Article

S-layer Protein-based Biosensors

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Abstract: The present Feature Paper highlights the application of bacterial surface (S-) layer proteins as versatile components for the fabrication of biosensors. One technologically relevant feature of S-layer proteins is their ability to self-assemble on many surfaces and interfaces to form a crystalline 2D protein lattice. The S-layer lattice on the surface of a biosensor becomes part of the interface architecture, linking the bioreceptor to the transducer interface, which may cause signal amplification. The S-layer lattice as ultrathin, highly porous structure with functional groups in a well-defined special distribution and orientation and an overall anti-fouling characteristics can significantly raise the limit in terms of variety and ease of bioreceptor immobilization, compactness of bioreceptor molecule arrangement, sensitivity, specificity, and detection limit for many types of biosensors. The present paper discusses and summarizes examples for the successful implementation of S-layer lattices on biosensor surfaces in order to give a comprehensive overview on the application potential of these bioinspired S-layer protein-based biosensors.

Keywords: biosensor; S-layer protein; crystalline 2D protein lattice; lipid membrane platform; linking matrix; bioreceptor; biomimetics

1. Introduction

Biosensor-related research has made tremendous progress over the past four decades, because the advance in electronics, nanolithography, nanobiotechnology, biomimetics and synthetic biology led to successful routes for combining biological systems with silicon technology. Biosensors are per definition devices, which use a biological recognition element retained in direct spatial contact with the transduction system [1] or, in simplified terms a device, that converts a physical or biological event into a measurable, mostly electrical signal [2]. Biosensors comprise in general of (Figure 1):

A) a biosensing element or bioreceptor, to which the analyte has a highly specific binding affinity.
B) an interface architecture, which provides an environment for the proper functioning of the biosensing element and where the specific biological event, which gives rise to a certain physical phenomenon takes place.
C) a transducer converting the physical phenomenon or chemical response resulting from the analyte's interaction with the biological element (e.g., physicochemical, optical, piezoelectric, electrochemical, etc.) into electrical signals. The latter can be reproducibly measured, quantified and processed [3].
D) an associated electronics comprising of signal amplifier, signal processor and an interface like a display, which finally allows a user-friendly visualization and evaluation of the data [4].

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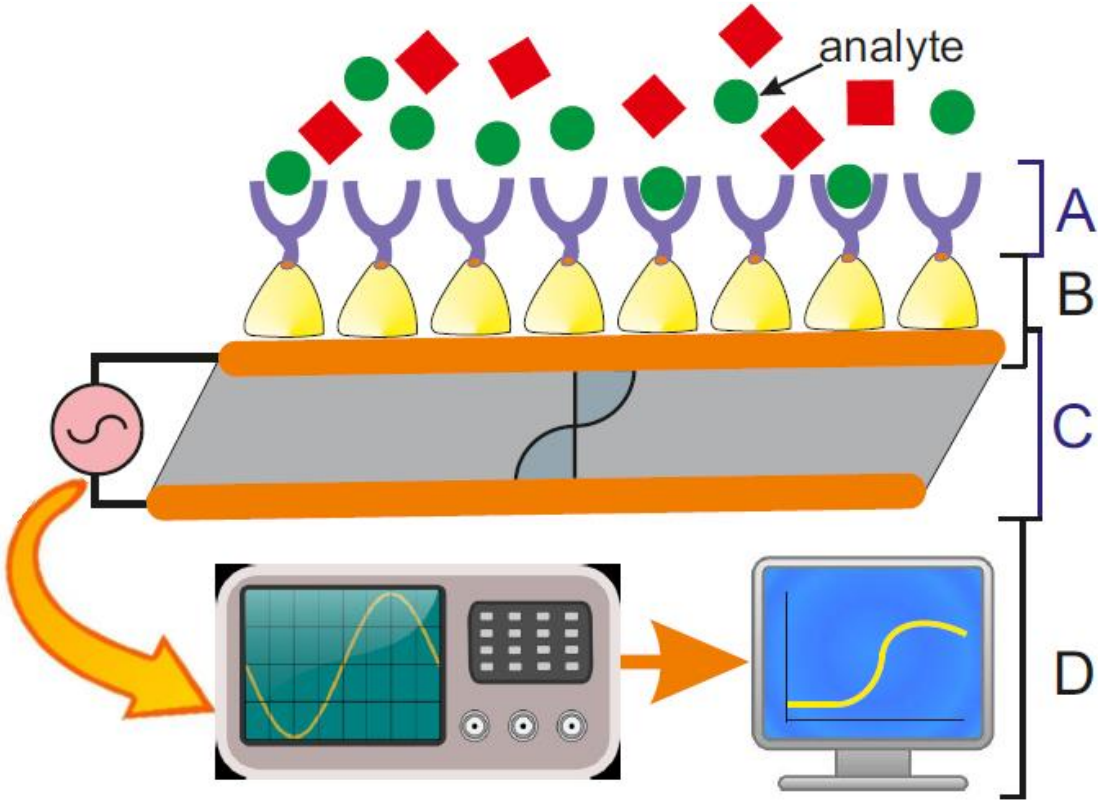

Figure 1: Elements and selected components of an S-layer protein-based QCM-D biosensor. A) A biosensing element or bioreceptor comprising of accessible functions like, e.g., an antibody to which the analyte binds with highly specific affinity. B) An interface architecture comprising of a QCM-D sensor surface covered by a recrystallized S-layer lattice, which provides an environment for the proper functioning of the biosensing element. Here the specific biological event takes place, which gives rise to a certain physical phenomenon. C) A transducer converting the physical phenomenon (piezoelectricity) resulting from the analyte's interaction with the biological element into an electrical signals. D) Associated electronics comprising of signal amplifier, signal processor and a display allowing a user-friendly visualization and evaluation of the data.

The biosensing element or bioreceptor is frequently a biologically derived or biomimetic material like living cell, tissue, enzyme, membrane protein (e.g., ion channel, receptor, pore-forming protein), membrane-active peptide (e.g., ionophore), antibody, nucleic acid, and biological sensitive elements created by genetic engineering. The analyte, which binds in a highly specific manner to the bioreceptor may be amongst others ions, nucleic acids and other organic molecules from cell cultures, human (blood, urine, saliva, tears, sperm, various secretions, etc.,) and food samples, and pollutants from environmental samples (e.g., air, water, soil, vegetation).

One of the most challenging tasks is to converge biological or biomimetic systems with silicon technology in order to generate the functional interface architecture [5]. Biological molecules may aggregate or even denature on the surface of electrodes, sensors or other mostly inorganic solid supports and hence, loose their function. In order to prevent the loss of function, very frequently an intermediate layer is generated in-between the biosensing element and the inorganic surface of (ion-sensitive) field-effect transistors, (interdigitated) microarray electrodes, metal-, polymer- or graphene-coated sensor chips, etc. This intermediate layer comprises either of polymers (e.g., polyethylene glycol, chitosan, agarose, hydrogel, or polyelectrolyte) [6, 7], self-assembled monolayers (SAMs; e.g., alkanethiol, dialkylsulfides, silanes, phosphonates) [8-11], or a monomolecular array of self-assembled protein subunits forming surface layers (S-layers) [12-16]. If a lipid membrane is desired as part of the biosensing element, an incomplete layer of so-called tether molecules replace the rigid SAM [17-19]. Incomplete it the sense that one wants to have only few tether molecules, which anchor the membrane to the surface. This arrangement, where the tether molecules are mixed

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with so-called spacer molecules ensures a certain retained fluidity of the lipid membrane [18]. The latter is very important if one wants to functionally reconstitute integral membrane proteins and/or membrane-active peptides in a tethered membrane. In general, a tether molecule is composed of a binding group to be anchored on a solid support (thiol, silane, chelation of Ni-ions with nitrilotriacetic acid, biotin, etc.), a hydrophilic backbone and a hydrophobic moiety to anchor the lipid membrane (alkyl chains, cholesterol, etc.) [20, 21]. Molecules like polymers (in particular polyethylene glycol), glyco-polymers, peptides and proteins are used so far to build up the hydrophilic part of the tether layers [22-24]. The challenge generating the intermediate layer is to combine multiple functions including: 1) to act as immobilization layer with a suitable binding to both, the inorganic support and the biological molecules (e.g., bioreceptors, matrix-forming lipids; 2) to allocate a binding matrix where immobilized molecules are arranged in a well-defined spatial and directed orientation; 3) to provide a reservoir for water and ions; and 4) to provide sufficient space and stability for the biosensing elements.

Moreover, some biosensors require an immobilization process of the bioreceptor to the sensor surface (metal, metal oxide, glass, polymer and other materials) using physical or chemical techniques [25]. This is in particular the case if one wants to rely on membrane proteins and membrane-active peptides as biosensing element because these biomolecules need a lipid membrane to adopt their functional structure and to deploy amplification properties. The immobilization of the biosensing element has the additional advantage to be assessable with the broad arsenal of surface-sensing techniques. Indeed, many biosensors rely on surface-sensitive techniques like surface plasmon spectroscopy (SPR) [26-28], surface acoustic wave (SAW), quartz crystal microbalance with dissipation monitoring (QCM-D) [29-32], electrochemical impedance spectroscopy (EIS) [33-36], cyclovoltammetry (CV) [37-39] or total internal reflection fluorescence microscopy (TIRFM) [40] as transducer. Important questions in this context are how one can create an intermediate layer with all the intrinsic properties listed above and how can the biosensing element be coupled to or integrated in this functional layer.

The present Feature Paper focusses on a promising approach to generate a particular type of protein-based intermediate layer, the so-called surface (S-) layer [41]. In the following, I present an introduction to bacterial S-layer proteins and their use for the immobilization of functional molecules and lipid membranes. Moreover, I also introduce S-layer fusion proteins and their utilization as components for the generation of biosensors. Finally, I discuss the application of S-layer lattices for the generation of functional lipid membrane platforms in detail and possible future directions for the application of these S-layer lattices in general are considered.

2. Bacterial S-layer proteins

The S-layer is defined as a "two-dimensional array of proteinaceous subunits forming the surface layer on prokaryotic cells" (Figure 2) [15]. They are found as outermost structure in hundreds of different species of almost every taxonomic group of walled bacteria and are an almost universal feature of archaea [15, 42-44]. As S-layers account for approximately 10% of cellular proteins in bacteria and archaea and since the biomass of prokaryotic organisms surpass the biomass of eukaryotic organism [45], S-layers can be considered as one of the most abundant biopolymers on Earth [15, 46]. Moreover, S-layers also represent the simplest biological protein or glycoprotein membranes developed during evolution [43, 47, 48].

High-resolution transmission electron microscopy (TEM) and atomic force microscopy (AFM) studies on the mass distribution of S-layer lattices revealed not only that the S-layer cover the entire cell surface as coherent layer [49-52], but demonstrated also the elegancy of these proteinaceous supramolecular bioarchitectures (Figure 2) [53]. Most S-layers are monomolecular assemblies of single subunit species with a molecular weight ranging between 40 kDa to 200 kDa. In general, bacterial S-layer lattices 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 to 35 nm [42, 54, 55]. Bacterial S-layers are generally 5 nm to 10 nm thick and reveal a rather smooth outer and a more corrugated inner surface. Furthermore, the S-layer lattice SbpA from *Lysinibacillus sphaericus* CCM

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2177 showed an outstanding antifouling characteristic of in the presence of highly concentrated protein solutions (e.g., 70 g L^{-1} human serum albumin), plasma and whole blood samples [56]. This finding is explained by the inherently (zwitterionic) neutral charge of the outer surface of SbpA. Moreover, S-layers are highly porous protein lattices (30% to 70% porosity) with pores uniform in size and morphology in the dimension of 2 nm to 8 nm [57-59]. Interestingly, many S-layers possess two or even more distinct classes of pores [42, 54, 55, 60, 61].

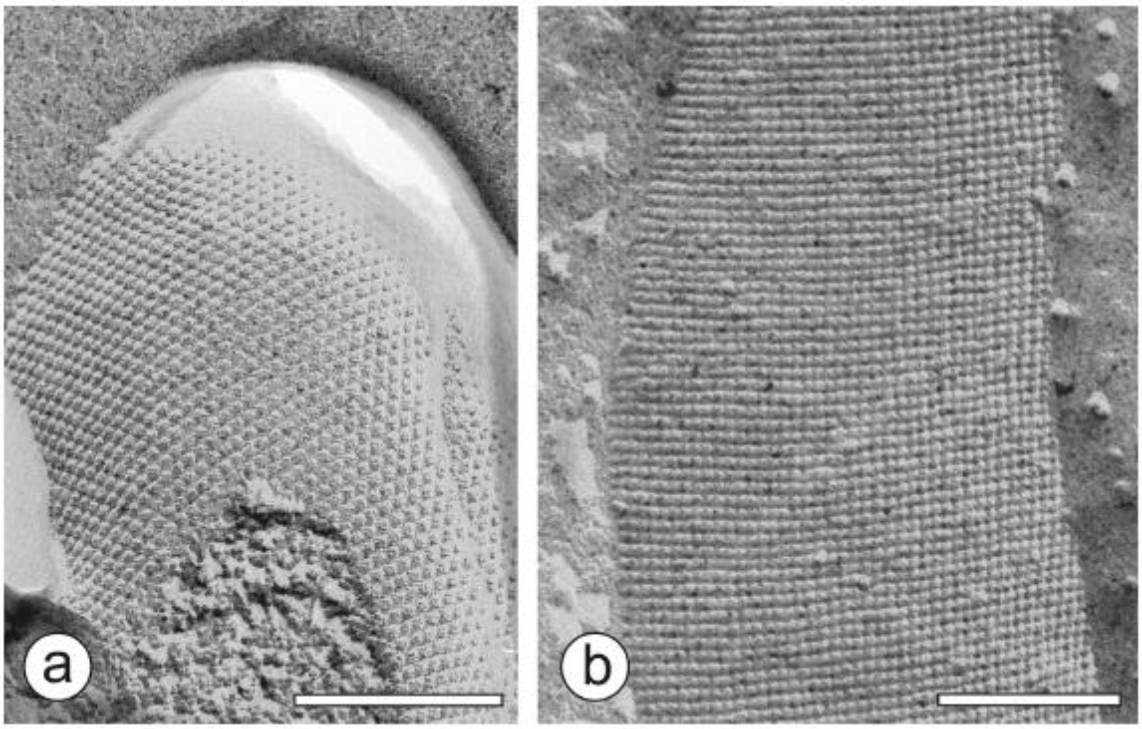

Figure 2: TEM image of a freeze-etched and metal shadowed preparation of (a) an archaeal cell (from *Methanocorpusuculum sinense*), and (b) a bacterial cell (from *Desulfotomaculum nigrificans*). Bars, 200 nm. Adopted from [15], copyright (2014) with permission from John Wiley & Sons Ldt.

A little is known about the specific biological functions of S-layers but it is now recognized that they can function as protective coats against, e.g., bdellovibrios, bacteriophages, and phagocytosis; can act as molecular sieve, molecule and ion traps; promoters for cell adhesion; immune-modulators; surface recognition; antifouling coatings; and virulence factors in pathogenic organisms [15, 58, 62, 63]. Moreover, the S-layer lattice is involved in the determination of cell shape and as a structure aiding in the cell division process in archaea, which possess S-layers as the exclusive envelope component external to the cytoplasmic membrane [64, 65]. Interestingly, ion-gating properties of microbial S-layer protein arrays have also been determined for the S-layer of *Deinococcus radiodurans* [66]. Ion transport appears to be mainly due to an electrical gradient inside the pores, presuming to originate from the negative charges present on this S-layer lattice. By evaluation of the gating characteristics of this nanoporous membrane toward various ionic species it turned out, that immobilized S-layers undergo a strong interaction with cations, in particular Ca^{2+}-ions.

One very important feature of S-layer proteins is the capability of isolated native or recombinantly produced subunits to self-assemble on surfaces or interfaces into crystalline arrays. These surfaces include glass, silicon oxide and nitride, mica, noble metals like gold, titan, platinum, but also stainless steel or many polymers as polystyrene, polyester, and cellulose, and on technically relevant materials like highly oriented pyrolytic graphite, graphene, or indium tin oxide [56, 67]. TEM [68-70] and AFM [71-73] 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 micrometers large S-layer patches [71, 74-76]. The growth of extended S-layers patches is favoured at low monomer concentrations due to the corresponding low number of nucleation sites. The individual patches are monocrystalline and separated by grain boundaries [71].

The formation of a coherent crystalline lattice depends on the used S-layer protein species, the environmental conditions of the subphase (i.e., ionic content and strength, pH-value) and on the surface properties of the interface. Interestingly, S-layer lattice can exhibit against cells in tissue cultures either cell adhesive (cytophilic) or cell repulsive (cytophobic) surface properties depending whether the inner or outer side, respectively, is exposed to the aqueous environment. 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 exposed outer, smooth cytophobic side) to an acidic (pH 4; resulting in an exposed inner rough, cytophilic surface pattern) condition [77].

While the reassembly of S-layer proteins at the air-water interface and at planar lipid films is well defined [69, 70, 78-80], the deliberate modification of the surface properties of a solid support allows to specifically control the reassembly process [71, 75, 81-83]. For example, the S-layer protein SbpA, which is currently one of the most detailed studied S-layer proteins for functionalizing solid supports, forms monolayers with a height of 9 nm on hydrophobic surface and double layers on hydrophilic silicon supports [71]. The height of the double layer structure is 15 nm indicating that the two layers are resting on each other like two interdigitated-toothed racks. Furthermore, in comparison to 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 (2D powder) [47].

In general, the S-layer is on the one hand side utilized as very precise immobilization matrix to present various biomolecules including bioreceptors in a unique manner [15, 84, 85]. On the other hand side, this protein-based intermediate layer constitutes also a versatile base plate for the generation of supported lipid membranes, which provide the essential environment for the reconstitution of functional membrane proteins and membrane-active peptides [41, 86, 87].

Although native S-layer proteins have already demonstrated their great potential as patterning elements and nanoscale building blocks, genetic approaches have opened up the possibility of modifying and tuning the natural properties of S-layer proteins [88, 89]. However, one has to take care that the S-layer proteins with inserted or fused foreign domains or proteins do not lose their capability to assemble into geometrically well-defined layers. The most relevant advantages of genetically engineered S-layers over less nanostructured approaches are the periodicity of functional domains in the nanometer range on the outermost surface of the S-layer lattice. To date, S-layer fusion proteins are produced through homologous expression and secretion by cells or inside a host organism, which is mostly *Escherichia coli* [89, 90]. Both strategies are suitable to produce bio-inspired materials with designed functional properties. Moreover, the possibility to fuse single or multifunctional domains of other proteins to S-layer proteins has led to a broad spectrum of applications ranging from fluorescent biomarkers, immobilized biocatalysts, vaccine, diagnostics, and sensor development, to biosorption of heavy metals and nanoparticle arrays [15, 50, 91, 92]. In this context, it is interesting to note that 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 entire composite S-layer supported lipid membrane (SsLM) [93-97]. Finally, the co-recrystallization of different S-layer fusion proteins will lead to a high flexibility for the variation of functional groups within a single S-layer array.

3. Modified S-layers as components in biosensors

Since S-layers represent one of the few examples in Nature, where proteins reveal the intrinsic capability to self-assemble into monomolecular lattices a considerable potential in biological and non-biological applications is evident. Previous studies demonstrated that S-layers represent an ideal patterning element for nanobiotechnological and biomimetic applications [41, 50, 52, 98-100]. Particularly, the repetitive physicochemical properties and isoporosity of S-layer protein lattices down to the sub-nanometer scale make them to unique matrices and building blocks for generating

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complex, supramolecular assemblies. The prime attractiveness of such 'bottom-up' strategies lies in their capability to generate uniform nanostructures, and the possibility to exploit such structures at the meso- and macroscopic scale. Most importantly, it has been shown that S-layers can be combined with all major species of biological or synthesized (macro)molecules. Particularly, cloning and characterization of genes encoding S-layer proteins opened new areas of applied S-layer research as the incorporation of single or multifunctional domains is now possible without loss of their self-assembly capabilities [88]. As S-layers are highly anisotropic structures with regard to their topography and the physicochemical properties of their inner and outer surface, it was essential to ensure the recrystallization of S-layer (fusion) proteins in defined orientation on solid supports (e.g., polymers, metals, semiconductors) and lipid membranes [71, 77]. Hence, the biomimetic approach to copy the physicochemical properties of the cell envelope structures might be among others a distinguished solution for the generation of supporting scaffolds for lipid membranes.

The recognition of biosensors started with the introduction of the first generation glucose oxidase (GOx) biosensor in 1962 [101], which is still the most widely used biosensor up to date [102-105]. Electrochemical biosensors, as exemplified by the glucose sensor, do not suffer the drawback of high sensor setup complexity and costs because of their close link to developments in low-cost production of microelectronic circuits and their easy interface with normal electronic read-out and processing. Other inherent advantages of electrochemical biosensors are their robustness, easy miniaturization, excellent detection limits also with small analyte volumes, and ability to be used in turbid biofluids with optically absorbing and fluorescing compounds [103, 106, 107].

Analysis of the mass distribution and permeability properties of isolated S-layer lattices of various Bacillaceae revealed that they function as isoporous molecular sieves with a pore size of 4 nm to 5 nm in diameter, corresponding to a molecular mass cut-off in the range of 30 kDa to 40 kDa [108-112]. S-layer ultrafiltration membranes (SUMs) are produced by depositing S-layer-carrying cell wall fragments or S-layer self-assembly products on microfiltration membranes, crosslinking the S-layer protein under a certain pressure and finally reducing the Schiff bases [109, 112]. Beside enzymes (GOx, invertase, peroxidase, glucuronidase, β-glucosidase, naringinase), also ligands (protein A, streptavidin, folate) or mono- and polyclonal antibodies have been immobilized on the SUMs [108].

Since S-layers constitute an immobilization matrix of only several nanometers thickness, the fabrication of unsurpassed thin sensing layers with densely packed functional biomolecules, in particular enzymes is possible [113-116]. The first amperometric sensor based on an S-layer lattice comprised of an SUM with covalently bound GOx [117]. The retained activity of the immobilized GOx was approximately 40%. In order to function as working electrodes, a layer of gold or platinum covered the enzyme loaded SUMs. The biosensor yields high signals (150 nA mm^{-2} $mmol^{-1}$ glucose), fast response times (10-30 sec), linearity range up to 12 mM glucose, stability under working conditions of more than 48 hours, and no loss of GOx activity after a storage period of 6 month. A further achievement with S-layer-based amperometric biosensors was the generation of a three-enzyme sensor for sucrose [118]. For this purpose, the enzymes invertase, mutarotase and GOx were immobilized on S-layer fragments isolated from *Clostridium thermohydrosulfuricum* L111-69 via aspartic acid as spacer molecules. After deposition of the modified S-layer fragments on microfiltration membranes, the surface of this multifunctional device was covered with gold by sputtering to function again as working electrode. Amperometric sucrose measurements based on the oxidation of hydrogen peroxide revealed a high signal level (1 μA cm^{-2} $mmol^{-1}$ sucrose), 5 min response time and a linear range up to 30 mM sucrose. In a further approach, a glucose sensor with an oxygen optode as transducer containing a ruthenium(II) complex, whose fluorescence is dynamically quenched by molecular oxygen was developed [119]. For fabrication of this fibre-optic biosensor the GOx was covalently immobilized as a monolayer on SUMs. The performance of the biosensor in terms of response time, linear range and stability was comparable to existing optodes. However, this system holds great potential for the development of micro-integrated optical biosensor due to its tiny size. A further improvement of this fibre-optic glucose sensor was to connect the GOx molecules in its tightest packing immobilized on the S-layer lattice with an optimum metallic contact, which must not disturb the protein structure [120]. Previously, platinum films were applied on

enzyme layers immobilized on S-layer protein by argon sputtering. However, this conventional method exhibits substantial limitations, e.g., a volume change of the S-layer/enzyme composite system when it is introduced into a conventional vacuum coating apparatus. This drawback was circumvented by pulse-laser-deposition method. The latter approach resulted in an enzyme activity of 70-80%, which constitutes a doubling of the activity compared to first amperometric sensor based on an S-layer lattice [114, 117, 120]. Hence, this example demonstrates that composite system consisting of the 2D-protein-layer/enzyme/metal arrangement can successfully serve as high efficient biosensors.

The continuous, stable, and accurate detection of glucose in blood is a challenging task because many blood components disturb the measurement. As S-layer lattices constitute highly hydrated, ultrathin biological antifouling materials [121], Picher et al. developed a lab-on-a-chip comprising of embedded amperometric sensors in four S-layer-coated micro-reactors, which can be addressed individually [56]. The S-layer had the function to provide an efficient antifouling coating, a highly-oriented immobilization matrix for the GOx and an effective molecular sieve. Moreover, the S-layer protein SbpA *from L. sphaericus* CCM 2177 readily formed monomolecular lattice structures at the various microchip surfaces (e.g., glass, polydimethylsiloxane, platinum and gold) within one hour. The microfluidic device operated in a feedback loop mechanism and was used to assess natural variations in blood glucose levels during hemodialysis and hence, to allow the individual adjustment of glucose. To ensure reliable and accurate detection of glucose in blood the lab-on-a-chip performed simultaneously blood glucose measurements, autocalibration routines, mediator-interferences detection, and background subtractions. The highly isoporous SbpA-coating eliminated unspecific adsorption events in the presence of human serum albumin, human plasma and freshly-drawn blood samples. Most important, the undisturbed diffusion of the mediator to the electrode surface enabled electrochemical measurements of glucose in concentrations between 0.5 mM to 50 mM [56]. Hence, this combination of biologically-derived nanostructured surfaces with micro¬chip technology constitutes a powerful tool for multiplexed analysis of complex samples.

The bioreceptor in cholesterol biosensors is very commonly the cholesterol oxidase (ChOx). A simple and reliable method to prepare reproducible and stable ChOx monolayers was to spread the ChOx at the water–air interface. Mixed films comprising of ChOx and S-layer proteins showed a long-term stability at the air-water interphase [122]. In a further study, the mixed film was transferred onto the surface of screen-printed carbon electrodes by the Langmuir–Blodgett technique [123]. The modified electrode surface was characterized by AFM and cyclic voltammetry (CV). AFM indicated the presence of deposited layers, which resulted also in a reduction of the surface roughness of the electrodes. As demonstrated by CV, the presence of S-layer proteins in the ChOx Langmuir-Blodgett film increased the oxidation peak intensity and reduced the oxidation potential. Therefore, these results showed the feasibility of producing a cholesterol biosensor based on the immobilization of a mixed film comprising of ChOx and S-layer proteins on screen-printed carbon electrodes [123].

For the development of oxygen sensors an oxygen sensitive Pt(II) porphyrin dye was covalently bound to the S-layer matrix [124]. The oxygen concentration was measured by phase modulation fluorimetry. Setups comprising low cost optoelectronic components like light emitting diodes and silicon photodiodes were constructed. For both sensor setups (planar and fiber optic) variations in the oxygen concentrations resulted in distinct and reproducible changes in the luminescence lifetime and intensity. The luminescence quenching efficiency of these sensors was found to be 1.5–1.9 (expressed as the ratio of signal under nitrogen and air) which compares well to other sensor systems using luminophores embedded in polymer matrices. These results demonstrated the application potential of S-layers as immobilization matrices in the development of biosensors [124]. The general principle for the construction of optical sensors by immobilization of various dyes, fluorophores and/or receptors on monomolecular S-layer protein coatings can be applied for sensing of manifold analytes.

In a recent study, a monomolecular S-layer lattice comprising of the S-layer protein SbpA conjugated with folate was recrystallized on a gold surface [39]. This biorecognition layer ensured the specific capture of human breast adenocarcinoma cells (MCF-7) via the recognition of folate

receptors, which are expressed on the surface of MCF-7. The fabricated acoustic and electrochemical sensors were able to distinguish between MCF-7 and human liver hepatocellular carcinoma (HepG2) cells as the latter do not express folate receptors. This biosensor offers several advantages including the small thickness of the SbpA lattice, which increases cells capturing efficiency. Moreover, there is no requirement to block the surface due to the antifouling properties of the S-layer lattice and no awareness of antibody immobilization as folate can be used as an alternative to the antibody for capturing target cells. Over all, evaluating of the developed biosensors by different techniques provides more information about the efficiency of the system. QCM-D measurements tracked the formation of SbpA-folate modified sensor and capturing of cancer cells efficiently in real-time and under controlled conditions. Although the QCM-D technique shows a limited detection range, it allows tracking the cell viability [39]. Hence, the cellular response to chemotherapeutic agents is worth to be investigated in further QCM-D studies. Indeed, electrochemical measurements confirm the selectivity and specificity of the developed biosensor and provide a simple, rapid, cost-effective and disposable analysis of cancer detection. Moreover, the development of efficient biosensors for accurate diagnosis helps to increase the cure and survival rates of patients with cancer and provides great promise for effective analysis with high selectivity and sensitivity.

The S-layer protein from *L. sphaericus* JG-A12 was bound on a gold surface in order to fabricate a uranyl (UO_2^{2+}) biosensor. Immobilization occurred either by binding of the cysteine of the S-layer protein to a SAM, which presented maleimide groups or to a mixed SAM presenting biotin, which bound neutravidin and the latter subsequently the biotinylated S-layer protein [125]. The new biosensor responds to picomolar levels of aqueous uranyl ions within minutes. In comparison to traditional SAM-based biosensors, the porous bioconjugated layer showed higher stability, longer electrode life span and a denser protein layer. The biosensors responded specifically to UO_2^{2+}-ions with a detection limit of 10^{-12} M and showed minor interference from Ni^{2+}, Cs^{+}, Cd^{2+} and Co^{2+}. Chemical modification of the phosphate and carboxyl groups of the S-layer protein prevented UO_2^{2+} binding, indicating that both moieties are involved in the recognition to UO_2^{2+} [125]. In future, it might be possible that S-layer protein isolates from bacteria surviving in other metal polluted sites may provide the sensing components for the fabrication of further biosensors for the detection of other metal ion.

4. Genetically engineered S-layers as components in biosensors

Their intrinsic self-assembly properties as well as their periodicity make S-layers to ideal building blocks for all kinds of detection systems like DNA-, protein-, allergy- or antibody-chips as well as label-free detection systems (for review see [15, 52, 126]).

The construction of S-layer-streptavidin fusion proteins carrying core-streptavidin either at the N-terminus or C-terminus allowed the generation of universal affinity matrices for the specific binding of biotinylated molecules like, e.g., proteins, allergens, antibodies, oligonucleotides, or nanoparticles [89, 127, 128]. Another application potential is in the development of label-free detection systems. The specific binding of functional molecules to the sensor chip functionalized with an oriented chimaeric S-layer can be measured directly by determining the change in mass on the chip. In addition, there is no need for any labeling if the applied transducer relies upon surface-sensitive techniques like QCM-D, SPR or SAW.

Proof-of-principle for label-free detection systems based on S-layer proteins was performed with the S-layer fusion protein incorporating the sequence of a variable domain of a heavy chain camel antibody directed against prostate-specific antigen (PSA) [129, 130]. After recrystallization of the S-layer fusion protein on gold chips, the monomolecular protein lattice was exploited as sensing layer in SPR biochips to detect PSA. A further application for this chimaeric S-layer fusion protein was to recrystallize them on silica microbeads. These S-layer fusion protein-covered microbeads were applied as biocompatible matrix at a microsphere-based detoxification system used for extracorporeal blood purification of patients suffering from autoimmune disease [131].

In another approach, the recombinant S-layer fusion protein rSbpA/ZZ incorporating two copies of the Fc-binding Z-domain, which is a synthetic analogue of the IgG-binding domain of protein A

from Staphylococcus aureus was constructed. Most important, the ZZ-domains remained exposed on the outermost surface of the S-layer fusion protein lattice. As determined by SPR measurements, the binding capacity of the self-assembled rSbpA/ZZ monolayer for human IgG was 5.1 ng/mm^2, which corresponded to 78% of the theoretical saturation capacity of a planar surface for IgGs aligned in the upright position [132]. rSbpA/ZZ has also been recrystallized on the surface of cellulose-based microbeads. Compared to commercial particles used as immunoadsorbents to remove autoantibodies from sera of patients suffering from an autoimmune disease, the IgG binding capacity of the S-layer fusion protein-coated microbeads was at least 20 times higher [132]. Hence, this novel type of microbeads should find application in the microsphere-based detoxification system. Recently, an efficient acoustic and hybrid three-dimensional-printed electrochemical biosensors based on rSbpA/ZZ for the detection of liver cancer cells was developed [37]. The biosensors function by recognizing the highly expressed tumor marker CD133, which is located on the surface of liver cancer cells. Detection was achieved by recrystallizing rSbpA/ZZ on the surface of the sensors. The fused ZZ-domain enabled immobilization of the anti-CD133 antibody in a defined manner. These highly accessible anti-CD133 antibodies were employed as a sensing layer for the efficient detection of HepG2 cells. The recognition of HepG2 cells was investigated in situ using QCM-D and CV, which confirmed the efficiency of the fabricated sensors to perform label-free and real-time detection of living cells. Most importantly, these sensors offer low-cost and disposable detection platforms for real-world applications. Hence, both fabricated acoustic and electrochemical sensing platforms can detect cancer cells and therefore may have further potential in other clinical applications and drug-screening studies [37].

For another field of research S-layer fusion proteins comprising of SbpA or SbsB, the S-layer protein from *Geobacillus stearothermophilus* PV72/p2 and peptide mimotopes such as F1, that mimics an immunodominant epitope of Epstein–Barr virus (EBV) were constructed [133, 134]. Diagnostic studies have been performed by screening 83 individual EBV IgM-positive, EBV-negative, and potential cross-reactive sera and resulted in 98.2% specificity and 89.3% sensitivity as well as no cross-reactivity with related viral diseases. This result demonstrated the potential of these S-layer fusion proteins to act as a matrix for site-directed immobilization of small ligands in solid phase immunoassays.

Finally, the laccase of *Bacillus halodurans* C-125 was immobilized on the S-layer lattice formed by SbpA either by covalent linkage of the enzyme or by construction of a fusion protein comprising the S-layer protein and the laccase (rSbpA/Lac) [135]. The specific activity of the free, immobilized, and fused laccase was proven because all showed laccase-like activity oxidizing 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), 2,6-dimethoxyphenol, syringaldazine, and hydroquinone. Interestingly, the S-layer part confers a much higher solubility on the laccase as observed for the sole enzyme. Comparative spectrophotometric measurements of the enzyme activity revealed similar but significantly higher values for laccase and rSbpA/Lac in solution compared to the immobilized state, respectively. However, laccase covalently linked to the SbpA monolayer yielded a four to five time higher enzymatic activity than rSbpA/Lac immobilized on a solid support. Combined QCM-D and electrochemical measurements revealed that the laccase immobilized on the SbpA lattice had an approximately twofold higher enzymatic activity compared to that obtained with rSbpA/Lac [135].

5. S-layer lattices for generation of functional lipid membrane platforms

The cell envelope structure of some archaeal species (e.g., *Sulfolobus* spp.) constitute simply of a cytoplasma membrane comprising of etherlipids and a membrane-anchored S-layer lattice (Figure 3 A) [44, 136]. In a biomimetic approach, this supramolecular cell envelope structure constitutes the building plan for SsLMs (Figure 3 B-F). It is assumed that the cell envelope structure of archaea is a key prerequisite for these organisms to be able to dwell under extreme environmental conditions such as temperatures up to 120 °C, pH down to 0, high hydrostatic pressure, or high salt concentrations [42, 137, 138]. Hence, S-layer lattices may therefore be very important to provide basic functions like mechanical and osmotic cell stabilization [139, 140]. As suitable methods for disintegration of archaeal S-layer protein lattices and their reassembly into monomolecular arrays on

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lipid films are not yet available, S-layer proteins from Gram-positive bacteria are used for the generation of SsLMs [41, 93, 95, 97, 98, 141]. In addition, a second S-layer acting as protective molecular sieve and further stabilizing scaffold and antifouling layer can be recrystallized on the top of the previously generated SsLM (Figure 3 E, F). These features make S-layer lattices to unique supporting architectures resulting in lipid membranes with nanopatterned fluidity and considerably extended longevity [41, 93-95, 97, 98, 142-144].

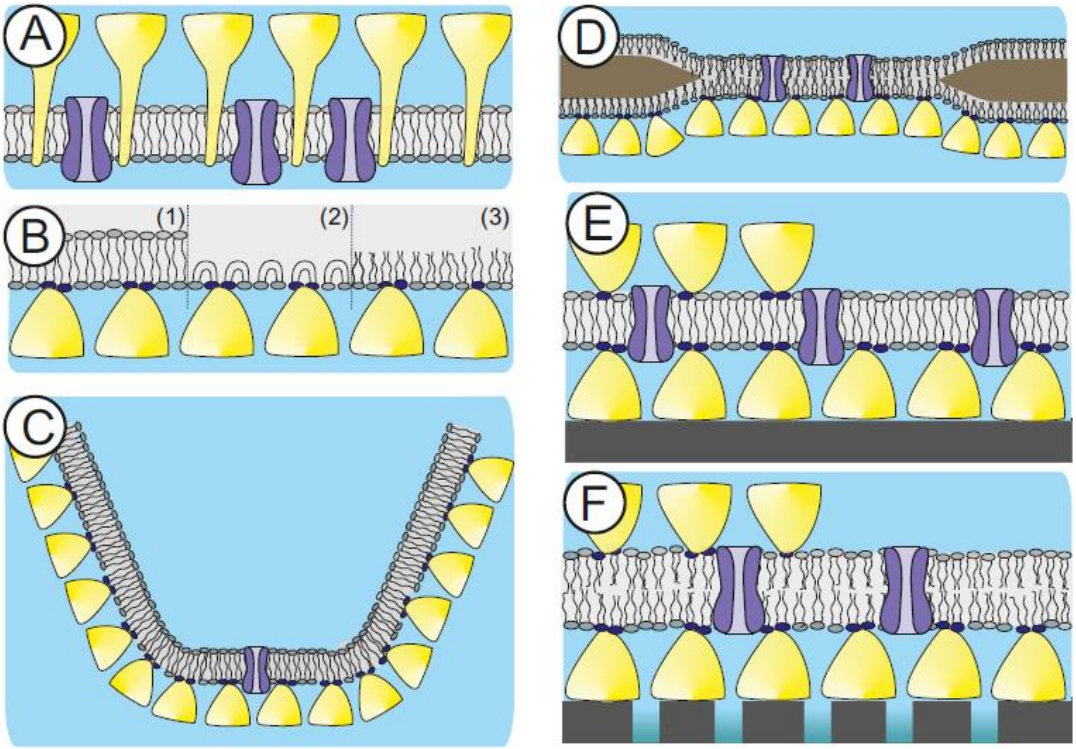

Figure 3: Scheme of natural and S-layer supported lipid membranes. Supramolecular structure of an archaeal cell envelope comprising of a cytoplasma membrane, archaeal S-layer proteins incorporated in the lipidic matrix and integral membrane proteins (A). Schematic illustrations of various S-layer-supported 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 side, a bacterial S-layer lattice can be attached on the external side of the solid supported lipid membrane. (F) Scheme of a bilayer lipid membrane generated on an S-layer ultrafiltration membrane. Optionally as shown on the left side, a bacterial S-layer lattice can be attached on the external side of the SUM-supported lipid membrane. In B to F, the head groups of the lipid molecules interacting with the S-layer protein are marked in dark. As indicated in C to F, all S-layer-supported model lipid membranes can be functionalized by biomolecules like membrane-active peptides and integral membrane proteins. Modified after [63], copyright (2004) with permission from Wiley–VCH.

The interaction of the S-layer proteins SbpA and SbsB with lipid molecules has been investigated in detail [78-80, 145]. It turned out that most probably negatively charged moieties on the S-layer protein interact via electrostatic interaction with the head groups of zwitterionic and/or positively charged lipids (Table 1). As natural, wild-type S-layer proteins possess frequently a so-called S-layer

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homologous domain, which interact with the secondary cell wall polymer, the latter can be coupled to the head group of a lipid and the lipid can be immobilized via a lectin-type like binding on the S-layer protein [18]. The recrystallized S-layer protein can also be chemically modified in order to bind lipids with head groups comprising of a primary amine group [146, 147], thiol group [148], or maleimide group (Table 1). Moreover, a linker like, e.g., streptavidin can be coupled to the S-layer protein, which allows a strong ligation of biotinylated lipids [149, 150]. Finally, the S-layer protein can be genetically engineered so that either a thiol group from an introduced cysteine, multiple histidines (His-tag), streptavidin, or a strept-tag is exposed on the S-layer lattice (Table 1). These modifications allow the coupling of lipids carrying a maleimide group [151-153], nickel(II)-nitrilotriacetic acid anchor [154], or biotin at their head group region [73, 155, 156], respectively.

In general, S-layer proteins can be recrystallized on either a lipid monolayer generated at the air-water interface (Figure 3 B), a preformed lipid membrane like a planar, freestanding lipid bilayer (Figure 3 C, D) or a spherical liposome or emulsome (Figure 4). Moreover, the lipid membrane can be generated on an already existing recrystallized S-layer lattice (Figure 3 E, F). The latter approach is the method of choice to generate functional lipid membrane platforms, which are, beside other applications, a straightforward approach in the development of biosensors [41, 144].

SsLMs prepared by the Langmuir-Blodgett/Langmuir-Schaefer technique without the need on an aperture has been compared to silane- and dextran-supported phospholipid bilayer [157]. Most probably due 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 to 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 [41, 93, 96-98]. This finding reflects also 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 excess to both membrane surfaces, and possessing an essentially unlimited ionic reservoir on each side of the bilayer lipid membrane (BLM; Figure 3 F). This is seen as basic requirement of experiments copying the in vivo situation (e.g., plasmatic / exoplasmatic side). However, the surface properties of porous supports, like roughness or great differences in pore size have significantly impaired the stability of attached BLMs [158]. Hence, a straightforward approach is the use of SUMs with the S-layer as stabilizing and smoothening layer between the lipid membrane and the porous support [159-161].

SUMs were produced by depositing S-layer fragments as a coherent layer on microfiltration membranes [109, 111, 162]. The mechanical and chemical stability of their composite structure is subsequently obtained by inter- and intramolecular cross-linking [109, 110, 162-164]. 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-nanometer range allowing to tune the molecular sieving as well as antifouling characteristics of SUMs [162, 163, 165]. Moreover, SUMs can be prepared with different net charges and hydrophilic or hydrophobic surface properties. That is why SUMs have been used as supporting and stabilizing structures for functional lipid membranes [15, 41, 94, 97, 144].

Whereas composite SUM-supported phospholipid bilayers were found to be highly isolating structures with a life-time of up to 17 hours [159-161], BLMs on plain microfiltration membranes revealed only a life-time of approximately 3 hours. The life time increased significantly to about one 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) [159, 160]. An even further increase in the stability of this composite supramolecular structure can be expected upon crosslinking of those lipid head groups, which are directly in contact with domains on the S-layer protein. Hence, the nanopatterned anchoring of the membrane is a promising strategy for generating stable *and* fluid lipid membranes [86].

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Table 1: Immobilization strategies. Summary of the to date investigated strategies to bind lipid molecules on S-layer protein lattices (modified after [41]).

Type	Reactive group	Crosslinker	Targeted group	References
Natural SPLs				
Electrostatic interaction	Negatively charges on SLP		Zwitterionic or positively charged lipids	[79, 80, 145]
Lectin-type like binding	S-layer homologous domain on SLP		Secondary cell wall polymer coupled to lipids	[18]
Chemical modification of SLPs				
Covalent bond	Carboxyl groups on SLP	Carbodiimide analogues	Primary amine group from lipids	[146, 147, 168]
Covalent bond	Primary amino groups on SLP	SMCC analogues	Thiol group from lipids	[148]
Covalent bond	Primary amino groups on SLP	SPDP/TCEP; insertion of thiol group in SLP	Maleimide group from lipids	Schuster et al., in preparation
Chemical binding of linker on SLPs				
Strong ligation	Streptavidin chemically coupled to SLP		Biotinylated lipids	[149, 150]
Genetically engineered SLPs				
Covalent bond	Thiol-group from introduced cysteine		Maleimide group from lipids	[151-153]
Multiple chelation	Multiple histidines (His6-tag) on SLP		Nickel(II)-NTA from lipids	[154]
Strong ligation	Streptavidin fused to SLP		Biotinylated lipids	[89]
Strong ligation	Strep-tag fused to SLP	Streptavidin	Biotinylated lipids	[72, 73, 155]

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.

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 antimicrobial peptide analogue PGLa(-) (Table 2) [159, 166, 167]. 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 is observed for the same membrane due to the valinomycin-mediated potassium transport [159].

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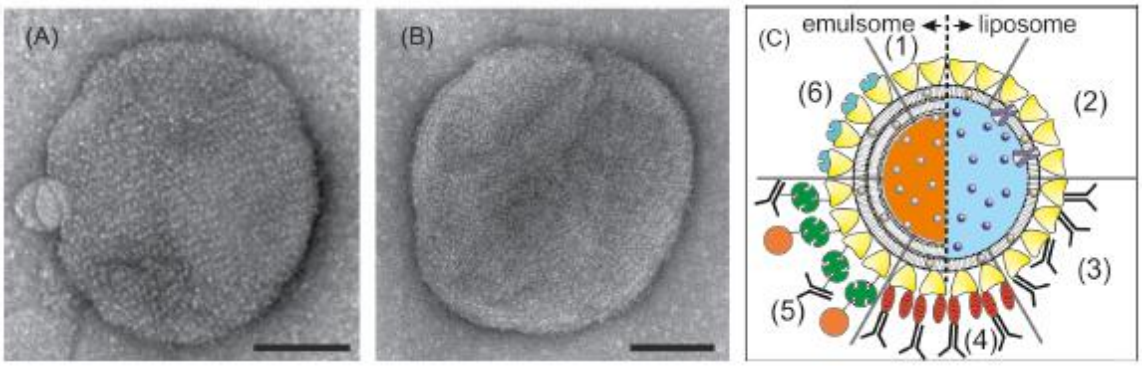

Figure 4: S-layer-coated liposomes and emulsomes. TEM images of emulsomes coated with the S-layer protein SbsB from Geobacillus stearothermophilus PV72/p2 (A) wildtype SbsB and (B) recombinant SbsB. The bars correspond to 100 nm. Adopted from [191], copyright (2013) with permission from Wiley–VCH. (C) Schematic drawing of (1) an S-layer coated emulsomes (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 immobilization matrix for functional molecules (e.g., human IgG) 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). Modified after [61], copyright (2002) with permission from Wiley–VCH.

Table 2: Membrane-active peptides. Summary of membrane-active peptides incorporated in S-layer supported lipid membranes (modified after [41]).

Membrane-active peptide	Source	Remarks	References
gramicidin A (gA)	*Bacillus brevis*	linear pentadeca peptide	[161]
alamethicin (Ala)	*Trichoderma viride*	linear, 20 amino acids	[159]
valinomycin (Val)	several *Streptomyces* strains, e.g., *S. tsusimaensis* and *S. fulvissimus*	cyclic dodecadepsi peptide, 12 amino acids and esters	[141, 159]
peptidyl-glycine-leucine-carboxyamide (PGLa) analogue	synthesized via protein chemistry	20 amino acid; negatively charged analogue of PGLa	[168]

SsLMs generated by the rapid solvent exchange technique were utilized 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 [168]. Hence, SsLMs constitute a promising platform for studying the interaction and insertion of membrane-active (antimicrobial) peptides [169, 170].

Incorporation of the membrane-active peptide gramicidin D could be demonstrated by measurements on single gramicidin D pores in all above mentioned SsLMs [161].

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) [159]. 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 membrane proteins in defined structures

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on, e.g., sensor surfaces is one of the most important concerns in designing biomimetic sensing devices [41, 171-173].

Membrane proteins have also been successfully reconstituted in SsLMs (Table 3). Reconstitution of α-hemolysin (αHL), moreover even single pore recordings could be achieved with SUM-supported phospholipid bilayers but no pore formation was observed with BLMs generated on the pure micro filtration membranes [160].

Table 3: Transmembrane proteins. Summary of transmembrane proteins reconstituted in S-layer supported lipid membranes (modified after [41]).

Transmembrane protein	Source	Remarks	References
α-hemolysin (αHL)	exotoxin from *Staphylococcus aureus*	pore-forming; homo-heptamer	[144, 160]
ryanodine receptor 1 (RyR1)	skeletal muscle cells	Ca^{2+}-release channel; homotetramer	[174]
nicotinic acetylcholine receptor (nAChR)	plasma membranes of neurons; on post-synaptic side of the neuromuscular junction	ligand gated ion channel; 5 subunits	[154]
voltage-dependent anion channel (VDAC)	located on the outer mitochondrial membrane; also produced by cell-free expression	porin, voltage gated; ion channel mono-meric but can cluster	[175]

The ryanodine receptor, RyR1, isolated from rabbit muscle cells was successfully reconstituted in SsLMs [174]. For this purpose, SsLMs were formed by the β-diketone ligand europium-triggered vesicle fusion technique [147] 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 of RyR1 to the bilayer or the S-layer lattice [174]. Nevertheless, further experiments like, e.g., combined QCM-D with EIS studies or patch clamp measurements on a chip have to be performed. Finally, SsLMs may constitute 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 the β-diketone ligand europium-triggered vesicle fusion technique [147] were incubated with the voltage-dependent anion channel (VDAC; Table 3). A significant decrease in membrane resistance could be observed but the membrane capacitance did not change significantly. Moreover, increasing VDAC concentration decreased the membrane resistance, which indicated increasing number of channels reconstituted spontaneously into the SsLM [175]. It is well known that VDAC reconstituted in artificial membranes forms a voltage-gated channel. At low membrane potentials (less than10 mV) VDAC is in the open state and switches to the closed state at high membrane potentials [176-178]. 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 compared to 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 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 [177, 179]. Moreover, it has been shown that the presence of the nucleotides nicotinamide adenine dinucleotide hydride (NADH) or nicotinamide adenine dinucleotide phosphate hydrogen induce channel closure and thus, the conductance of the VDAC channels is significantly reduced [180-183]. Indeed, addition of

NADH to the SsLM with reconstituted VDAC caused a significant increase of the membrane resistance, which is a strong evidence for the blocking of VDAC channels by NADH molecules [175].

All the before mentioned examples for the functional reconstitution of membrane-active peptides and membrane proteins in SsLMs are currently proof-of-principle studies. However, there is a strong desire to use them in particular to probe function of membrane proteins, e.g., in drug screening applications. In this context it is interesting to note, that membrane proteins currently comprise more than 50% of all drug targets and many of these proteins are directly involved in charge transfer processes across the membrane [184, 185]. A direct electrical readout of ionic currents generated by ion channels, for example, bears the advantage of an amplification of the readout signal of membrane functions without need for labelling compared to current state-of-the-art techniques for membrane protein screening.

Vesicular lipid structures like unilamellar liposomes, comprising of a closed, spherical lipid mono- or bilayer with an aqueous inner space and emulsomes, comprising of a solid fat core surrounded by lipid layers are mainly used as drug targeting and drug delivery systems [186, 187]. However, these lipid nanoparticles can also be used as biosensors for diagnosis purposes if the drug is replaced or supplemented by a radiotracer, contrast agent or a fluorescent dye. The use of molecular imaging to measure non-invasively the in vivo distribution of nanomedicines becomes increasingly important [188]. Labeling the nanoparticle gives also an indication of delivery to the target tissue. In this context, it is worth to mention that liposomes and emulsomes can be covered by an S-layer lattice (Figure 4 A, B) [150, 189-192]. In addition, the S-layer lattice may be functionalized with, e.g., antibodies in order to detect cancer cells (Figure 4 C). Moreover, S-layer-coated and with labelling agents loaded liposomes or emulsomes may be used for molecular imaging to detect, e.g., inflammations in a body.

6. Conclusions & Outlook

One of the most challenging progress created by the development and research at the intersection of biological and engineering sciences are biosensors, which are becoming one of the most popular scientific areas. Significant progress in issues like miniaturization, functional sensitivity, simplified read-out, multiplexing, and utilization of newly discovered physical phenomena pushed further the development of smart devices. Moreover, semiconducting technology has proceeded in a way that in the field of biosensors a rapid infiltration of new (bio)nanotechnology-based approaches occurred. Due to bottom-up self-assembly processes at the nanometer scale, the traditional separation between transducers and bioreceptors is not valid any more. Indeed, by an integrative approach, the interface architecture take part in the recognition event and the receptors become active transducing elements of the biosensors (Figure 1).

The present Feature Paper describes the successful implementation of cell envelope components like, e.g., phospho- and etherlipids and in particular bacterial S-layer proteins in biosensors. S-layer proteins can be self-assembled to become part of the interface architecture and thereby connecting the bioreceptor to the transducer interface. The recrystallized S-layer lattice provide significant advantages over other coatings, which can be summarized as following: 1) S-layers are very thin structures (5 nm to 10 nm). This becomes very important if one uses surface-sensitive phenomena as transducer as it is indicated in Fig. 1. Because these techniques have a limited measuring range, the sensitivity decreases with increasing intermediate layer thickness. 2) S-layers are highly porous structures (30% to 70% porosity). This becomes very important if one uses electrochemical methods as transducer. Due to the electrolyte-filled pores, there is much less limitation of ions and the S-layer lattice itself shows a negligible resistance and capacitance. 3) The S-layer lattice presents functional groups in a well-defined special distribution and orientation. This property allows to bind bioreceptors densely packed and highly directed on the interface. 4) The S-layer lattice shows an anti-fouling, self-cleaning surface where almost no biomolecules stick to it. This characteristic is favourably when the measured signal corresponds to the bound and adsorbed biomolecules on the sensor surface. Finally, 5) the S-layer lattice can completely cover areas in the cm^2-range by a one-step process. Hence, a coherent proteinaceous coating can functionalize the whole surface of

commonly used transducers. Although only little material is necessary, a possible drawback of using S-layer proteins is the need for the scale-up of the cultivation of bacteria and the isolation, subsequent purification and storage of S-layer proteins. However, recombinant production of S-layer proteins may help to overcome this issue. Moreover, physicists have to consider adopting certain protocols to meet the demands of biological materials.

As previously mentioned, S-layer lattices are because of their structural features highly suitable to immobilize biosensor-relevant molecules like enzymes, dyes, fluorophores and receptors. In another approach, the bioreceptor may be fused to the S-layer protein. The S-layer protein recrystallizing part ensures a layer presenting the bioreceptor molecules like enzymes, antibodies, IgG-binding domains and peptide mimotopes in a tight packing and rectified orientation. The S-layer lattice is used also as an anchoring scaffolding and/or ion reservoir in the generation of lipid membrane platforms. In contrast to tethered lipid membranes, where a precisely balanced mixture of tether and spacer molecules have to be assembled on the sensor surface, only one type of biomolecules, the S-layer protein is sufficient to provide few repetitive anchoring points for the lipid membrane.

Moreover, biologically inspired lipid membrane-based platforms enabled unprecedented signal amplification down to single-molecule sensitivity. This was achieved by the creation of mechanically and chemically stable membrane platforms with a high longevity. A further crucial property of membrane platforms is their ability to host membrane-associated and -integrated biomolecules like membrane-active peptides, ionophores, pore-forming proteins, ion channels or (G-protein coupled) receptors in a functional form. All these, in many cases highly sensitive biomolecules distinguish themselves by operating at very low concentrations of, e.g., ligands. For instance, ion channels and G-protein coupled receptors are highly sophisticated nanomachines that successfully solve the problem of selective and efficient amplification of a binding event.

Membrane platforms can be miniaturized in a chip format and allow very sensitive recording of single protein activity by, e.g., voltage-clamp electrochemical setups. Such systems are promising for drug discovery since they directly measure membrane protein functionality when they are exposed, e.g., to drug candidates. At present, biosensor research is not only driving the ever-accelerating race to construct smaller, faster, cheaper and more efficient devices, but may also ultimately result in the successful integration of electronic and biological systems. Any advancement in this field will have an effect on the future of diagnostics and health care. Personalized and preventive medicine, bedside diagnostics, and drug discovery will all benefit from the novel electronic sensing technologies.

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Conflicts of Interest: The author declares no conflict of interest.

Abbreviations

2D	two-dimensional
αHL	α-hemolysin
AFM	atomic force microscopy
BLM	bilayer lipid membrane
CD133	tumor marker
ChOx	cholesterol oxidase
CV	cyclovoltammetry
EBV	Epstein-Barr virus
EIS	electrochemical impedance spectroscopy
GOx	glucose oxidase
HepG2	human liver carcinoma cells

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IgG	immunoglobulin G
MCF-7	human breast adenocarcinoma cell
nAChR	nicotinic acetylcholine receptor
NADH	nicotinamide adenine dinucleotide hydride
PGLa(-)	negatively charged analogue of peptidyl-glycylleucine-carboxyamide
PSA	prostate-specific antigen
QCM-D	quartz crystal microbalance with dissipation monitoring
RyR1	ryanodine receptor/Ca2+ release channel
rSbpA/Lac	recombinant fusion protein comprising of SbpA and laccase
rSbpA/ZZ	recombinant fusion protein comprising of SbpA and two copies of the Fc-binding Z-domain (a synthetic analogue of the IgG-binding domain of protein A from *Staphylococcus aureus*)
SAM	self-assembled monolayer
SAW	surface acoustic waves
SbpA	S-layer protein of *Lysinibacillus sphaericus* CCM 2177
SbsB	S-layer protein of *Geobacillus stearothermophilus* PV72/p2
S-layer	two dimensional arrays of proteinaceous subunits forming surface layers on prokaryotic cells
SPR	surface plasmon resonance
SsLM	S-layer supported lipid membrane
SUM	S-layer ultrafiltration membrane
TEM	transmission electron microscopy
TIRFM	total internal reflection fluorescence microscopy
VDAC	voltage-dependent anion channel

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