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Colloids and Surfaces A: Physicochem. Eng. Aspects 321 (2008) 163–167
COLLOIDS AND SURFACES A
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Surfaces functionalized with self-assembling S-layer fusion proteins for nanobiotechnological applications

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Received 10 July 2007; received in revised form 11 October 2007; accepted 21 December 2007
Available online 4 January 2008

Abstract

The fabrication of supramolecular structures and devices requires molecules that are capable of interlocking in a predictable well defined manner on surfaces required for nanobiotechnological applications. Thus, molecular self-assembly systems which exploit the molecular scale manufacturing precision of biological systems are prime candidates for supramolecular engineering. In this context, crystalline bacterial cell surface layer (S-layer) proteins of prokaryotic organisms represent a unique self-assembly system which can be exploited as patterning element for a biomolecular construction kit involving all major species of biological molecules, for example, glycans such as S-layer-specific heteropolysaccharides, lipids, and nucleic acids.

One of the most fascinating properties of native or recombinant S-layer proteins is their capability to self-assemble in suspension (as flat sheets or cylinders), into monomolecular protein lattices on artificial surfaces (e.g. silicon wafers, noble metals, plastics) or on Langmuir lipid films and liposomes. Functional groups (e.g. carboxyl groups, amino or hydroxyl groups) or genetically incorporated functional domains (e.g. streptavidin) are repeated with the periodicity of the S-layer lattice at a distance resembling the lattice constants, leading to regular arrays of bound functional molecules or nanoparticles. Thus, genetically and/or chemically modified S-layer proteins can be exploited as building blocks and templates for generating functional nanostructures at meso- and macroscopic scale for both, life and non-life science applications.

Keywords: Bacterial surface (S-layer) proteins; Self-assembly; S-layer fusion proteins; Nanobiotechnology; Molecular construction kit; Functionalized surfaces; Biochip development

1. Introduction

Being composed of a single protein or glycoprotein species with molecular weights ranging from 40 to 200 kDa, bacterial cell surface layers or S-layers represent the simplest biological membrane developed during evolution [1–5]. Bacterial S-layers are generally 5–20 nm thick, whereas those of archaea reveal a thickness up to 70 nm. The proteinaceous subunits of S-layers are aligned either in lattices with oblique (p1, p2), square (p4) or hexagonal (p3, p6) symmetry. Depending on the lattice type, one morphological unit is composed of either one, two, three, four or six identical subunits with a center-to-center spacing of approximately 5–30 nm. Most S-layers show a smooth outer, and a more corrugated inner surface. S-layers are highly porous protein lattices with a surface porosity of 30–70%. In many S-layers, two or even more distinct classes of pores of identical size and morphology are present, with diameters in the range of 2–8 nm. In bacteria, the S-layer subunits are linked to each other and the underlying cell envelope layer by non covalent forces. At least for S-layer proteins of Bacillaceae, a cell wall targeting domain on the N-terminal region was found to be responsible for anchoring the S-layer subunits to the underlying rigid cell envelope layer by binding to a heteropolysaccharide, termed secondary cell wall polymer (SCWP) [6–10]. In most S-layer proteins, three S-layer-homology (SLH) motifs, each consisting of 50–55 amino acids, from which 10–15 are conserved, form the functional SLH-domain which specifically recognizes a distinct type of pyruvylated SCWP as the proper anchoring structure [7,11,12]. Complete solubilisation of S-layers into their constituent subunits and release from the bacterial cell envelope can be achieved by treatment with high concentration of hydrogen-bond breaking agents (such as urea or guanidine hydrochloride), by applying chelating agents or by changing the pH. During removal of the disrupting agent, the S-layer subunits

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doi:10.1016/j.colsurfa.2007.12.038

frequently reassemble in suspension, leading to self-assembly products which show the shape of flat sheets or open-ended cylinders which represent either monolayers or double layers or recrystallize into two-dimensional lattices on different solid supports such as such as silicon wafers, gold chips (Fig. 1), and silanized glass or plastic materials, as well as on Langmuir lipid films or liposomes, and at the air–water interface [5,13–15].

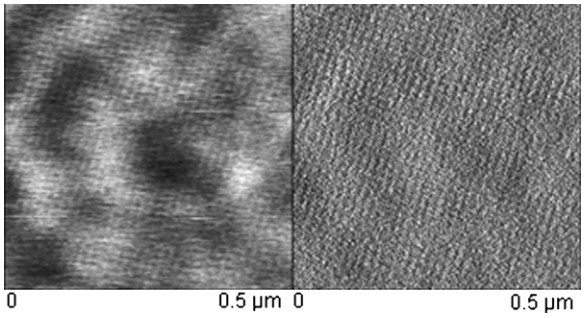

Fig. 1. Contact mode AFM image of S-layer protein SbpA of *B. sphaericus* CCM 2177 recrystallized into a square lattice structure on a gold chip precoated with SCWP. Image is acquired under 100 nm NaCl. Left: height mode. Right: deflection mode.

2. Surfaces functionalized with self-assembling S-layer fusion proteins and their application potential in nanobiotechnology

To date, one big challenge in nanobiotechnology is the technological utilization of self-assembly systems [15–19]. For that purpose, S-layer technology was advanced by the construction of functional S-layer fusion proteins that comprised (i) the N-terminal cell wall anchoring domain, (ii) the self-assembly domain, and (iii) a fused functional sequence. This construction principle refers to the so-called C-terminal S-layer fusion proteins, in which the N-terminal SCWP-binding domain was exploited for oriented binding and recrystallization on artificial supports coated with SCWP [20–22]. This biomimetic approach copies the principle governing S-layer lattice formation on bacterial cell surfaces. For some specific applications, N-terminal fusion proteins, particularly based on SbsB, the S-layer protein of *Geobacillus stearothermophilus* PV72/p2, were constructed, which attached with their outer surface to liposomes and silicon wafers, so that the N-terminal region with the fused functional sequence remained exposed to the external environment [23]. Concerning the first construction principle, most S-layer fusion proteins were based on the S-layer proteins SbpA of *Bacillus sphaericus* CCM 2177, SbsB of *G. stearothermophilus* PV72/p2, SbsC of *Geobacillus stearothermophilus* ATCC 12980 or *Geobacillus stearothermophilus* NRS 2004/3a. These chimaeric proteins incorporating either the sequence of core streptavidin [20,21,23], the major birch pollen allergen Bet v1 [24–26], two copies of the Fc-binding Z-domain of a synthetic analogue of the B-domain of protein A [27], enhanced green fluorescent protein (EGFP) [28], the hypervariable region of heavy chain camel antibodies (cAb) recognizing lysozyme or prostate-specific antigen (PSA) [22,29], short peptide sequences for oriented binding of silver or cobalt nanoparticles, or (hyper)thermophilic enzymes for the development of biocatalysts [30,31] were cloned and expressed in *E. coli* (Table 1).

In order to ensure crystallization of S-layer fusion proteins on solid supports in defined orientation with the functional domains exposed on the lattice surface, the lectin-type binding between the SCWP and the N-terminal of the S-layer protein can be exploited for biochip development (Fig. 2). Proof of principle was already provided by the production of a universal matrix based on the streptavidin–biotin interaction formed by an oriented recrystallization of S-layer-streptavidin heterotetramers on gold chips [20,21]. For this purpose, minimum-sized core streptavidin (118 amino acids) was fused either to N- or C-terminal positions of rSbsB or to a C-terminally truncated form of rSbpA. As biological active streptavidin occurs as tetramer, heterotetramers consisting of one chain fusion protein and three chains core streptavidin were prepared by applying a special refolding procedure. Hybridization experiments with biotinylated and fluorescent labeled oligonucleotides using surface-plasmon-field-enhanced fluorescence spectroscopy indicated that a functional sensor surface could be successfully generated by recrystallization of heterotetramers on gold chips [20,21]. Such promising structures could be exploited for the development of DNA or protein chips as required for many nanobiotechnological applications.

Table 1
Functional S-layer fusion proteins for biotechnological applications

S-layer fusion protein	Functionality	Reference
rSbsB/core streptavidin	Binding of biotinylated ligands, biochip development	[23]
rSbpA/core streptavidin		[21]
rSbpA/Bet v1 (major birch pollen allergen)	Vaccine development, treatment of type 1 allergy	[26]
rSbsC/Bet v1		[25]
rSbpA/ZZ	IgG-binding domain	[27]
rSbpA/EGFP	Coating and functionalization of liposomes	[28]
rSbpA/cAB (heavy chain camel antibody against PSA)	Detection of prostate-specific antigen	[22]
rSbpA/AG4 and AGP35	Oriented binding of metal nanoparticles	–
rSbpA/CO2P2		
rSbpA/LamA (hyper)thermophilic enzymes	Development of biocatalysts	[31]
rSgsE/RmlA		[30]

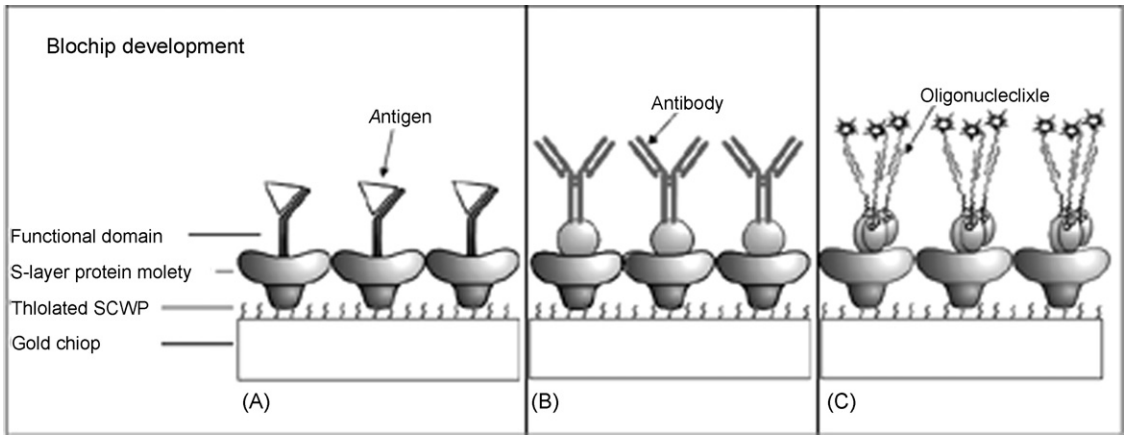

Fig. 2. Schematic drawing of functional S-layer fusion proteins recrystallized on gold chips precoated with SCWP. After recrystallization into a crystalline protein monolayer of an S-layer fusion proteins carrying (A) a heavy chain camel antibody, (B) a IgG binding domain or (C) core streptavidin at the C-terminus, (A) prostate-specific antigen (PSA), (B) human IgG or (C) biotinylated oligonucleotides and hybridized fluorescently labelled oligonucleotides could bound specifically.

3. S-layer protein lattices as template for the formation of nanoparticle arrays

The current challenge in the development of the next generation of nanoelectronic devices will require entirely new materials and fabrication technologies. The ultimately high requirements in the synthesis of molecular functional units can only be met when novel concepts based on state-of-the-art top down lithographic procedures are combined with bottom-up approaches using principles learned from nature. The bioinspired synthesis of inorganic materials, such as metallic or semiconducting nanoparticles, has already attracted much attention over the last two decades. In particular, the broad base of knowledge about the binding of biological molecules has paved the way for investigating the potential of S-layer proteins and their self-assembly products as catalysts, templates and scaffolds for the generation of ordered nanoparticle arrays.

Based on the investigation of mineral formation by bacteria in natural environments [32] S-layer lattices can be used in wet chemical processes for the precipitation of metal ions from solution [33–37]. In this approach, self-assembled S-layer structures were exposed to metal–salt solutions, such as tetrachloroauric (III) acid ($HAuCl_4$), followed by slow reaction with a reducing agent such as hydrogen sulfide (H_2S) [34,35,37] or by exposure to an electron beam [33,36]. Nanoparticle superlattices were formed according to the lattice spacing and symmetry of the underlying S-layer. As determined by electron diffraction, most often the nanoparticles were crystalline but their ensemble was not crystallographically aligned. The wet chemical approach was used in the formation of CdS, Au, Pd, Ni, and Pt nanoparticle arrays.

The binding of preformed nanoparticles into regular arrays on S-layers is an attractive alternative to the wet chemical approach since it allows to better control particle size and hence particle distances both of which are important for studying and exploiting quantum phenomena. Based on the work on binding biomolecules, such as enzymes or antibodies, onto S-layers it has already been demonstrated that metallic and semiconducting nanoparticles can be bound in regular arrangements on S-layers [38–40]. The pattern of bound molecules usually resembles the lattice parameters of the S-layer matrix. A major breakthrough in the regular binding of metallic and semiconducting nanoparticles was achieved by the successful design and expression of S-layer-streptavidin fusion proteins which allowed a specific binding of biotinylated ferritin molecules into regular arrays [23].

4. S-layer stabilized lipid membranes and liposomes

S-layer stabilized lipid membranes mimic the supramolecular assembly of archaeal cell envelopes. The latter are composed of a cytoplasmic membrane and a closely associated archaeal S-layer as the exclusive wall component [3,5,41,42]. In this biomimetic architecture artificial lipids replace the natural lipids of the cytoplasmic membrane and isolated or recombinant S-layer proteins derived from Bacillaceae are attached either on one or both sides of the lipid membrane (Fig. 3). Closed S-layer lattices can be generated for instance at Langmuir lipid monolayers, planar lipid membranes, liposomes, or lipid coated nanocapsules [41–44].

An interesting feature of S-layer supported lipid membranes is its widely retained nanopatterned fluid characteristic and therefore, this type of lipid membrane is also referred to as “semifluid membrane” [45]. But most important, attached S-layer lattices reveal no impact on the hydrophobic lipid alkyl chains. Thus, S-layer lattices constitute unique supporting scaf-

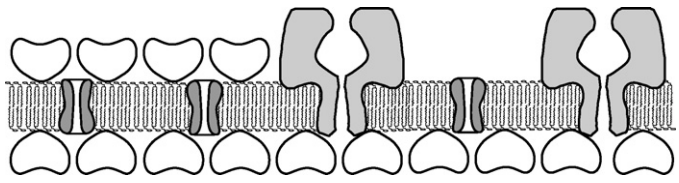

Fig. 3. Using the supramolecular building principle of archaeal cell envelope structures, biomimetic membranes can be generated. The archaeal cytoplasmic membrane is replaced by a phospholipid bilayer and S-layer proteins derived from Bacillaceae are recrystallized on one or on both sides to form closed S-layer lattices on the lipid membrane. Integral membrane proteins like pore-forming proteins or ion channels can be reconstituted into the S-layer supported lipid membranes.

foldings for lipid membranes with life-times of approximately 1 week. Furthermore, reconstitution of transmembrane proteins like α-hemolysin and the membrane-active peptides alamethicin, gramicidin A, and valinomycin can be performed in order to assess and utilize its functionality.

Functional reconstitution of membrane proteins in fluid and stable lipid membranes is of utmost importance as currently more than 60% of consumed drugs act on membrane proteins [41,46]. Therefore the biomimetic approach to generate stabilized lipid membranes with functional membrane proteins has attracted lively interest in recent years posing the challenge to apply membrane proteins as key elements in drug discovery, diagnostics, protein–ligand screening, electronic or optical devices, and biosensors [41,44,46].

5. Conclusion

The wealth of information accumulated on the structure, chemistry, assembly, genetics and function of S-layers has led to a broad spectrum of applications for life and material sciences. Isolated native S-layer proteins and S-layer fusion proteins incorporating functional sequences self-assemble into monomolecular crystalline arrays on a great variety of solid substrates and on various lipid structures including plane membranes and liposomes. Moreover, the specific lectin-type binding interactions between S-layer lattices and an accessory SCWP have opened possibilities to build monolayers with defined orientations of functional domains on surfaces and/or interfaces as well as multilayer lattices with the constituent subunits in accurate superposition [47]. S-layers have proven to be particularly suited as building blocks and patterning elements in a biomolecular construction kit involving all major classes of biological molecules (proteins, lipids, glycans, nucleic acids and combinations of them) enabling innovative approaches for the controlled “bottom-up” assembly of functional supramolecular structures and devices [48]. The broad application potential of S-layer proteins in nanobiotechnology is based on the fuctionalization of different artificial surfaces for bio chip development or the generation of nanoparticle arrays as required for optic and electronic memory devices.

Acknowledgements

This work was supported by the Austrian Science Fund (FWF), projects, P17170-B10, and P18510-B12, by the EU project NAS-SAP, and by the US Air Force Office of Scientific Research (AFOSR), projects F49620-03-1-0222 and FA9550-07-0313.

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