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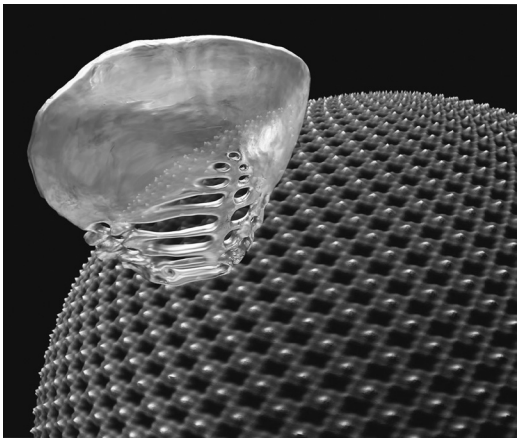
# Curiosity and Passion for Science and Art



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# FOREWORD

Science and art are two sides of a single, fabulous coin. They both are rooted in humankind's natural curiosity, creativity, imagination, and honesty. These characteristics eventually lead not only to new insights and new knowledge but also to the expression of humans' deepest feelings toward nature and their fellow men. This trait is shared by all cultures, no matter how primitive or advanced.

Science and art each require, as a starting point, the careful observation and recording of natural phenomena. Whether it be landscapes or test tubes, the macro- or the microcosmos, scientists and artists study and record and reproduce nature in order to gain insights. With such insights, scientists make discoveries of biological and physical phenomena within the beauty of nature, while artists arouse human feelings through their most profound expressions.

Both scientific and artistic endeavors arise from a passion for understanding nature and for expressing human desire. The pursuit of science and art is not done with the aim of making money, but for the pleasure of finding things out and of expressing humans' innermost feelings. Science and art are not for the half-hearted, high-fliers, or career-minded seekers. They both need enduring practice and sustained patience — persistence even after numerous failures. It is often said that, in science, most experiments fail, and initial observations are difficult to repeat. Experimental work is routine and repetitious, but breakthroughs and new knowledge depend on reproducible results. Likewise, artistic breakthroughs often come after years of dedicated work.

It is my belief that art and science are the greatest legacy our ancestors left us, from stone carvings, cave paintings, and primitive tools to today's inventions that amplify human abilities and express the human mind. All else — kings, queens, religions, and empires — become irrelevant.

Uwe Sleytr has pursued science and art simultaneously with passion and achieved greatness. In this unique book, you will read about his relentless quest for an exceedingly important class of surface proteins — which he termed *S-layer proteins* — on numerous bacteria and nearly all archaea. Calculated by the mass

or weight of the living system on Earth, S-layer proteins are the single most abundant protein on the planet. Moreover, most important, S-layers represent a unique structural basis and patterning element for generating more complex supramolecular structures. Accordingly, they fulfill key requirements as building blocks and patterning elements for the production of new materials and nanoscale devices in molecular nanotechnology, nanobiotechnology, synthetic biology, and biomimetics.

At the same time, Sleytr has passionately pursued his interest in art, from early drawings and paintings like Gustav Klimt when he was in his early 20s, to his sculptures of amazing gold-coated clay masks, to his latest photography of optically distorted mask images and computer color mixing. Through these image distortions Sleytr expresses his vision of an unpredictable future of human beings and society possibly mediated by advances in synthetic biology and self-enhancement through genome editing.

As both an accomplished scientist and artist, Uwe Sleytr is able to present both kinds of visions. It is a great pleasure to read about his lifelong pursuit and interests in a single book. I hope that you will find it a fascinating book for your interest in both science and art.

*Shuguang Zhang*
*Massachusetts Institute of Technology*

# Contents

# CURIOSITY AND PASSION FOR SCIENCE

*Curiosity and imagination is more important than knowledge.*
— Albert Einstein

*Learn the rules like a pro, so you can break them like an artist.*
— Pablo Picasso

## 1.1. My Interests During My Formative Years

For as long as I can remember, I have been curious. People who knew me from an early age characterized me as a child constantly asking questions of "why" and "how." Over the years, this attitude has not changed. As a scientist my curiosity merged with a passionate persistence for unraveling nature's secrets.

I was very fortunate in growing up with parents who not only responded to my questions but also encouraged my curiosity by asking me questions themselves. My father was an artist with a strong interest in life sciences, who made his living primarily as a lithographer, and my mother was the owner of part of a bookstore. That provided the ideal intellectual environment for stimulating many of my later interests.

As an example, I had the privilege of being permitted to use my father's light microscope when I was about ten years of age. To make the invisible visible and, under proper guidance, to discover mysteries of the unseen world of the microcosmos was fascinating to me. This very early realization that we are surrounded by and composed of constituents we cannot see opened my mind and triggered my later strong interest in microbiology, including molecular and cell biology.

From that age on, I collected and bred butterflies by searching for caterpillars and feeding them with the proper plants until they pupated. Another passion was my aquarium. I bred tropical fish (zebrafish, guppies, and Siamese fighting fish) and I spent many hours just observing the behavior of the different species. I remember many other activities from that period of my childhood: fishing and diving in a lake close to our home, or simply watching my surroundings, merging all my senses with nature. Looking back at all these activities and impressions, they were important for stimulating my lifelong curiosity and for determining my interests.

I am convinced that at that early stage, one's neuronal network can be programmed and imprinted in such a way that curiosity and imagination are established as an important part of one's nature, personality, and desires.

During high school, my interests focused primarily on natural sciences and art. At the end of this essay, I will give more attention to the relevance of art in my life. I was fortunate, in that both my teachers and my parents encouraged me toward both arts and science. I remember that my art teacher predicted that I would end up at the fine arts university, and my natural sciences teachers predicted that I would study biology. But, in the end, I selected Food and Biotechnology at the University of Natural Resources and Life Sciences in Vienna as my field of study. I decided that my artistic pursuits should continue at a leisure level. An essential reason for choosing these particular academic studies was the balanced mix of applied biology, chemistry, and engineering. Moreover, in comparison with related areas offered by other universities in Vienna, this area of study had a strong emphasis on general and applied microbiology, which always majorly attracted my attention.

My Ph.D. work, which started after my military service in 1966, concerned the characterization of thermophilic bacteria which cause infections in extraction plants at beet sugar factories. Because I had access to an electron microscopy facility, I took the opportunity to study the ultrastructure of a variety of relevant *Bacillus* and *Clostridium* species capable of growing at temperatures of around 70°C. Starting with negative staining and ultrathin sectioning techniques, I soon encountered the intrinsic artefact problem with these specimen preparation procedures. At that time a new cryopreparation technique called "freeze-etching" was developed by a group at ETH Zurich, which replaced the chemical fixation of specimens by cryofixation.

## 1.2. Freeze-Etching Methods

Freeze-etching or freeze-fracturing as a preparative technique for ultrastructural investigation involves the production of a heavy metal–carbon replica of the fracture plane through a frozen specimen. Replica formation under high vacuum can immediately follow the cryofracturing process, or take place after a controlled freeze-drying ("etching") step at –100°C for removing a thin layer of ice from the fracture face and thus laying bare structures originally not exposed by the fracturing process itself. The most important advantages of the freeze-etching technique, as compared with other specimen preparation methods for electron microscopy, are: (1) no chemical fixation is necessary; (2) the specimen is not dehydrated; (3) the fracture follows a plane of weakness through the specimen and thus a three-dimensional surface is revealed — this commonly follows the line of cell membranes.

Happily, our university acquired one of the first commercial instruments in the mid-1960s: a Balzers BA 360 M freeze-etching unit from Balzers AG, Balzers, Liechtenstein. Even better, I could learn the technique at ETH Zurich, where the development of the technique took place. Until then very few results had been published involving this new technique, and very controversial interpretations were published concerning the fine structures exposed by fracturing and etching.

When I first examined freeze-etching replicas of different thermophilic bacteria using transmission electron microscopy, I noticed that all surfaces of intact cells exposed by the etching process revealed a regular pattern.[6–8] Apart from the relevance of this newly observed structure, the very appealing esthetical aspect fascinated me and I wanted to learn more about the nature of these bacterial cell surface layers (termed S-layers). I immediately contacted well-recognized microbiologists to get possible suggestions as to how this surface structure could be interpreted, and I received advice to apply freeze-etching to *Escherichia coli* and *Bacillus subtilis*, which at that time were considered the classical experimental strains in microbiology. Although different strains of both species did not show S-layers, my fascination for this elegant two-dimensional crystal structure persisted and I continued to study the S-layer lattices originally observed on the surface of the thermophilic organisms I had on hand. Moreover, due to the fact that in *E. coli and B. subtilis* S-layers were missing, very few microbiologists who were engaged in structural studies of bacteria became interested in S-layer research. I was almost alone in this new field for quite a while.

After finishing my Ph.D. thesis, I was appointed as an assistant professor in the Department of Food Technology in 1970, where I continued my studies of thermophilic *Bacillaceae* and, in particular, S-layers. Concurrently, I became very much involved in the improvement of freeze-etching methods and the interpretation of results. At that time low temperature techniques for electron microscopy were based on the pioneering work of several groups, but the actual breakthrough in the application of freeze-fracturing replication techniques, for both biological and nonbiological materials, came with the development of commercial freeze-etching apparatus.[37] The relevant difference among the various techniques was that the freeze-fracturing process was carried out either under high vacuum conditions[2] or under liquid nitrogen.[5] The latter required a subsequent step in which the specimen is transferred to a high vacuum chamber for an optional etching process and replica formation. For all techniques,[37] the specimens are first rapidly frozen at a speed of several thousand Kelvins per second either by immersion or by spraying into a suitable coolant, or on a smooth metal block at liquid nitrogen temperature (77 K) or lower.

Groups involved in freeze-etching studies soon demonstrated that under optimal conditions this cryofixation leads to vitrification (amorphous ice) of the specimen water or formation of ice crystals sufficiently small (pseudovitrification)

so as to avoid structural damage. Nevertheless, I soon realized that despite optimal specimen freezing, the different preparation steps (pretreatment of the specimen before freezing, cleaving, etching, heavy metal–carbon replica formation) can each lead to the production of specific artefacts, and that it would be of great importance to understand more fully the factors involved in structural changes during the processing. In this context, the ability to produce and retain replicas from both halves from a frozen–fractured specimen (described as the complementary, or double, replica technique) has proven a valuable asset in both analyzing membrane structures[9,20,21] and helping to interpret the nature and mechanism of artefact formation.[21,36,51] Most relevant, Walter Umrath and I found that the evaluation of corresponding fracture moieties has proven to be an important tool for discriminating between surface and central fractures in membrane systems. At that time, a fundamental controversy still existed concerning the fracture site in frozen membranes.[3,4] The appearance of two nonetchable, morphologically different membrane structures in corresponding fracture moieties of bacterial protoplasts and membrane systems in yeast cells was unequivocal proof that cleavage of biological membranes took place along a central plane by separating the lipid bilayer.[9] In this context, it is worth mentioning that, in collaboration with Walter Umrath and Tony Robards, we evaluated complementary fracture faces, and demonstrated that plastic deformation of cell components occurs in some specimens when fractured even at temperatures as low as 4 K. These data clearly demonstrated that even at temperatures very near 0 K a fracturing process can liberate sufficient energy to allow local plastic deformation to occur.[20,21,25,36]

Our research on possible artefact formations led to the design of a new freeze-etching device with Walter Umrath from Leybold-Herraeus in Cologne, which was commercialized under the name EPA 100.[30,37] When developing this device, we had in mind that there should be a minimum of condensable gases around the specimen during the exposure of the fracture plane for etching and replica formation, and a maximum of automation and flexibility for the different preparation procedures, such as freeze-cleaving under vacuum. There should also be opportunities for contamination-free transport from liquid helium (4 K) and/or liquid nitrogen (77 K) into vacuum if freeze-cleaving or handling of specimens under liquid gases or extraction liquids at low temperatures was necessary. As an essential innovation for improving the vacuum condition in the chamber and to prevent condensation contamination by residual gases on the fracture faces (during etching) before replica formation, the specimen was entrapped during the whole procedure in a liquid-nitrogen-cooled cold trap.[30,37] This freeze-etching device was successfully introduced into the market and delivered excellent results, but in the course of a restructuring process within the Leybold-Herraeus company the production of electron-microscopical preparation devices, including the EPA 100, was terminated and the essential technological innovations, including

patents (particularly the introduction of the optically tight cold shroud around the specimen area), were transferred to competitors.

Much later, out of long-standing friendship and scientific collaboration on the use of low temperatures in ultrastructure research, Tony Robards and I (Fig. 1) wrote the textbook *Low Temperature Methods in Biological Electron Microscopy* for the series *Practical Methods in Electron Microscopy* (Ed. Audrey M. Glauert), published by Elsevier. After its publication in 1985,[383] I left cryotechniques and focused my interest exclusively on studies concerning the structure, chemistry, genetics, morphogenesis, function, and potential applications of S-layers.

**Fig. 1.** With Anthony W. Robards (*left*) in 1985.

## 1.3. Early S-Layer Work

In retrospect, the most important progress in S-layers took place during the period I spent at the Strangeways Research Laboratory and the Medical Research Council (MRC) Laboratory for Molecular Biology (LMB) in Cambridge, England, between late 1972 and 1975, supported by EMBO and MRC fellowships. During this stay, I particularly remember the occasions when I met the MRC-LMB Director Max Perutz, who enjoyed talking with me because I came from Vienna, the city where he grew up. He communicated to me a deep understanding for a young scientist driven by curiosity and a desire for discoveries. It was during these discussions that I acquired an understanding of the relevance of serendipity in science and the importance of motivating scientists who have specialized in unrelated areas to work closely together to solve complex questions occurring in biology. This requires elimination of the existing barriers between the different disciplines and, most important, a commitment to sharing success among the players. Later on, when I had the opportunity to establish my own team, as

head of the Center for Ultrastructure Research, the Ludwig Boltzmann Institute for Molecular Nanotechnology, and the Department of Nanobiotechnology in Vienna, I succeeded by following this clear-sighted advice.

At the same time that I visualized S-layers on the cell surface of intact cells of a variety of the Gram-positive *Bacillaceae*, using freeze-etching, Audrey Glauert's group at the Strangeways Research Laboratory in Cambridge (Fig. 2) published data on a regular array of molecules as a component of the outer membrane of the Gram-negative *Acinetobacter* species using negative staining.

**Fig. 2.** Audrey M. Glauert (1925–2014).

Furthermore, Glauert and her colleague Margaret J. Thornley observed that the S-layer lattice from *Acinetobacter* cell walls could be detached and disintegrated into its constituent subunits by incubation with one-molar urea or with water after treatment with EDTA. After removal of the urea they reaggregated into the same ordered array at the air–water interface in the presence of magnesium ions. The detached subunits were characterized as acidic proteins of molecular weight ~ 65 kD.[17,18] Soon after arrival in Cambridge and with access to a freeze-etching unit in Nigel Unwin's lab at the MRC Laboratory, I could confirm that in the *Acinetobacter* species the regular array, without any doubt, is located on the cell surface.[12,19] In a subsequent, more detailed freeze-etching study, I showed that intact cells can fracture along a central plane in the hydrophobic region of both the outer membrane and the plasma membrane and that healthy cells release small vesicles composed of both the regular array of protein and the outer membrane.[15] Back then we were not aware of the particular relevance of the observation that vesicles are released from the cell surface of growing Gram-negative bacteria. Today it is known that this extracellular secretion involving vesicles is the major

mechanism by which Gram-negative pathogens communicate with and intoxicate host cells.

Although less commonly used today, freeze-etching is still the most suitable technique for identifying S-layers on intact cells of a specific organism. In addition, other electron-microscopic techniques involving thin sections, freeze-dried and metal-shadowed, negative-stained and frozen hydrated preparations, as well as the more recent atomic force microscopy, are available for the identification and structural characterization of S-layers.

In the course of our freeze-etching studies of S-layers, Audrey Glauert and I found by chance that bacterial flagella, known to be composed of a helical array of flagellin subunits, reveal in longitudinal fractures a hollow central core suitable for constituent flagellin molecules to travel from the site of synthesis to the distal end of the flagella during growth.[13]

S-layer lattices generally exhibit several space group symmetries: (i) oblique (p1, p2), (ii) square (p4), and (iii) hexagonal (p3, p6) with center-to-center spacings of the morphological units which are 4–35 nm in size.[209,371] Because these lattices were shown to be curved, or otherwise distorted, the images obtained with the different preparation techniques were very noisy. I then developed, together with Tony Crowther from the MRC Lab, a simple computer procedure for image-averaging to reduce the noise. The averaged images revealed for the first time that the morphological units of the hexagonal and tetragonal arrays of two different *Bacillaceae* species are composed of six and four subunits, respectively.[35] Nowadays, much more elaborate averaging techniques, including 3D reconstruction techniques for biological macromolecules, are in use (Jachim Frank; see Vol. 2 in this series).

During my stay in Cambridge (and later back in Vienna, in collaboration with Paul Messner, Dietmar Pum, and Margit Sara), I performed numerous *in vitro* self-assembly studies of isolated S-layer subunits and detailed structural analyses, particularly of lattice orientation and lattice faults (e.g. dislocations and disclinations) of freeze-etched intact (potentially living) cells to elucidate the dynamic process of the incorporation and reassembly of new subunits into (closed) S-layer lattices during cell growth and cell division.[24,26,34,40,46,59,64,93,106] In this context, I remember very stimulating discussions with Aaron Klug and Tony Crowther in the cafeteria of the LMB concerning the interpretations of my electron micrographs. I had calculated that under optimal growth conditions approximately 500 S-layer subunits per second are incorporated on a cell surface. Moreover, it could be confirmed that the only requirement for maintaining a highly ordered macromolecular array with no gaps on the surface of a growing and dividing cell is a continuous synthesis of a surplus of proteinaceous subunits and their translocation to sites of lattice growth.[118,141] We obtained conclusive information concerning the development of coherent S-layer lattices on growing

cell surfaces by reconstitution experiments with isolated S-layer subunits on cell surfaces from which they had been removed (homologous reattachment) or on those of other organisms (heterologous reattachment).[26,33,34,40]

Later, back in Vienna, together with Karin Gruber and Sylvie Lortal, we could show using labeling experiments with fluorescent antibodies and colloidal gold/antibody marker methods that different ways for S-layer extensions exist for Gram-positive bacteria[74,92,109] in comparison with Gram-negative bacteria. We confirmed that the information for the dynamic lattice formation and lattice orientation resides in the S-layer proteins themselves, and this is not affected by any pattern in the supporting layer;[26,46,49,54] and further, that the inter-subunit bonds in the S-layer lattice are stronger than those binding the crystalline array to the supporting envelope layer. Altogether, these characteristic properties are seen as a major requirement for the continuous recrystallization of the coherent S-layer lattice into a low free energy arrangement of subunits during cell growth and division.

In contrast to bacteria, where the S-layer is attached to an outer membrane (Gram-negatives) or the rigid petidoglycan-containing layer (Gram-positives), in most archaea, S-layers assemble as the exclusive wall component in close association with the plasma membrane[209,371] and consequently are strongly associated with maintenance of the cell shape.[28] Our freeze-etching analysis of cell morphology and lattice fault distribution in the archaea *Thermoproteus tenax*, *Thermoproteus neutrophilus*,[59] and *Methanocorpusculum sinense*[106] provided strong evidence that the S-layer lattice is not only involved in the maintenance of the cell shape, but must also be involved in cell fission. We concluded that the cell division process is determined by the ratio between the increase in protoplast volume and the increase in the actual S-layer surface area during cell growth. We postulated that this elementary mode of cell fission represents a common feature in lobed archaea, which possess S-layers as the exclusive wall component.[106]

## 1.4. S-Layer Glycobiology

In the 1970s, Cambridge, England was also the right place to become involved in more detailed studies of the chemical composition of S-layers. Since at that time considerable data had accumulated on the ultrastructure, isolation, and assembly of the S-layers of *Clostidium thermosaccharolyticum* (later renamed *Thermoanerobacterium thermosaccharolyticum*) and *Clostridium thermohydrosulfuricum* (later renamed *Thermoanaerobacter thermohydrosulfuricus*), I decided to use this organism as a system model. I still remember clearly the difficulties in producing enough biomass from both anaerobic organisms for proper chemical analysis of their S-layers and the increasing complaints of colleagues about the hydrogen sulfide production

of "my bugs." In collaboration with Kareen Thorne, I could characterize the tetragonal (p4) and hexagonal (p6) ordered arrays as glycoproteins of molecular weight 140 kD, both composed of predominantly acidic amino acids and with an acidic isoelectric point after isoelectric focusing. Most relevant, we could demonstrate that both proteins are glycosylated. Using then state-of-the-art paper chromatography techniques for qualitative sugar determination, we considered glucose, galactose, mannose, and rhamnose as glycocomponents.[31] Much later, a more accurate carbohydrate analysis was possible with nuclear magnetic resonance.[72,97,111,114,124,145]

At the time Kareen Thorne and I published our first results, it was also reported that S-layers from archaea have covalently attached glycan chains.[28] In fact, S-layer proteins were the first glycoproteins to be detected in prokaryotes.[56,75,320,371] Since then, S-layer glycoproteins from several other prokaryotic organisms have been isolated and studied in detail, leading to the awareness of the wide distribution of S-layer glycoproteins among bacteria and archaea.[320,371] Accumulated data confirm that a common feature of almost all bacterial S-layer glycoproteins is the presence of long-branched homo- or heterosaccharides with 50–150 glycoses which constitute about 15–50 repeating units. The monosaccharide constituents of bacterial S-layer glycans include a wide range of neutral hexoses, 6-deoxyhexoses, and amino sugars. In some glycoproteins this spectrum is extended by rare sugars which are otherwise characteristic constituents of lipopolysaccharide (LPS) O-antigens of Gram-negative bacteria. The typical linkages of the S-layer glycan chains to the protein moiety are O-glycosidic linkages to serine, threonine, and tyrosine. In contrast, N-glycans were shown to be characteristic of archaea. It is also evident that S-layer glycoprotein lattices are composed of a mixture of variably glycosylated S-layer protein species. Thus, it can be concluded that the glycosylation event contributes decisively to the variation potential of S-layer proteins.

In these early studies of glycosylated S-layers, it was also important to demonstrate that the carbohydrate residues are exposed on the cell surface.[90] The most recent studies with Bernhard Schuster of the relevance of S-layer glycosylation to cell surface properties by comparing glycosylated and nonglycosylated S-layers have provided evidence that the carbohydrate chains can determine the amount of water bound to the cell surface.[379] Since it has been shown that the self-cleaning capability of biological surfaces relies not only on the wettability but also on the structure of the water molecules near the substrate, we were tempted to speculate that the infiltration of the nanoporous S-layer lattice with the lubricating water might exhibit some kind of S-layer-specific "nano-pitcher-plant effect."[296,379] Thus, based on accumulated data, one may presume that the common intrinsic features of S-layer lattices are biocompatible, antifouling, and/or self-cleaning structures due to the combination of polyzwitterionic and polyhydrophilic characteristics,

with a nanopatterned interface with roughness on a nanometer scale, and a lock-in-place hydration layer facing the surface.[365,375]

The latter can be even modulated by the presence of surface-exposed glycan residues. This data on the general features of S-layer protein or glycoprotein lattices could explain the widespread occurrence of these monomolecular arrays in the world of prokaryotic organisms and justify the energy cost for their synthesis. Finally, this general feature is not in contradiction with more specific functional properties observed, such as in pathogenic organisms or specific cell–cell or cell–tissue interactions.[371]

Finally, investigations in collaboration with Margit Sara on the glycosylation of S-layer proteins, and studies of the structural–functional relationship of distinct segments of S-layer subunits, also led to an interesting incidental finding. Studies of a great variety of S-layer proteins from *Bacillaceae* revealed the existence of specific binding domains on the N-terminal part for sugar polymers, the so-called secondary cell wall polymers (SCWPs), which are covalently linked to the peptidoglycan of the cell wall proper.[180,192,197,202,214,228,261,269,289,298,320,359] Elucidating the mechanism involved in specific binding of S-layer proteins to supporting envelope layers was seen as essential for understanding both the dynamic process of assembly of S-layer lattices during cell growth, and the modification of surfaces with SCWPs. Most relevant, coating surfaces with isolated SCWPs facilitated recrystallization of functionalized S-layers (such as S-layer fusion proteins) in a defined orientation, which is of particular importance for a variety of nanobiotechnological applications.[313,371] In this context, I would also like to refer to a variety of studies with Jose Luis Toca-Herrera analyzing the surface dependence on the self-assembly kinetics and nanocrystal formation of S-layer proteins. Particularly valuable information was obtained by combining quartz crystal microbalance with dissipation monitoring and atomic force microscopy.[268,270,301,302,304,327,339,350,360,364]

## 1.5. S-Layers as Molecular Sieves

The first high resolution electron-microscopic studies of negatively stained S-layer preparations with Tony Crowther from the LMB in Cambridge provided some knowledge of the mass distribution in monomolecular arrays.[35] More detailed information on the dimensions and morphology of the pores running through the S-layer lattice came from three-dimensional reconstructions derived from images from specimen tilt series. To determine the effective size of pores in S-layer lattices of different *Bacillaceae* species, Margit Sara and I performed permeability studies according to the space technique.[66] We could show that S-layer lattices allowed free passage for molecules with a molecular weight of up to 30 kD with a sharp exclusion limit between the molecular weights of 30 kD and 45 kD, suggesting a

limiting pore diameter in the range of 3–4.5 nm, which clearly resembled the pore dimensions determined by high resolution transmission electron microscopy. Most relevant for an *in vivo* molecular sieving function, S-layers represent highly porous protein lattices exhibiting a porosity of 30–70%. Moreover, permeability studies of S-layers from *Bacillaceae* demonstrated that the surface and pore area of the protein meshwork have a very low tendency for nonspecific adsorption of (macro)molecules.[63,67,79,87,98,105,112,123,125] This characteristic property of S-layers was seen as essential for maintaining an unhindered exchange of nutrients and metabolites between the cell and its environment. We also noticed that the S-layer in Gram-positive bacteria masks the net negative charge of the supporting peptidoglycan-containing rigid layer and hence has significant influence in determining interactions between living cells and their environment.[65,73]

S-layers possessing the characteristic feature of isoporous molecular sieves led to the development of a completely new type of ultrafiltration membrane (UF).[63,76] It is worth mentioning that polymeric UFs have a surface porosity which is usually lower than 10% and reveal a pore size distribution with pores differing by as much as an order of magnitude. In order to produce UF membranes with a crystalline isoporous active filtration layer, isolated S-layer fragments were deposited under pressure on commercial microfiltration membranes. The chemical and mechanical stability of this composite structure was subsequently obtained by inter- and intramolecular cross-linking. Most remarkable, the chemical and thermal resistance of these membranes was shown to be comparable to that of polyamide membranes.[67] The S-layer ultrafiltration membranes (SUMs) produced by S-layers from a great variety of *Bacillaceae* revealed the same cutoff value characteristics as the S-layer sacculi derived from intact bacterial cells. Further, in comparison with conventional UFs produced from amorphous polymers, SUMs revealed a much lower unspecific protein adsorption (membrane fouling), in conformity with native S-layer lattices. This feature is considered to be particularly important for tuning membrane-mediated separation processes. Moreover, since S-layer lattices provide precisely controlled spatial distribution and orientation of physicochemical properties in the subnanometer range, functional groups such as amino, carboxyl, and hydroxyl groups both on the surface and within the pore area could be used for very accurate chemical modifications for changing the membrane cutoff values and molecular sieving properties.[79,86,123,125] These unique features of the porous protein meshwork and the possibility for chemical modifications provide the basis for the use of S-layers as a patterning element for immobilizing biologically active molecules such as enzymes and ligands,[88,122,136,144,146,147,160,240,276,282,355,367] antigens or antibodies in a geometrically defined dense repacking order,[170,173,253,329,371] and the use of S-layers as a patterning element for molecular imprinting,[382] for the production of ordered nanoparticle arrays,[185,318] for biomineralization,[321,361] and for conjugate vaccines.[116,121,131,137,161,164,169,189,371]

## 1.6. Composite S-Layer Lipid Structures

Looking back, the development of methods to recrystallize isolated S-layer subunits into large scale coherent protein lattices at an air–water interface or on lipid films, which I started with Dietmar Pum[127,150] and later we continued with Mathias Lösche[168] and Bernhard Schuster,[176] initiated a broad spectrum of basic and applied research projects. Moreover, later with Seta Küpcü, we adapted the supramolecular principle of S-layer-stabilized planar lipid membranes to liposomes[156] and emulsomes.[377]

It must be remembered that with this approach we were attempting to copy the building principles of S-layer-supported lipid membranes (SsLMs) from the cell envelope structure of archaea. Since the cell envelopes of most archaea dwelling under extreme environmental conditions, such as (i) temperatures of up to 120°C, (ii) pH down to zero, (iii) high salt concentrations, and (iv) high hydrostatic pressure, are exclusively composed of a cytoplasmic membrane and a closely associated or even partially integrated S-layer glycoprotein lattice, they must integrate the basic functions of mechanical and osmotical cell stabilization. Altogether, these observations inspired the concept of exploiting this supramolecular principle for stabilizing planar or vesicular functional lipid membrane systems.[150,156,247,255,257,279,297,299,319,370,378,380,381] Because suitable methods for the disintegration of archaeal S-layer protein lattices and their reassembly into monomolecular arrays on lipid films were not (and still are not) available, we used S-layers from *Bacillaceae* to copy the supramolecular building principle of archaeal envelopes.[279,299,319,336,352]

Moreover, S-layer proteins can be used as biofunctional surfaces[313] and constitute a fascinating structure for hosting and stabilizing functionalized lipid membranes.[156,204,243,255,286,287,297,319,330,356,362] SsLMs consist of either a tetraether lipid monolayer (as observed in archaea) or an artificial phospholipid bilayer that replaces the cytoplasmic membrane of bacteria or membranes of eukaryotic organisms. Using bacterial S-layers, we determined that well-defined contact points exist between the S-layer protein and the attached lipid membranes, affecting 5% of the lipid molecules while still enabling the remainder to diffuse freely in the lipid film.[201] Thus, we referred to these nanopatterned lipid membranes as "semifluid membranes."[150,205,212,213] The stability of the composite SsLMs could be further enhanced by covalent linkage of the phospholipid head groups to the S-layer lattice.[344,348] SsLMs revealed a broad application potential in the format of planar, free-standing, and planar-surface-attached lipid membranes. The last included SsLMs attached to S-layer ultrafiltration membranes[227] and membranes with a second S-layer assembled on top of the previously generated SsLM as a protective molecular sieve and further stabilizing scaffold.[225]

From all these studies, it is now evident that SsLMs act as interfacial

architectures mimicking the supramolecular principle of cell envelopes, which have been optimized through billions of years of evolution in the most extreme habitats, conferring stability and lifespans superior to other approaches for generating functional solid-supported biomimetic membranes (such as tethered bilayer lipid membranes).[212,225,257,259,273,279,311] Most relevant, we could demonstrate that in SsLMs membrane-active peptides[193,245,257,358,376] and even more important, (complex) integral membrane proteins[191,193,227,234,374,380] can be incorporated in a functional state. Since the results from the human proteome project suggest that more than 30% of proteins are membrane or membrane-associated proteins like pores, ion channels, membrane-anchored enzymes, and G-protein-coupled receptors, we foresee broad applications for SsLM systems, particularly in the area of (lab-on-a)biochip technology. With SsLMs, it might become possible to create artificial noses or tongues, where many receptor proteins have to be exposed and read out simultaneously.[370]

In analogy with the reproduction of archaeal cell envelope structures, we have reproduced the supramolecular concept of animal and human virus envelopes, which are composed of a lipid bilayer membrane, stabilized by incorporated densely packed glycoproteins, for stabilizing and functionalizing liposomes[156,196,206,218] and emulsomes[362,377,378] by assembling a coherent S-layer lattice on their surface. Both formats possess great application potential as drug targeting and drug delivery systems.[319,370,371,378] In this context, it should be remembered that S-layers can be used for stabilizing nanocapsules composed of polyelecrolytes.[258]

## 1.7. S-Layers as Patterning Structure for Various Uses

The regular structure, physicochemical repetitive uniformity down to the subnanometer scale and morphogenetic potential of S-layers stimulated efforts to modify S-layer proteins through genetic manipulations, and to incorporate single or multifunctional domains of other proteins while maintaining the self-assembly capability. Although native S-layer proteins have already demonstrated their great potential as patterning structures for immobilizing functional molecules and nanoparticles in a defined orientation and spatial distribution,[59,122,123,185,195,255,264,272,274,275,309,318,331] genetic approaches opened up the possibility of modifying and changing the natural properties of S-layer proteins, thus leading to bioinspired materials with designed functional properties. Without prior knowledge of the structural details of the S-layer lattices at the atomic level, our team at the Department of Nanobiotechnology, involving among others Margit Sara, Eva-Maria Egelseer, Nicola Ilk, Andreas Breitwieser, and Carina Huber, initially worked on the basis of trial and error, but later on benefitted from selected structural predictions.[226,229,248,306,307,324,326,337,341,346,354] Soon after we

started this approach,[239] it turned out that most of our studied S-layer proteins were tolerating insertions or fusions with foreign proteins or domains while retaining the ability to assemble into well-defined coherent crystalline arrays on a great variety of surfaces and interfaces.[295,303,352,353] The genetically-fused functionalities include: (i) ligands like streptavidin,[240,276,281] (ii) protein A and protein G,[233,260] (iii) enzymes,[290,293,329,347,367] antigens,[265,291,315,343] (iv) camel antibodies,[246,263] (v) fluorescent proteins,[262,285,325,328,335] and (vi) various peptides.[294,300,303,310,316,334] Most important, we could demonstrate that as observed with chemically immobilized macromolecules, the S-layer lattice acted as a protein cushion, thus preventing denaturation of the functional moiety of the fusion proteins, as frequently observed upon direct binding to solid supports.[353,371,372] S-layer fusion proteins have led to new types of (i) affinity structures, (ii) microcarriers, (iii) enzyme membranes, (iv) vaccines, targeting, (v) delivery, (vi) encapsulation systems, (vii) diagnostic devices and (viii) biosensors, showing a significant improvement in the signal-to-noise ratio.[329,352,353,371]

## 1.8. A Few Final Remarks Concerning the History of S-Layer Research

It is now more than 60 years since Houwink[1] demonstrated by electron microscopy a "monomolecular monolayer" in the cell wall of an unidentified *Spirillum*. Since then, such striking lattices have been observed in more and more preparations of prokaryotic cell envelopes. Today, S-layers have been identified in hundreds of different species of almost every taxonomical group of walled bacteria and are a ubiquitous feature of archaea. Consequently, S-layers are now recognized as an important class of secreted proteins and as the simplest protein membrane developed in biological evolution. Their construction is based on a single (glyco) protein subunit with the intrinsic ability to assemble into isoporous lattices. Moreover, as the biomass of prokaryotic organisms surpasses the biomass of eukaryotic organisms, S-layer proteins, which account for approximately 10% of cellular proteins, can be considered to be one of the most abundant biopolymers on our planet.[371]

In the early 1970s, there were approximately three laboratories actively working with bacterial S-layers because such studies required high resolution electron microscopy, preferential cryotechniques, complemented by biochemical and molecular biological methods. (Selected milestones in basic and applied S-layer research are listed in Table 1 of Ref. 371).

At that time, such regular arrays were not always called S-layers, but were instead referred to as: (i) paracrystalline arrays, (ii) regular-structured layers (RS layers), (iii) planar crystalline layers, or (iv) surface layers (S-layers).

The term "S-layer" was introduced in 1976[33] and became generally accepted at the First International Workshop on Crystalline Bacterial Cell Surface Layers, in

Vienna, Austria (August 1984). S-layers were then defined as "Two-dimensional arrays of proteinaceous subunits forming surface layers on prokaryotic cells".

Figure 3 shows attendees of that S-layer workshop, which I organized with the help of Paul Messner, Dietmar Pum, and Margit Sara, who back then were assistant professors at the Centre for Ultrastructure Research at the University of Natural Resources and Life Sciences, Vienna.

The second international meeting on S-layers, which likewise took place in Vienna, was organized as an EMBO Workshop by our group as well (from August 31 to September 2, 1987). A compilation of the 34 scientific contributions was published by Springer in 1988 as the book *Crystalline Bacterial Cell Surface Layers* (Springer-Verlag, Berlin, Heidelberg, New York; U.B. Sleytr, P. Messner, D. Pum, M. Sara, eds.).[384]

**Fig. 3.** First International Workshop on Crystalline Bacterial Cell Surface Layers, in Vienna, Austria, 1984. *From left to right*: Bill Kay (partially hidden), Terry Beveridge, Harald Engelhardt, [unknown], Susan Koval, [unknown], Leo März, Jim Chalcroft, Dietmar Pum, Robert Glaeser, Sven Hofmoller, Martin Kessel, Karin Gruber, Helmut König, Günter Wolf, Kari Lounatmaa, [unknown, hidden], Paul Messner, Margit Sara (partially hidden), Gerhard Meister, [unknown], Wolfgang Baumeister, [unknown], Uwe Sleytr.

The next international workshop on bacterial S-layers was organized in the format of a NATO–Advanced Research Workshop (AWR) from September 27 to 30, 1992, in London Ontario, Canada (Fig. 4). The organizing committee consisted of the two workshop directors, Susan F. Koval and Terry J. Beveridge, as well as Helmut König, Uwe B. Sleytr, and Trevor J. Trust. This third workshop quite clearly demonstrated how rapidly S-layer research was progressing. A compilation of research presented at the meeting was published in 1993 as a 37-chapter volume in *NATO Advanced Science Institute Series A: Life Sciences*, Vol. 252, with the

title *Advances in Bacterial Paracrystalline Surface Layers* (T.J. Berveridge and S.F. Koval, eds.; Plenum, New York).[387]


**Fig. 4.** NATO S-layer workshop, 1992, London, Ontario, Canada. The organizers were Terry Beveridge (first row, second from right; kneeling) and Susan Koval (second row, seventh from right; in plaid dress).

The last workshop, on "Structure, Biochemistry and Molecular Biology of Microbial S-Layers," took place in Rothenburg ob der Tauber from September 16 to 20, 1996, and was organized by Helmut König (Fig. 5). The scientific contributions were published in a special issue of *FEMS Microbiol. Rev.* (1997) **20:** Vols. 1 and 2 (International Workshop on Structure, Biochemistry, Molecular Biology and Applications of Microbial S-Layers; H. König and P. Messner, eds.). Since this last focussed meeting of the S-layer research community, new data on S-layer research have been predominantly presented at more general international meetings of the microbiology research community or with regard to the application potential of S-layer systems at meetings focused on nanobiotechnology, self-assembly systems, or synthetic and structural biology.

It is evident from a review of the current literature[371] that enormous advances in studies of the structure, chemistry, genetics, biosynthesis including glycosylation, morphogenesis, and function of bacterial S-layers and their application potential have been achieved.

**Fig. 5.** S-layer workshop, 1996, in Rothenburg ob der Tauber, Germany. *From left to right, first row:* Paul Messner, Uwe Sleytr, Terry Beveridge, Susan Koval, Helmut König (organizer), Lori Graham. *Second row:* Bill Kay, Conway de Macaris, Margit Sara, Christina Schäffer, Seta Küpcü, Eva-Maria Egelseer. *Third row:* Michael Unger, John Smit, Günter Allmaier, Barbara Wetzer, Dietmar Pum. *Fourth row:* Fuller, Peter Pouwels, Sylvie Lortal, Grogow-Thomas, Gerhard Schroll.

Many applications of S-layers depend on the capability of isolated subunits to reassemble into monomolecular arrays in suspension or on suitable surfaces (e.g. polymers, metals, silicon wafers) or interfaces (e.g. lipid films, liposomes, emulsomes). S-layers also represent a unique structural basis and patterning element for generating more complex supramolecular structures involving all major classes of biological molecules (e.g. proteins, lipids, glycans, nucleic acids, or combinations of them). The biomimetic approach, copying the supramolecular building principle of S-layer-associated plasma membranes developed by archaea, offers manifold application potential as far as to novel technologies for the generation of functional lipid membranes and biomimetic virus envelopes. Thus,

S-layers fulfill key requirements as building blocks and patterning elements for the production of new supramolecular materials and nanoscale devices as required in molecular nanotechnology, nanobiotechnology, synthetic biology, and biomimetics.

Nevertheless, many open questions remain, particularly concerning our understanding of the biological significance and evolutionary aspects of S-layers. Most importantly, S-layers are generally part of complex envelope structures and consequently in functional terms should not be considered as an isolated layer. Therefore, it is also tempting to speculate that a simple S-layer like protein membrane with the ability for dynamic growth could have fulfilled all necessary barrier functions and morphogenetic potentials required by a self-reproducing system during the early stage of biological evolution.[46]

For me, one of the most challenging open questions in S-layer research concerns the elucidation of the unique antifouling characteristics and the hydrodynamic surface properties of the (glyco)protein lattices. It has to be assumed that S-layers modify the structure of the first "locked-in" water layer(s). But final proof for this assumption will require studies of the structural details of hydrated S-layers at the atomic level.