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PROSPECTS

Two-Dimensional Protein Crystals (S-Layers): Fundamentals and Applications

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Abstract Two-dimensional crystalline surface layers (S-layers) composed of protein or glycoprotein subunits are one of the most commonly observed prokaryotic cell envelope structures. Isolated S-layer subunits are endowed with the ability to assemble into monomolecular arrays in suspension, on surfaces or interfaces by an entropy-driven process. S-layer lattices are isoporous structures with functional groups located on the surface in an identical position and orientation. These characteristic features have already led to applications of S-layers as (1) ultrafiltration membranes with well-defined molecular weight cut-offs and excellent antifouling characteristics, (2) immobilization matrices for functional molecules as required for affinity and enzyme membranes, affinity microcarriers and biosensors, (3) conjugate vaccines, (4) carriers for Langmuir-Blodgett films and reconstituted biological membranes, and (5) patterning elements in molecular nanotechnology. © 1994 Wiley-Liss, Inc.

Key words: Crystalline bacterial surface layers, S-layers, ultrafiltration membranes, immobilization matrix, conjugate vaccines, biomembranes, biosensors, affinity microparticles

Crystalline bacterial surface layers (S-layers) can be found as outermost cell envelope component in organisms of almost every taxonomic group of walled eubacteria and archaeobacteria [for compilation, see Sleytr et al., 1988; Messner and Sleytr, 1992]. They are composed of a single protein or glycoprotein species with molecular weights ranging from 40,000 to 200,000 [Sleytr et al., 1988]. S-layers completely cover the cell surface (Fig. 1) and can exhibit either oblique, square or hexagonal lattice symmetry (Fig. 2) with spacings of the morphological units in the range of 3–30 nm. With respect to their inner and outer faces, they are highly anisotropic structures in their topography and physicochemical properties. Most S-layers are 5–15 nm thick and possess pores of identical size and morphology in the 2- to 6-nm range. In many S-layers, two or even more distinct classes of pores could be observed. Comparison of amino acid analyses and genetic studies on S-layers from a broad spectrum of organisms has shown that the crystalline arrays are usually composed of weakly acidic proteins. The contents of hydrophobic amino acids is generally high and the cysteine or methionine content low. An interesting feature of many archaeobacteria and selected eubacteria is their ability to glycosylate their S-layer proteins [Messner and Sleytr, 1991]. The carbohydrate moieties strongly resemble O-antigen polysaccharides of gram-negative eubacteria. They are polymers of linear or branched repeating sequences of two to six monosaccharide units, which include a wide range of hexoses, deoxy- and amino-sugars, uronic acids, or even sulfate or phosphate residues. The chain length may vary from a few sugars up to approx. 150 monosaccharide residues. The glycan chains can be linked to the protein moiety by N- or O-glycosidic linkages.

The subunits of most S-layers interact with each other and with the supporting envelope layer through noncovalent forces [Sleytr and Messner, 1989]. Generally, a complete disintegration of S-layers into the constituent subunits can be obtained by treatment of intact cells or cell wall fragments with high concentrations of H-bond breaking agents (e.g., guanidine hydrochloride). On removal of the disintegrating agent (e.g., by dialysis) isolated S-layer subunits from numerous bacteria have shown the ability to

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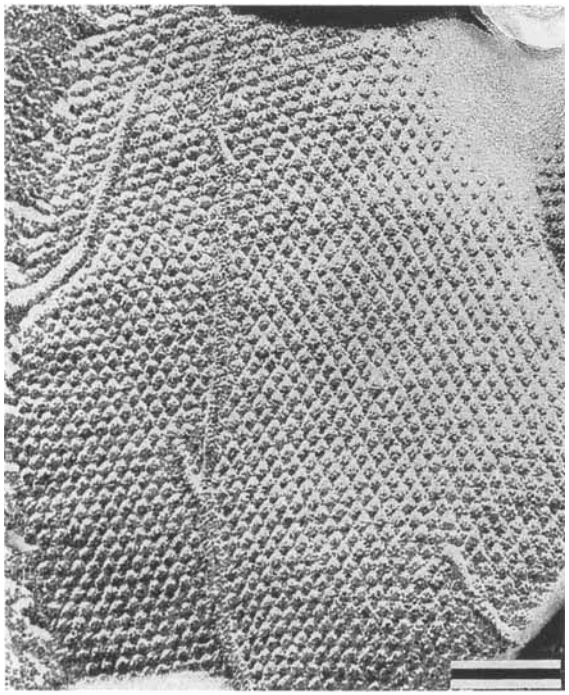

Fig. 1. Electron micrograph of a freeze-etched preparation of intact cells of *Methanocorpusculum sinense* showing a hexagonal (p 6) lattice. Bar = 100 nm.

assemble into two-dimensional arrays either in suspension or on suitable surfaces or interfaces [Sleytr and Messner, 1989].

Studies on the self assembly process in the absence of a template have shown that subunits can recrystallize into flat sheets, open-ended cylinders, or closed vesicles. The size of the self-assembly products can reach 10–15 μm. With some S-layer subunits different assembly routes can be obtained by changing the assembly conditions (pH, temperature, ionic strength, presence or absence of divalent cations). In addition to monolayers also double layers can be formed.

S-layer subunits also have the ability to recrystallize on solid supports such as silicon, glass, mica, carbon, metals, or synthetic polymers upon removal of the disrupting agent used for their isolation [Sleytr et al., 1992; Pum and Sleytr, 1993]. Depending on the surface properties both of the supporting layer (e.g., charge or hydrophobicity) and the S-layer (glyco)protein, a specific orientation of the subunits can be obtained.

The considerable body of knowledge accumulated on structure, chemistry, assembly and physicochemical properties of S-layers has led to a broad spectrum of applications, particularly in the field of biotechnology, biomedicine and mo-

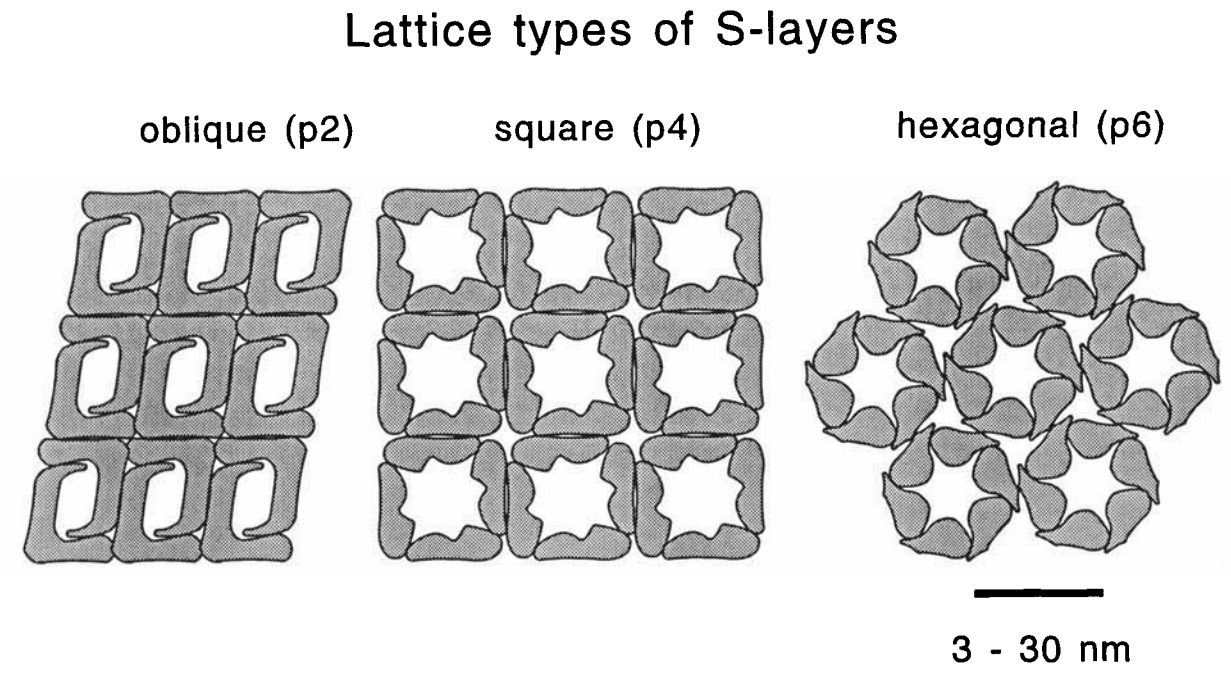

Fig. 2. Schematic diagram of the major space groups found for S-layer lattices. The unit cells which are the building blocks are composed of di-, tetra-, or hexamers.

lecular nanotechnology [for recent reviews, see Sleytr et al., 1992; Pum and Sleytr, 1993].

S-LAYERS AS NATURE-TAILORED ULTRAFILTRATION MEMBRANES

The observation that S-layers function as isoporous molecular sieves led to the development of a completely new type of ultrafiltration membrane [Sára and Sleytr, 1988; Sára et al., 1990], in many aspects superior to amorphous membranes composed of synthetic polymers. For the production of S-layer ultrafiltration membranes (SUMs) S-layer fragments are deposited on commercial microfiltration membranes showing either a spongy structure and a variable pore size or a smooth surface and a well defined pore size, such as radiation track membranes. The mechanical and chemical resistance of the composite structure is obtained by introducing inter- and intramolecular covalent linkages between the individual S-layer subunits. Moreover, since S-layers are periodic structures composed of identical subunits, functional groups on the polypeptide chain (e.g., carboxyl, amino, or hydroxyl groups) are located on each constituent subunit in an identical position and identical orientation. This uniformity of reactive groups on both the surface of the S-layer lattice and within the pore area provides possibilities for a broad spectrum of chemical modifications for obtaining differently charged or hydrophilic and hydrophobic membranes [Küpcü et al., 1991]. Different surface properties of ultrafiltration membranes are of particular interest for minimizing membrane adsorption (fouling) which leads to a decrease of membrane performance. By immobilizing molecules of different size in the pore areas, SUMs with different molecular sieving properties and cut off values were obtained [Sára et al., 1993a]. In this context, it is of particular interest to note that native S-layers possess excellent antifouling characteristics. This is seen as an essential requirement to prevent pore plugging and to maintain an unhindered exchange of molecules up to a defined molecular weight between the cell and its environment.

S-LAYERS AS MATRIX FOR IMMOBILIZATION OF FUNCTIONAL MACROMOLECULES

S-layer lattices reveal identical physicochemical properties on each molecular unit area in a nanometer scale. Consequently they represent unique structures for a defined binding of differently sized macromolecules. With glycosylated S-layers not only the protein moiety but also the carbohydrate residues are available for the immobilization. High resolution electron microscopy studies revealed that molecules immobilized on both the protein and the carbohydrate chains of S-layer subunits are frequently very regularly arranged, reflecting the periodicity of the supporting S-layer lattice [Sára and Sleytr, 1992; Sára et al., 1993a,b].

In order to obtain an immobilization matrix resistant toward acid, alkali, hydrogen bond breaking agents (e.g., guanidine hydrochloride) and organic solvents the S-layer subunits are most commonly first crosslinked with glutaraldehyde. Carboxyl groups are activated with carbodiimide, whereas hydroxyl groups are frequently treated with periodate or activated with cyanogenbromide. After glutaraldehyde fixation frequently a considerable proportion of the amino groups of the S-layer proteins remains unmodified. They can be converted into sulphhydryl groups upon reaction with the monofunctional imidoester iminothiolane. The introduced sulphydryl groups are subsequently available for immobilization of molecules involving disulfide bonds.

Depending on the field of application S-layers can be used in different forms as immobilization matrix, for example, as S-layer ultrafiltration membrane, as plane, cylindrical or vesicular self-assembly products, or as "cup-shaped" cell wall fragments. The latter can be obtained by breaking rod-shaped cells by controlled ultrasonication (Fig. 3). Thermophilic and mesophilic Bacillaceae, grown under defined culture conditions, give cell wall fragments with a peptidoglycan containing layer completely covered by an S-layer lattice on both sides (Fig. 3).

The possibility to immobilize functional molecules on different types of S-layer materials has led to a broad spectrum of applications including bioanalytical mono- and multienzyme sensors including optodes [for reviews, see Pum et al., 1991; Pum and Sleytr, 1993], enzyme and affinity membranes [Sára et al., 1993a,b] and affinity microparticles (AMp) [Sára et al., 1993b]. Cup-shaped AMp (Fig. 3) with a densly packed monomolecular layer of Protein A were capable of adsorbing IgG from different solutions including serum or hybridoma cell culture supernatants up to the theoretical saturation capacity. The AMp were able to withstand high centrifu-

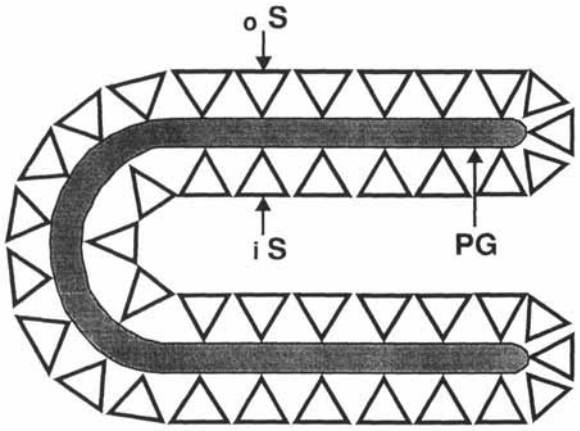

Fig. 3. Schematic drawing of "cup-shaped" cell wall fragment. The peptidoglycan layer (pg) is completely covered on both surfaces with a mirror-symmetrically arranged S-layer lattice (os, is).

gation and sheer forces and even the harshest cross flow conditions. No attrition of the AMp, Protein A leakage or a decrease in the IgG-binding capacity was observed after several runs. Since the pores in the S-layer lattices of the AMp had a size of 4–5 nm, the immobilization of Protein A and adsorption of IgG was restricted to the outer surface of the S-layer. This excluded mass transfer problems usually encountered with affinity matrices prepared from amorphous polymers where more than 90% of the ligands are immobilized in the interior.

S-LAYER FOR VACCINE DEVELOPMENTS

During the past few years, particular attention has been paid to S-layers present on cell envelopes of pathogenic bacteria. Detailed studies on the fish pathogenic bacterium *Aeromonas salmonicida* [Kay and Trust, 1991] and the human pathogen *Campylobacter fetus* [Blaser and Gotschlich, 1990] revealed that the S-layer is a particularly important virulence factor. To fight the respective diseases the S-layers are used for developing vaccines. Since S-layers have also been detected on numerous other pathogens of humans and animals, including *Chlamydia* spp., *Treponema* spp., *Campylobacter* spp., *Rickettsia* spp., *Wolinella* spp., *Bacteroides* spp., *Bordetella pertussis, Cardiobacter hominis, Aeromonas* spp., and a great number of *Bacillus* spp. and *Clostridia* spp. [for reviews, see Sleytr et al., 1988; Messner and Sleytr, 1992], it can be expected that they will also prove relevant as virulence factors.

Another line of development is directed to the use of S-layers as immobilization matrix and adjuvant for weakly immunogenic antigens and haptens [Messner et al., 1992; Malcolm et al., 1993a,b; Smith et al., 1993]. As vaccine carriers both glycosylated and nonglycosylated S-layer lattices have been used. Studies with a variety of carbohydrate haptens (e.g., "tumor-associated" antigens, blood group oligosaccharides, bacterial capsular polysaccharides) revealed that characteristic differences with respect to T- and B-cell responses can be elicited if coupling is performed to native or glutaraldehyde-crosslinked S-layer vesicles. Haptens covalently linked to native S-layers induced effective class switching from IgM to IgG subclasses after secondary and tertiary immunization. On the other hand, chemically crosslinked S-layer conjugates were shown to prime hapten-specific delayed-type hypersensitivity (DTH) responses in mice upon challenge 1 week after primary intramuscular immunization. These results indicate that the DTH responses are mediated by T-helper lymphocytes. Preliminary results also confirmed that a strong hapten specific DTH response can be elicited by oral/nasal immunization. This shows that S-layers have good potentials as a unique carrier for conjugate vaccines. In comparison to the commonly applied tetanus and diphtheria toxoids S-layers may have an additional advantage. Secondary and tertiary immunization could be performed using the same hapten(s) coupled to different immunologically non-cross-reactive S-layers. This would circumvent the tolerance problems frequently observed with toxoid carriers. It is also interesting to note that S-layer vaccines have an intrinsic adjuvant property.

S-LAYERS AS CARRIERS FOR LANGMUIR-BLODGETT FILMS AND BIOLOGICAL MEMBRANES

Recently, it could be demonstrated that large scale recrystallized S-layers can be used as supporting and stabilizing structure for Langmuir-Blodgett films and reconstituted biological membranes [Pum and Sleytr, 1993; Pum et al., 1993]. Such composite structures mimic the molecular architecture of those archaeobacterial cell envelopes that are exclusively composed of an S-layer and a closely associated plasma membrane [Sleytr and Messner, 1989; Messner and Sleytr, 1992]. Thorough investigations of structure and function of membrane associated or integrated

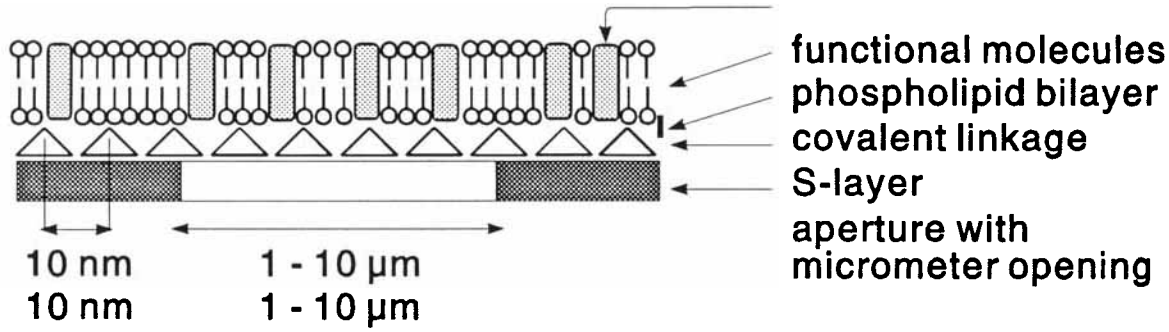

Fig. 4. Schematic illustration of an S-layer supported lipid membrane incorporating functional molecules. Holes and apertures up to 10 µm in diameter can be covered with these stable composite structures.

proteins are often hampered by the short lifetime of the monomolecular or bimolecular lipid films. We have shown that S-layer supported Langmuir-Blodgett films and reconstituted biological membranes can cover holes or apertures up to 10 µm in diameter (Fig. 4) and maintain their structural and functional integrity for a much longer period of time in comparison to unsupported structures (e.g., black lipid membranes) [Pum et al., 1993]. The reason for that is the reduction or inhibition of horizontal vibrations which are the main cause for desintegration of unsupported lipid membranes. Particularly after crosslinking the S-layer proteins alone or with molecules from the lipid layer from the subphase with glutaraldehyde very stable composite structures could be achieved. Subsequently, lipid layers can be deposited on such "semifluid membranes" by standard Langmuir-Blodgett techniques or by fusion of lipid vesicles [Pum and Sleytr, 1993].

Functional molecules (e.g., ion channels, carriers, pore-forming proteins, proton pumps, light harvesting receptor molecules) can be incorporated into S-layer stabilized lipid layers using well established procedures. Spread over apertures, functional studies on membrane-associated or integrated molecules can be combined with structural studies by transmission electron microscopy and scanning tunneling or atomic force microscopy [Pum et al., 1993]. It is expected that the technology of S-layer supported lipid membranes will lead to a broad spectrum of applications in biotechnology, development of biosensors and molecular nanotechnology [Pum and Sleytr, 1993].

FUTURE DIRECTIONS

S-layers are the simplest biological membranes developed during evolution. It is particularly their construction principle involving a single constituent protein or glycoprotein subunit that allows a deeper understanding of the "molecular logic" involved in the morphogenesis of the two-dimensional lattices [Sleytr and Messner, 1988; Sleytr et al., 1993]. An astonishing spectrum of applications for S-layers have already emerged and many others should be found. S-layers which have been recrystallized on solid supports or which have been transferred to them from interfaces have been suggested as patterning elements for nanolithography and for structuring microelectronical or microoptical devices [for reviews, see Pum et al., 1991; Pum and Sleytr, 1993]. Preliminary studies have shown that S-layer lattices are particularly suitable as immobilization matrices for a precise positioning of functional molecules with an electrically polarized scanning probe tip of a scanning force microscope [Sleytr et al., 1992; Pum and Sleytr, 1993]. A defined deposition of electro-organic molecules (electron mediators) on S-layer lattices could help to solve the problem of molecular wiring and/or molecular adressibility in bioelectronics.

Another line of development is directed toward genetic manipulations on S-layer proteins. Incorporation of peptide stretches of well known functional domains of other proteins could lead to completely new affinity- and enzyme-membranes, ion-selective binding matrices, microcarriers, biosensors, diagnostics, or vaccines.

A broad spectrum of future developments could be thought of for S-layers particularly in combination with lipid membranes. S-layer-stabilized liposomes would allow to develop new strategies for trapping or encapsulating molecules. "S-layer liposomes" should be very stable structures in which surface recognition, delivery and biodegradation systems could be incorpo-

rated. Large scale reconstituted S-layers or S-layer/lipid composite structures should provide ideal matrices for designing biosensors, biocompatible surfaces and bioabsorbable systems for tissue regeneration.

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