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Liposomes coated with crystalline bacterial cell surface protein (S-layer) as immobilization structures for macromolecules

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Abstract

Isolated subunits from the crystalline cell surface layer (S-layer) of *Bacillus coagulans* E38-66 were recrystallized on positively charged liposomes. The liposomes were composed of dipalmitoylphosphatidylcholine/cholesterol and stearylamine. The natural arrangement of the S-layer subunits on the bacterial surface is as an oblique (p2) lattice. The subunits attached to positively charged liposomes by their inner face (which bears a net negative charge) in an orientation identical to the lattice on intact cells. The S-layer protein, once recrystallized on liposomes, was crosslinked with glutaraldehyde and subsequently used as a matrix for the covalent attachment of macromolecules. The high stability of S-layer-coated liposomes and the possibility for immobilizing biologically active molecules on the crystalline array may offer potential in various different liposome applications.

Keywords: Liposome; Surface layer, crystalline; S layer; Cell surface protein; Immobilization; (*B. coagulans*)

1. Introduction

Two-dimensional crystalline surface layers (S-layer) represent the outermost cell envelope component in many bacteria (for reviews see [1–7]). High-resolution electron microscopical studies showed that S-layer lattices exhibit oblique (p2), trigonal (p3), square (p4) or hexagonal (p6) symmetry with spacings of the morphological units of 3 to 30 nm. The lattice type, the centre-to-centre spacing of morphological units and the molecular weight of subunits are strain specific feature of S-layers. S-layer lattices are composed of single proteinaceous or glycoproteinaceous monomers (M_r 40 000–200 000) which generally contain high proportions of acidic and hydrophobic amino acids, and lysine. Isolated S-layer subunits from many organisms reassemble into regularly structured lattices in the presence or absence of supporting layers or interfaces [8,9]. Such S-layer self-assembly products are either monolayers or double layers, and can have the shape of flat sheets, spheres, or open-ended cylinders. Because of their two-dimensional crystalline structure, and because they are composed of identical proteins or glycoproteins, carboxylate and amine groups of S-layers occupy identical positions and display identical orientations on each S-layer subunit [10,11]. The well-defined surface structure and the uniformly distributed functional groups are the basis for using S-layers as immobilization matrices suitable for various biologically active molecules, such as enzymes and affinity ligands [11–14]. S-layer proteins have also been used as matrices and carrier-adjuvants for weakly immunogenic antigens and haptens [15–17].

Freeze-etching preparations from whole cells of *B. coagulans* E38-66 revealed an oblique S-layer lattice which completely covered the cell surface. The base vectors were: $a = 9.4$ nm, $b = 7.4$ nm, $\gamma = 80°$ [18]. On SDS-gels, isolated S-protein from *B. coagulans* E38-66 exhibited an apparent molecular weight of 97 000 [19].

More recently it was shown that isolated S-layer subunits from various *Bacillus* strains, including *B. coagulans* E38-66, have the ability to recrystallize into closed monolayers at the air/water interface or on solid supports pretreated with poly(L-lysine) [9,18]. For S-layer subunits from *B. coagulans* E38-66, it was further demonstrated that binding to the poly(L-lysine) coated surfaces occurred via the more hydrophobic, charge-neutral outer face [18].

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Large-scale recrystallization of S-layer subunits was also possible on phospholipid monolayers consisting of dipalmitoylphosphatidylcholine or dipalmitoylphosphatidylethanolamine [9]. Contrary to the behaviour at the air/water interface, the subunits were attached to the phospholipid layers by their inner face which is negatively charged and which, in intact cells, is associated with the peptidoglycan layer [9,18,20,21].

In this study, we have investigated the recrystallization of the S-protein of *B. coagulans* E38-66 on liposomes and the applicability of the resulting composite structures as carrier for functional macromolecules.

2. Materials and methods

2.1. Organism, growth conditions, cell wall preparation and isolation of S-protein

Bacillus coagulans E38-66 was kindly provided by the Österreichische Zuckerforschungsinstitut, Tulln, Austria. Growth of the organism in continuous culture was performed as described previously [22]. Cell walls were prepared according to the procedure of Sleytr and Glauert [23] except that cells were sonicated [24]. S-protein from cell wall fragments was extracted with guanidinium hydrochloride (GHCl; 5 M in 50 mM Tris-HCl buffer pH 7.2) for 2 h at 20° C [22]. The peptidoglycan-containing cell wall layers were separated from the extracted S-protein by centrifugation at $40\,000 \times g$ for 20 min. Subsequently, S-layer containing GHCl-extracts were dialyzed against 2 mM $CaCl_2$ solution in distilled water for 2 h at 20° C. The S-layer self-assembly products were sedimented for 15 min at $40\,000 \times g$ at 4° C. The clear supernatant was carefully removed and used for recrystallization on liposomes. The S-protein content of supernatants was adjusted to 1 mg per ml with a 2 mM $CaCl_2$ solution. The purity of the extracted S-protein was checked by SDS-PAGE [25].

2.2. Preparation of positively charged liposomes

Unilamellar liposomes were prepared by using the ethanol injection method [26]. The molar ratio of dipalmitoylphosphatidylcholine (DPPC) (Avanti Polar Lipids, Birmingham), cholesterol and stearylamine (Sigma, St. Louis) was 10:5:1. The lipid mixture was dissolved in absolute ethanol heated above the transition temperature of DPPC (T_m) of 41° C, and then incubated at 37° C for 30 min. 250 μl of this solution containing 22 μmol DPPC was rapidly injected with a Hamilton syringe into 3.3 ml KCl solution (160 mM in distilled water) at 37° C. The opalescent solution was cooled to 4° C and sedimented for 30 min at $40\,000 \times g$ at 4° C. To remove the residual ethanol, the pellet was resuspended once in distilled water and sedimented again. The size distribution of liposomes and their integrity was examined by electron microscopy.

2.3. Recrystallization of S-protein on positively charged unilamellar liposomes

1.5 ml of the solution containing 1 mg per ml S-protein was combined with 1 ml of the liposome suspension and samples were incubated at 22° C in a Test Tube Rotator, Type 3025 (GFL, Burgwedel, Germany) with a rotation speed of 10 min^{-1} for 2 h. Recrystallization experiments were performed in 2 mM $CaCl_2$ over a pH range of 5–9, the pH being adjusted with 0.1 M NaOH. Analogous recrystallization experiments were performed in liposome suspensions without removing the ethanol solvent. Recrystallization of S-protein on liposomes was examined by electron microscopy.

2.4. Electron microscopy

Negative staining with unbuffered uranyl acetate (2% in distilled water) and ultrathin sectioning was performed as described before [24]. For freeze etching, small drops of wet pellets obtained by sedimentation of the liposomes at $28\,000 \times g$ for 15 min were deposited on gold discs and frozen by rapid immersion in liquid nitrogen-cooled Freon 22. Freeze fracturing, etching and replication were performed in a Balzers BAF 400 instrument (Balzers, Liechtenstein) [24].

Specimens were examined in a Philips EM 301 transmission electron microscope (TEM) at 80 kV, using a 30 μm objective aperture.

2.5. Crosslinking of the S-protein with glutaraldehyde

After recrystallization of the S-protein on liposome surfaces, crosslinking with glutaraldehyde was performed in order to obtain a more stable, two-dimensional, crystalline protein structure. Crosslinking with glutaraldehyde (2.5% in 0.1 M phosphate buffer, pH 7.2) was performed at 20° C for 20 min. The formation of covalent bonds between S-layer subunits was confirmed by extracting the liposomes with an SDS-solution (5% in distilled water) for 10 min at 100° C. After centrifugation at $28\,000 \times g$ for 15 min at 20° C, the clear supernatant was applied to SDS gels.

2.6. Activation of carboxylate groups from the S-protein with carbodiimide and covalent attachment of ferritin

After crosslinking the recrystallized S-protein with glutaraldehyde and washing with distilled water, free carboxylate groups on the S-protein were activated with 1-ethyl-3,3′-(dimethylaminopropyl)carbodiimide (EDC) [27] for 60 min at 20° C. Subsequently, activated samples were incubated over night with 1 ml of ferritin solution (Sigma, St. Louis, MO, 1 mg per ml distilled water) while rotating the tubes at 20° C. After removing excess of ferritin by centrifugation at $28\,000 \times g$ and washing under the same condi-

tions for 15 min at 20° C, covalent attachment ferritin to the S-protein was examined by the TEM.

3. Results

3.1. Liposome preparation

Liposomes can be prepared from a variety of lipids by several methods [28]. For our study, we used the ethanol injection method and a molar ratio of DPPC/cholesterol/stearylamine of 10:5:1. Cholesterol was incorporated into bilayers of DPPC in order to obtain stable membranes, an important feature if liposomes are to be exposed to biological fluids. Stearylamine as hydrophobic amine was added to confer to the liposomes a net positive surface charge. The type, diameter and size distribution of the liposomes were examined by electron microscopy using negative staining preparations. As shown in Fig. 1a, the liposomes are of the unilamellar (single bilayer) type which was also confirmed in cross fractures of freeze fractured preparations (not shown). The diameter of the vesicles ranged from 100 to 300 nm. To guard against preparation artefacts such as specimen shrinkage, or damage which might occur in the course of negative staining, the integrity of liposomes was also examined in freeze-etched preparations. According to the electron microscopical data, the liposomes were stable for a least one week in KCl solution (not shown).

3.2. Recrystallization of S-protein on liposomes

During dialysis of GHCl-S-protein extracts against 2 mM $CaCl_2$ for 2 h at 20° C, approx. 20% of total S-protein reassembled into flat sheets or open-ended cylinders with a maximum size of 5 μm as previously shown [18]. According to thin-section preparations and optical diffraction analyses of negatively stained preparations, all self-assembly products were monolayers [18]. After removing the self-assembly products by centrifugation, the clear supernatant contained about 80% of the original S-protein [29]. These non-assembled S-layer subunits were used for the recrystallization experiments on liposomes. The following observations were made using negative stained preparations. Within 30 min after adding the S-protein to the liposomes, patches of oblique S-layer lattice characteristic for *B. coagulans* E38-66 had formed on the surface of the lipid vesicles (Fig. 1b). After 1 h incubation, amorphous regions were still visible between the differently-oriented, crystalline arrays. This indicated that binding of the S-layer subunits to the liposome surface had occurred but rearrangement into regular arrays was not yet complete. After one more hour, the liposomes were completely covered with a coherent layer of the oblique S-layer lattice (Fig. 1c and d). Most S-layer patches attained a size of 50 nm and displayed different orientations on individual liposomes, indicating that crystal growth had started at numerous nucleation sites [21]. The S-layer coated, 100–300 nm-sized liposomes maintained their spherical shape, indicating that the S-layer lattice is flexible enough to follow the spherical surface structure during growth. Recrystallization of coherent S-layers was observed in a pH range of 5–9.

Freeze-etched preparations revealed that the oblique S-layer lattice on liposomes has an orientation identical with the one observed on the surface of intact cells (Fig. 1e).

Using ultrathin-section preparations, we demonstrated that the S-layer lattice on the surface of the liposomes is a monolayer with a thickness of 4–5 nm (Fig. 2a). This corresponds exactly to the dimension determined for S-layers on intact cells of *B. coagulans* E38-66 and S-layers reconstituted on to solid supports [18]. Since no crystallization of S-layer subunits has been observed on liposomes prepared without stearylamine (not shown), it can be concluded that binding and crystallization of S-protein on the liposomes requires electrostatic interactions between the amine groups exposed on the bilayer and the carboxylate groups on the inner face of the S-layer subunits.

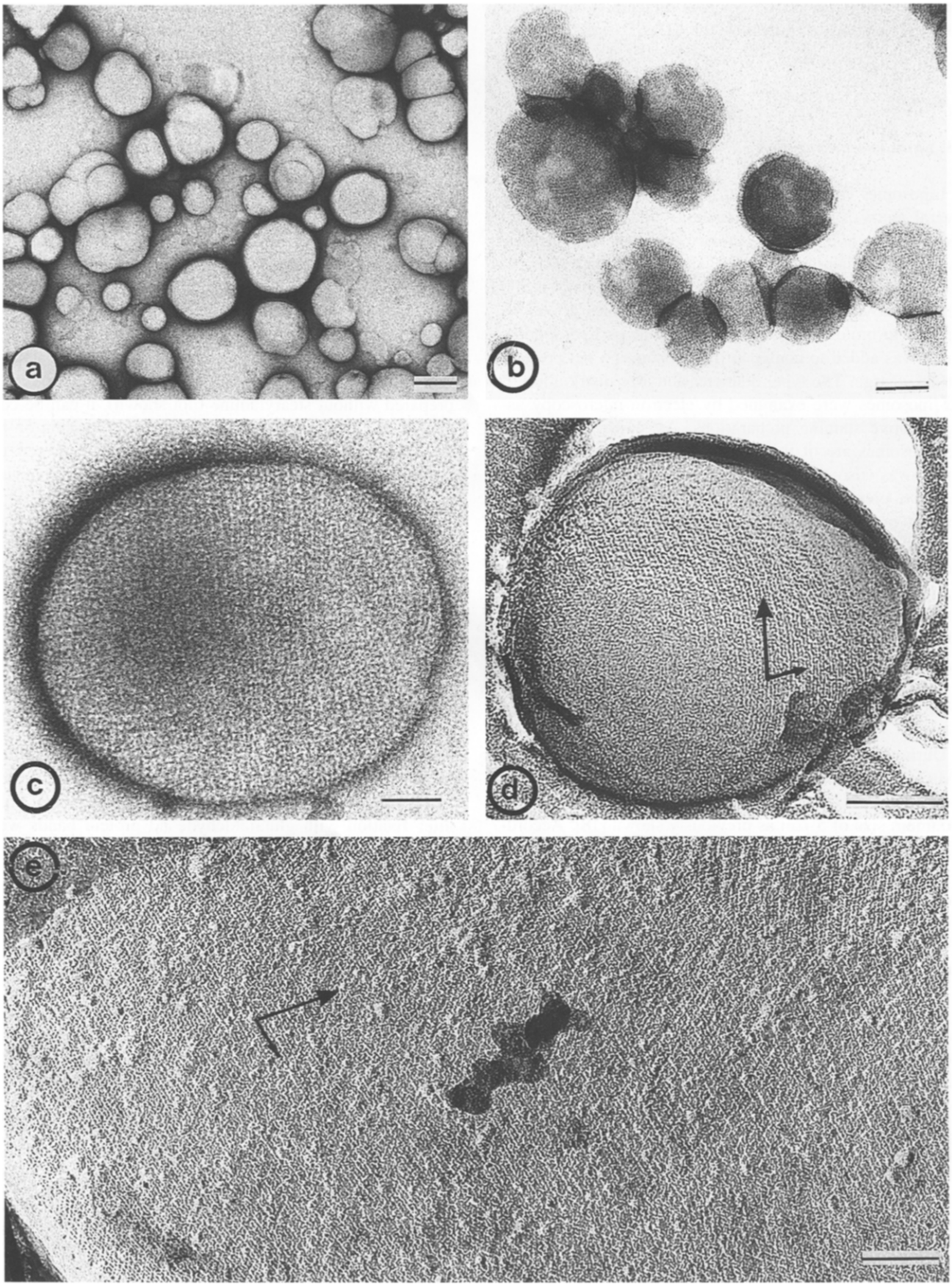
3.3. Crosslinking of S-protein on liposomes

In previous studies, it was demonstrated that charged amine and carboxylate groups are required for the structural integrity of the native S-protein of *B. coagulans* E38-66 [30]. To avoid disintegration of the recrystallized S-layer lattices during pH shifts, as required for many procedures to activate functional groups for the covalent attachment of macromolecules, the S-protein was crosslinked with glutaraldehyde. On 10% SDS gels, liposomes coated with native S-protein yielded one protein band with an apparent molecular weight of 97000. Since after fixation with glutaraldehyde no protein bands were detected on SDS gels (data not shown), complete crosslinking of S-layer subunits must have occurred.

3.4. Activation of carboxylate groups of the S-protein and covalent attachment of ferritin

In previous studies [11–14,31] it was shown that EDC selectively and efficiently activates carboxylate groups of S-proteins in aqueous media. With this procedure polymerization of the immobilized protein can be excluded because activation and immobilization occur during separate steps.

To demonstrate that S-layers recrystallized on liposomes can act as a matrix for covalent attachment of macromolecules, ferritin was chosen as a model system because it can easily be visualized by electron microscopical procedures. In previous experiments ferritin (p*I* 4.3) has been shown not to adsorb to the S-protein of *B. coagulans* E38-66 crosslinked with glutaraldehyde [18]. Covalent binding of ferritin molecules to activated carbox-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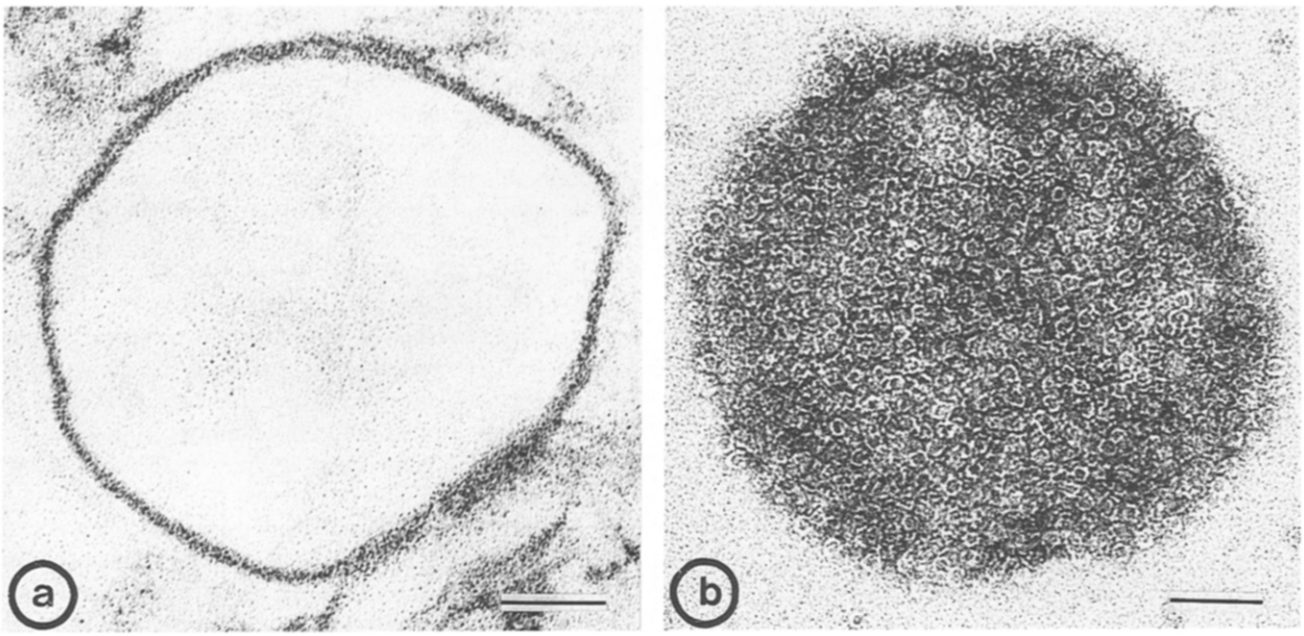

Fig. 2. (a) Thin-section preparation of S-protein coated liposome, after crosslinking with glutaraldehyde. The coherent S-layer monolayer can be detected. (b) Negative-staining of liposome coated with S-protein and covalent attachment of electron dense ferritin molecules. Bars, 50 nm in panels a and b.

ylate groups of glutaraldehyde-treated S-layers was demonstrated by negative staining. As shown in Fig. 2b the covalently bound ferritin molecules are arranged as monolayer on the oblique S-layer lattice on the liposome surface. Each ferritin molecule (diameter 12 nm) covers at least two morphological units of the oblique S-layer lattice, which are composed of dimers [18].

4. Discussion

In the course of structural, chemical and assembly studies crystalline bacterial S-layers have been shown to be highly anisotropic with respect to their inner and outer face (for reviews see [1,3–5,7,32]). For S-layers from gram-positive eubacteria, including the *Bacillus coagulans* strain examined in this study, the S-layer subunits were shown to be linked to each other and to the underlying peptidoglycan layer by non-covalent interactions including hydrogen bonding, direct electrostatic interactions between free amine and carboxylate groups, salt bridges, and hydrophobic bonds [8]. Furthermore, during disassembly and recrystallization studies on native and isolated S-layers the bonds generating the two-dimensional protein arrays were shown to be stronger than those binding the S-layer to the supporting envelope structure [33,8]. These characteristics are seen as essential for a dynamic assembly and a continuous recrystallization process of coherent S-layer lattices on the surface of growing bacterial cells [23].

The S-layer of *B. coagulans* E38-66 was selected for coating liposomes because: (i) the inner face of the crystalline array exhibits a net negative charge due to excess free carboxylate groups [30], (ii) the outer face is more hydrophobic than the inner face [18], and (iii) S-protein from this organism recrystallize with their inner surface into large coherent monolayers on DPPE and DPPC monolayers spread on Langmuir-Blodgett (LB) troughs and with their outer surface at the air/water interface [9].

The liposomes used in the present study exhibited a net positive surface charge due to incorporation of stearylamine into the bilayers. Consequently, binding of the S-layer subunits occurred as predicted from LB experiments, [9] with the inner negatively charged surface of the S-protein binding to the bilayer. Lattice growth on the surface of the liposomes was observed in the pH range from 5 to 9 when the amine groups of stearylamine are

Fig. 1. (a) Negative staining of liposomes composed of dipalmitoylphosphatidylcholine/cholesterol and stearylamine and produced by ethanol injection method. The size distribution ranged from 100 to 300 nm. (b) Negative-staining of liposomes coated with patches of S-protein. (c) Negative-staining of S-protein from *B. coagulans* E38-66 recrystallized on positively charged liposomes exhibiting an oblique (p2) S-layer lattice. (d) Freeze-etching preparation of liposome which exhibits a complete covering with an oblique (p2) lattice. The vector pair indicates the orientation of the S-layer on the liposome which is identical to the crystalline array on intact cells (compare with Fig. 1e). (e) Freeze-etched preparation of whole cells from *B. coagulans* E88-66, exhibiting an oblique (p2) S-layer lattice with a typical crazy paving appearance. The vector pair indicates the orientation of the lattice. Bars, 200 nm in panel a, 100 nm in panels b, d, e and 50 nm in panel c.

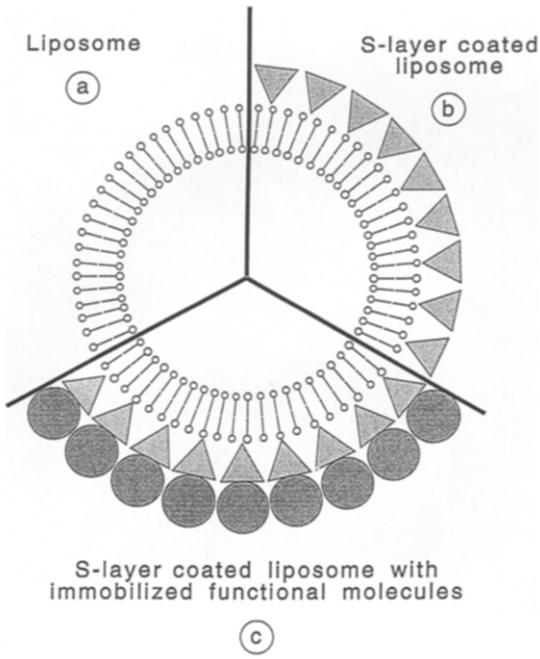

Fig. 3. Schematic drawing of a liposome (a), coated with an S-layer lattice (b) which is used as matrix for immobilizing functional macromolecules (c).

positively charged. Since recrystallization of S-layer subunits was not observed in the absence of stearylamine, it can be concluded that the defined attachment of the S-layer protein to the liposomes was governed primarily by electrostatic interactions. In the course of S-layer growth, no changes in the overall morphology of the liposomes occurred (Fig. 1b–d) which can be explained by the high flexibility of the *B. coagulans* E38-66 S-layer lattice. This notion is supported by the observation that, in freeze-etched preparations of intact cells of *B. coagulans* E38-66, numerous lattice distortions occur and numerous lattice faults are present at the rounded surfaces of the cell poles [36]. S-layer-like proteins which possess hydrophobic domains acting as an anchor in the lipid matrix were previously shown to induce remarkable morphological changes in the lipid vesicles [34].

Langmuir-Blodgett techniques revealed that lipid films associated with S-layers from gram-positive eubacteria are much more stable than unsupported mono- or bimolecular lipid layers [9,21]. Such S-layer-supported films strongly resemble the cell envelopes of simple walled archaebacteria (Fig. 3) which are exclusively composed of S-layers and closely associated lipid membranes [4,5,7,35–38]. Since many of these organisms exist under the most extreme environmental conditions (e.g., high temperature, low pH, high salt concentration), two-dimensional arrays of proteins must have a considerable stabilizing effect on lipid membranes. The reason for that could be the reduction in horizontal vibrations, considered to be the main cause for disintegration of unsupported lipid membranes.

Crosslinking of membrane-associated S-protein with glutaraldehyde led to even more stable composite structures. This stabilizing effect, first observed on LB-lipid films [21], was exploited in the present study for increasing the mechanic stability of the S-layer lattice during the process of covalent binding of ferritin. Since glutaraldehyde solutions usually contain monomers and polymers of different lengths, different distances of two amine groups either on the S-layer lattice or on the liposome membrane will not be a hindrance for cross-linking. It can be expected that stearylamine molecules (of the outer monolayer of liposomes) covalently linked to the S-protein will modulate the lateral diffusion of the free lipid molecules [10]. Such postulated changes in the fluidity of liposome membranes could be important if lipid vesicles are used as encapsulation or delivery systems.

The immobilization and chemical modification potential of S-layer lattices has been well studied in context of using S-layers as isoporous ultrafiltration membranes and as matrices for immobilizing functional molecules [11,31]. Since S-layers are highly porous structures with well-defined topographies, functional groups such as carboxylate, amine and hydroxyl groups occur on each constituent protein subunit (including the pore areas) in identical positions and orientations [39]. These unique features allow binding of molecules to the crystalline array in a reproducible and geometrically well-defined manner. Since liposomes have a broad application potential as carriers and drug delivery systems, the possibility for modulating the surface properties, particularly the surface charge, appears very important. In comparison to naked liposomes, S-layer coated vesicles will enable a more defined and wider choice of surface properties. It is also known that charged liposomes bind to many cell types more effectively than neutral liposomes. In the present study, ferritin was used as a model system for demonstrating the suitability of S-layers attached to liposomes as immobilization matrices because this macromolecule does not penetrate into S-layer pores [20] and can be easily detected by electron microscopical procedures.

It is hoped, that the possibility of recrystallizing S-layers on liposomes and of using the protein lattice as a matrix for modifying the surface properties of the liposomes and to immobilize functional macromolecules will open a wide spectrum of applications in basic and applied liposome research.

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