I. Basic and applied S-layer research: an overview

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Abstract

S-layers are crystalline monomolecular assemblies of protein or glycoprotein, which represent one of the most common cell surface structures in Archaea and Bacteria. As porous lattices completely covering the cell surface they can provide prokaryotic cells with selection advantages by functioning as protective coats, as structures involved in cell adhesion and surface recognition, as molecule or ion traps, and molecular sieves. In Archaea, which possess S-layers as exclusive cell wall component, the (glyco)protein lattices function as cell shape determining/maintaining framework. Studies on structure, chemistry, genetics, assembly and function of S-layers revealed a considerable application potential for the regular arrays in biotechnology, biomimetics, biomedicine and molecular nanotechnology.

Keywords: Crystalline surface layers; S-layers; Biomimetics; Molecular nanotechnology

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1. Introduction

Electron microscopical and chemical studies reveal considerable diversity in the architecture of prokaryotic cell envelopes. Since most organisms have to survive in very competitive habitats this observed diversity, particularly that of the molecular architecture of the outermost envelope layer, reflects very

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1 This review is part of a series of reviews dealing with different aspects of bacterial S-layers; all these reviews appear in Volume 20/1-2 (June 1997) of FEMS Microbiological Reviews, thematic issue devoted to bacterial S-layers.
specific adaptations to environmental and ecological conditions [1].

One of the most remarkable features of prokaryotic cell envelopes is the presence of monomolecular arrays of protein and glycoprotein subunits referred to as S-layers [2,3]. Their identification on selected organisms was originally considered to represent a rather unique cell wall component [2,4]. After approximately 30 years of research S-layers are now recognized as one of the most commonly observed bacterial cell surface structures. They have now been identified in hundreds of different species belonging to all major phylogenetic groups of Bacteria and represent an almost universal feature of Archaea [5,6]. Morphological, chemical and morphogenetic studies have shown that S-layers represent the simplest type of biological membrane developed during evolution [7]. With a few exceptions they are composed of a single glyco(protein) species endowed with the ability to assemble into closed two-dimensional arrays on the cell surface during all stages of cell growth and cell division.

The considerable body of knowledge accumulated on the general principles of S-layers, particularly their structure, chemistry, genetics and morphogenesis, has led to a broad spectrum of applications in biotechnology, vaccine development, diagnostics, biomimetics and molecular nanotechnology [6].

2. Structure, chemistry, assembly and molecular biology

High resolution electron microscopical studies revealed, that S-layer lattices can have oblique (p1, p2), square (p4) or hexagonal (p3, p6) symmetry with a center-to-center spacing of the morphological units of approximately 3 to 35 nm. Amongst Archaea p6 lattices were shown to be predominant [5,6]. S-layers are generally 5 to 25 nm thick and reveal a rather smooth outer surface and a more corrugated inner surface. Since S-layers are monomolecular assemblies of identical subunits they exhibit pores of identical size and morphology. In many S-layers two or even more distinct classes of pores could be observed. Pore sizes were determined to be in the range of approximately 2 to 8 nm and pores can occupy 30 to 70% of the surface area [6,8–12]. In many species of Bacteria the S-layers of individual strains exhibit great diversity with respect to lattice symmetry and center-to-center spacing of the morphological units. In some organisms, two or even more superimposed S-layer lattices were identified [6]. Freeze-etching preparations (Fig. 1) have demonstrated that the S-layer lattices completely cover the
Table 1

- \( M_o \) of constituent subunits in the range of 40,000 to 200,000
- Weakly acidic proteins (pI = 3–5). Exception: *Methanothermusa* (pI = 8.4) and lactobacilli (pI > 9.5)
- High amount of glutamic and aspartic acid (\( \sim 15 \text{ mol}\% \))
- High lysine content (\( \sim 10 \text{ mol}\% \))
- Hydrophobic amino acids (\( \sim 40-60 \text{ mol}\% \))
- No or only a low content of sulfur-containing amino acids
- Hydrophilic and hydrophobic amino acids do not form extended clusters
- In most S-layer proteins 40% of the amino acids are organized as \( \beta \)-sheet and about 20% occur as \( \alpha \)-helix
- Aperiodic foldings and \( \beta \)-turn content may vary between 5% and 45%
- Posttranslational modifications of S-layer proteins include (i) deavage of N- or C-terminal fragments, (ii) glycosylation and (iii) phosphorylation of amino acid residues

Cell surface at all stages of cell growth and division in both Archaea and Bacteria [2,12,13]. Most S-layers are composed of a single homogeneous protein or glycoprotein species. Characteristic chemical and molecular biological properties of S-layers are summarized in Table 1 [5,6,14–16]. A remarkable feature of both Archaea and Bacteria is their ability to glycosylate their S-layer proteins [17–19]. Studies on the carbohydrate moieties revealed that the glycan chains are polymers of two to six monosaccharide units which include a wide range of hexoses, deoxy- and amino sugars, uronic acids and even sulfate or phosphate residues as constituents. Most remarkably, S-layer glycans from different bacillaceae revealed similarities with the structure of the O-antigens of lipopolysaccharides of Gram-negative Bacteria [20,21].

Comparative studies on the distribution and uniformity of S-layers including structural, molecular biological and genetic studies revealed that, although their overall amino acid composition is comparable in general, the homology between S-layer lattices of different bacterial species or even strains within a species is generally very low [5,6,14–16]. This strongly indicates that, at least in Bacteria, S-layers are non-conserved structures and only of limited taxonomical value for higher taxa. However, with the additional information of amino acid sequence and glycan structure differentiation even at species level is possible.

Although S-layers are now recognized an important class of secreted proteins up to now only approximately 35 S-layer genes have been cloned and sequenced [15,16]. There is also only little information available on the regulation of S-layer protein synthesis, the translocation of S-layers across the bacterial cell envelope components, and about protein domains involved in inter- and intramolecular interactions. This lack of knowledge is primarily due to the difficulties in cloning S-layer genes in a form in which they are stably expressed at high levels.

It can be calculated that approximately \( 5 \times 10^5 \) S-layer protein monomers are needed to cover an average-sized rod-shaped prokaryotic cell. Thus, at a generation time of about 20 min, at least 500 copies of a single polypeptide species have to be synthesized, translocated to the cell surface, and incorporated into the S-layer lattice per second. Consequently, S-layer protein expression must be very efficient and the synthesis must be coordinated with cell growth. Nevertheless, some organisms are overproducers and shed S-layer material into the medium. S-layer protein production can be directed by single or multiple promoters in front of the S-layer gene, yielding very stable mRNAs [15,16]. Still very few data are available on S-layer protein secretion. Although most S-layer proteins seem to be secreted over the general secretory pathway it appears, that some organisms use specific pathways [15,16]. In a few cases S-layer protein variations for an individual bacterial species has been reported [22–24]. This phenotypical variation of S-layer proteins is determined by one or more silent S-layer genes where only one gene is expressed at a given time. Particularly in pathogenic organisms such antigenic variation was shown to be a mechanism for creating a heterogeneous population capable to survive the bactericidal activity of the immune system [22].

Once secreted the subunits of most S-layers interact with each other and with the supporting envelope layer through non-covalent forces. Particularly differences in the net surface charge and hydrophobicity of the inner and outer surface have been shown to be responsible for the proper orientation of the S-layer subunits and their insertion in the course of lattice growth [2,7,13,25]. Depending on the cell envelope design S-layer subunits may specifically interact with components of the plasma membrane (in
Gram-negative Archaea), the peptidoglycan or secondary cell wall polymers (in Gram-positive Bacteria), other polymers such as pseudomurein (in Gram-positive Archaea) or components of the outer membrane (in Gram-negative Bacteria) [1,6,9,26,27]. Most recently defined domains of S-layer proteins were identified to be involved in specific interactions with cell envelope components [16,24,28–32].

Numerous studies have also been performed to elucidate the dynamic process of assembly of S-layer lattices on intact bacterial cells during cell growth and cell division [7,13,25,33–36]. It is now evident that S-layers are 'dynamic closed surface crystals' with the intrinsic capability to assume continuously a structure of low free energy during cell growth. Analysis of the distribution of lattice faults in Archaea, which possess an S-layer as exclusive wall component, provided evidence that they are involved in lattice extension, maintenance of cell structure and in the fission process [13,37,38].

### 3. Functional aspects

Unless other cell surface components (e.g. glyocalyces, capsules) or sheaths are present, S-layers as the outermost envelope component represent an important interface between the cell and its environment [1]. Since prokaryotes carrying S-layers are ubiquitously found in the biosphere and because S-layers represent one of the most abundant of cellular proteins it became obvious, that the porous protein lattices reflect specific adaptations to ecological conditions and selection criteria. Particularly with Bacteria this is stressed by the frequently observed phenomenon, that under optimal growth conditions in continuous laboratory cultures, S-layer deficient mutants or variants outgrow the wild type strains. S-layers are also generally part of more complex envelope structures and must not be considered in functional terms as isolated supramolecular structures.

The elucidation of the functional significance of S-layers is still fragmentary and many of the functions assigned to S-layers are still hypothetical and not based on firm experimental data. It is also obvious that no general function for all S-layers will be determined. A survey of the major functional principles of S-layers in Archaea and Bacteria is given in Table 2 and in references [1,3,5,8,9,14,27,33,39–43]. From a very general point of view S-layers have shown to function as (i) protective coats, molecular sieves in the ultrafiltration range, and as molecule and ion traps, (ii) target structure which promotes cell adhesion and surface recognition and (iii) framework determining cell shape in Gram-negative Archaea. It is also quite obvious that S-layers can be multifunctional.

Particular attention has been paid to S-layers present on pathogenic organisms. The most detailed studies have been performed on Aeromonas salmonicida, Aeromonas hydrophila [44,45] and Campylobacter fetus [46,47]. Since the presence of S-layers have been reported on many other pathogens of animals and humans (e.g. a broad spectrum of Bacillus spp., Clostridium spp., Chlamydia spp., Treponema spp., Campylobacter spp., Rickettsia spp., Wolinella spp., Bacteroides spp., Bordetella pertussis, Cardiobacter hominis, Aeromonas spp. — for compilation see ref. [5,6]) it can be expected that further

### Table 2

- Determination of cell shape and cell division (in archaea that possess S-layers as the exclusive wall component)
- Protective coats
  - Prevents predation by *Bdellovibrio bacteriovorus* (in Gram-negative bacteria)
  - Phage resistance by S-layer variation
- Prevention or promotion of phagocytosis
- Adhesion site for exoenzymes
- Surface recognition and cell adhesion to substrates
- S-layers function as physicochemical and morphological well-defined matrices
- Masking the net negative charge of the peptidoglycan-containing layer in Bacillaeae
- Isoporous molecular sieves
- Molecular sieves in the ultrafiltration range
- Delimiting in Gram-positive bacteria a compartment (periplasm)
- Preventing non-specific adsorption of macromolecules
- Virulence factor in pathogenic organisms
- Important role in invasion and survival within the host
- Specific binding of host molecules
- Protective coat against complement killing
- Ability to associate with macrophages and to resist the effect of proteases
- Production of S-layers which do not immunologically cross-react (S-layer variation)
- Fine grain mineralization
studies will demonstrate their significance as virulence factors.

4. Application potential

The considerable body of knowledge accumulated on structure, chemistry, morphogenesis, genetics and physicochemical surface properties of S-layers has led to a variety of applications during the last decade which are summed up in Table 3 and reviewed in references [14,48–55].

The broad application potential of S-layers is based on the unique intrinsic features of porous monomolecular arrays. Many applications also depend on the capability of isolated S-layer subunits to assemble into regular arrays in suspension or on suitable surfaces or interfaces upon removal of the disrupting agent (e.g. guanidine hydrochloride) used for their isolation [7,13,49]. Since S-layers are periodic structures composed of a single (glyco)protein species they exhibit identical physicochemical properties on each molecular unit down to the subnanometer scale and possess pores identical in size and morphology. Most important, functional groups (carboxyl, amino or hydroxyl groups) are aligned on the surface and within the pore areas of S-layer lattices in well defined position and orientation. This uniformity provided possibilities for a broad spectrum of chemical modifications for obtaining differently charged or hydrophilic and hydrophobic S-layer ultrafiltration membranes with very accurate nominal molecular mass cut-offs [56–59].

A broad spectrum of applications involves the use of S-layer lattices in suspension or attached to supports as immobilization matrix for binding monolayers of functional molecules (e.g. antibodies, ligands, hapten, immunogens, enzymes) in an unsurpassed reproducible way. Scanning force microscopy and high resolution electron microscopy demonstrated that the immobilized molecules frequently even reflect the periodicity of the S-layer lattice. This technology is used for the production of bioanalytical sensors, enzyme and affinity membranes, immunoassays, dipsticks and conjugate vaccines [48–56].

Another line of applications exploits the capability of S-layer subunits to recrystallize at macroscopic scale into coherent lattices on Langmuir-Blodgett lipid films [49,60] or on liposomes [61]. These composite membranes mimic at macroscopic scale the supramolecular structure of the cell envelopes of Gram-negative Archaea. Since functional molecules can be incorporated into S-layer stabilized lipid membranes this technology has the potential to initiate a broad spectrum of development in areas such as diagnostics, sensor technology and electronic or optical devices.

Cloning and characterization of genes encoding S-layer proteins, particularly homologous and heterologous expression experiments, opened new areas of

Table 3
- Biomimetic S-layer ultrafiltration membranes (SUMs) with defined physicochemical surface properties and molecular sieving characteristics
- SUMs, S-layer self-assembly products or S-layer microparticles as matrices for controlled immobilization of functional molecules
  - Covalent binding of enzymes for amperometric and optical bioanalytical sensors
  - Immobilized monoclonal antibodies for dipstick-style immunoassays
  - Immobilized protein A for escort-particles in affinity cross-flow filtration for isolation and purification of antibodies
  - Immobilized antibodies for preparation of microparticles for ELISA
- Supporting structures for functional lipid membranes at meso- and macroscopic scale
  - 'Semi-fluid' lipid membranes mimicking cell envelopes of Gram-negative archaea
- S-layer coated liposomes
  - Immobilization of functional molecules on S-layer coated liposomes (e.g. addressor molecules for drug-targeting)
  - Entrapping of functional molecules (drug-delivery)
  - Artificial viruses
  - S-layer coated liposomes with immobilized antigens and hapten for vaccination
- Conjugate vaccines
  - S-layers as carriers with intrinsic adjuvant property for immobilization of antigens and hapten
- Vehicle for producing fusion proteins
  - Homologous and heterologous expression of self-assembling fusion proteins (incorporation of functional domains as required for affinity matrices, enzyme membranes, vaccines, biosensors and diagnostics)
- Biomimetic templates and nonnatural resist for semi-conductor technologies
- Matrices for controlled biomineralization
- Matrices for nanofabrication of metallic point patterns
applied S-layer research [15,16]. In S-layer proteins domains have been identified which allow foreign epitope insertion without hindering self-assembly into regular arrays [16,62]. Incorporation of peptide stretches of well known functional domains will lead to new types of affinity and enzyme membranes, ion-selective binding matrices, micro (affinity) carriers, biosensors, diagnostics, vaccines, biocompatible surfaces or bioabsorbable systems for tissue regeneration [48].

Recently, it was demonstrated that S-layers can be employed in nanostructure technologies. S-layers recrystallized on silicon wafers were patterned by deep ultra-violet radiation and used as novel nanonatural resist, as matrix for controlled biominalization and support for functional lipid membranes [42,49,63–65]. Further, it was shown that metal oxide coated S-layers can be employed as masks for nanostructuring semi-conductor surfaces [66]. Such nanopatterning procedures could lead to new optoelectronic materials and devices.

5. Conclusions and perspectives

S-layers are now recognized as one of the most common envelope surface structures in Archaea and Bacteria. Detailed studies on structure, chemical composition, genetics, morphogenesis, surface and permeability properties revealed that S-layers are the simplest biological membranes developed during evolution. On growing cells S-layers exhibit the unique feature of a dynamic closed (glyco)protein crystal.

With the exception of a few pathogenic organisms and the Gram-negative Archaea still very little is known about the functional significance of S-layers. This is primarily explained by the fact that applied laboratory cultures rarely reflect the organisms natural habitat, involving unbalanced growth and a great variety of yet unknown selection criteria characteristic for complex ecologies. The unique feature of S-layers, particularly their self-assembly characteristics, their structural and physicochemical repetitive uniformity down to the subnanometer scale, and their isoporosity, make them structures at the ultimate resolution limit for the molecular functionalization of surfaces and interfaces. Biomimetic approaches copying the supramolecular concept of S-layer stabilized plasma membranes (developed by Archaea in the most extreme ecosystems) should lead to technologies exploiting functional lipid membranes at macroscopic scale. Although many applications for S-layers have already been demonstrated many further areas in which these unique biomaterials are of relevance may yet emerge. It is expected that in the near future particularly modifications of S-layer protein by recombinant DNA technologies will significantly influence the development of applied S-layer research.

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References


