of the most important concerns in designing biomimetic sensing devices.

4.4. Liposomes

S-liposomes (Fig. 2g) are biomiometic structures with remarkably high mechanical and thermal stability (Mader et al., 1999) and the possibility for immobilizing or entrapping biologically active molecules (Mader et al., 2000). Thus, S-liposomes may reveal a broad application potential, particularly as carrier and/or drug delivery systems, as artificial virus envelopes in, e.g. medicinal applications and in gene therapy (Mader et al., 2000; Pum et al., 2006; Schuster et al., 2006; Sleytr et al., 2007a,b; Ilk et al., 2008; Kepplinger et al., 2009). Furthermore, an S-layer fusion protein carrying the sequence of the enhanced green fluorescent protein at the C-terminus was recrystallized on liposomes. Because of the intrinsic fluorescence of this S-layer fusion protein, the uptake of S-liposomes into eukaryotic cells could be easily visualized (Ilk et al., 2004).

5. Conclusions and perspectives

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, 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 meso- and macroscopic scale (Figs. 4 and 5). In this biomimetic architecture, artificial 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. Furthermore, amphiphilic lipid tether molecules, with one end bound to the support and the opposite hydrophobic end reaching through the pores of the S-layer lattice, may facilitate membrane formation and provide more nanopatterend anchors for modulating the fluidity and enhancing the stability of this composite architecture (Fig. 5).

An important strategy to design stable membranes on surfaces and interfaces concerns the application of SCWPs as constituent of the molecular construction kit. The use of SCWPs enables reassembly of S-layer lattices on surfaces and lipid films in predetermined orientation and is thus, essential for many nanobiotechnological applications (Sára et al., 1998; Egelseer et al., 2008). Moreover, SCWPs alone or linked to lipid molecules provide useful linkers or spacers between S-layer lattices and inorganic supports (Fig. 5) (Sleytr et al., 2000). SCWP-linked phospholipids incorporated in the outer leaflet of the lipid membrane will allow the recrystallization of a second S-layer on the top of the composite architecture. Most important, this S-layer lattice on the top is clear separated from the lipid membrane. Thus, previously reconstituted transmembrane proteins will not be affected by the top layer composed of glycoproteins which serves as a nanoporous filter with antifouling characteristics and as protecting layer for, e.g. mechanical challenges (Fig. 5). This biomimetic approach is one of the best examples for synthetic biology and mimics structural and functional aspects of many bacterial and archaeal S-layer lattices (Sleytr and Beveridge, 1999).

The very accurate nanopatterned fluidity and maintenance of lateral fluidity (mobility) of membrane-active peptides and membrane proteins (Schuster et al., 2001, 2003) in these biomimetic membranes provides significant advantage in comparison to other supported membrane systems. Moreover, the combination of S-layer proteins and the specific interacting SCWP's enables a defined variation of the distance between the S-layer lattice and the solid support (Schuster et al., 2006; Sleytr et al., 2007a,b) which is seen as basic requirement for the incorporation of membrane proteins with domains exposed on the inner and/or outer membrane surface and for the assembly of multi-component membrane functions (e.g. G-protein coupled receptors, kinases, etc.).

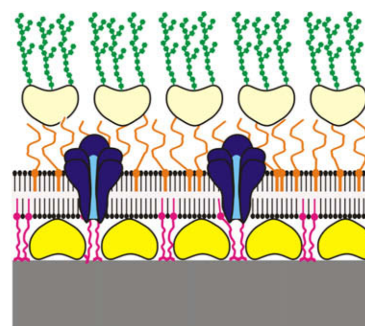

Fig. 5. Schematic drawing of a lipid membrane on an S-layer (yellow) covered porous or solid support (grey). The lipid membrane (black) is bound by incorporated tethered lipid molecules (magenta) reaching through the S-layer pores to the substrate. In this lipid membrane, transmembrane proteins (blue) can be reconstituted. Finally, a further proteinaceous lattice composed of S-layer glycoproteins (with carbohydrate moieties in green) can be recrystallized on the top via SCWP-linked phospholipids (orange) which are anchored in the outer leaflet of the lipid membrane.

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 (Fig. 5) should significantly help to overcome this problem.

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

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