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S-layer-coated liposomes as a versatile system for entrapping and binding target molecules

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Abstract

In the present study, unilamellar liposomes coated with the crystalline bacterial cell surface layer (S-layer) protein of *Bacillus stearothermophilus* PV72/p2 were used as matrix for defined binding of functional molecules via the avidin– or streptavidin–biotin bridge. The liposomes were composed of dipalmitoyl phosphatidylcholine, cholesterol and hexadecylamine in a molar ratio of 10:5:4 and they had an average size of 180 nm. For introducing specific functions into the S-layer lattice without affecting substances encapsulated within the liposomes, crosslinking and activation reagents had to be identified which did not penetrate the liposomal membrane. Among different reagents, a hydrophilic dialdehyde generated by periodate cleavage of raffinose and a sulfo-succinimide activated dicarboxylic acid were found to be impermeable for the liposomal membrane. Both reagents completely crosslinked the S-layer lattice without interfering with its regular structure. Biotinylation of S-layer-coated liposomes was achieved by coupling *p*-diazobenzoyl biocytin which preferably reacts with the phenolic residue of tyrosine or with the imidazole ring of histidine. By applying this method, two biotin residues accessible for subsequent avidin binding were introduced per S-layer subunit. As visualized by labeling with biotinylated ferritin, an ordered monomolecular layer of streptavidin was formed on the surface of the S-layer-coated liposomes. As a second model system, biotinylated anti-human IgG was attached via the streptavidin bridge to the biotinylated S-layer-coated liposomes. The biological activity of the bound anti-human IgG was confirmed by ELISA.

Keywords: Crystalline bacterial surface layer; S-layer; Liposome; Immobilization

1. Introduction

Crystalline bacterial cell surface layers (S-layers) represent the outermost cell envelope component of many bacteria and archaea [1–3]. S-layers are composed of identical protein or glycoprotein subunits

Abbreviations: anti-human IgG-(BS^3)SLs, biotinylated anti-human IgG bound to biotinylated (BS^3)SLs via StA; BS^3, bis(sulfosuccinimidyl)suberate; BS^3SLs, SLs treated with BS^3; (BS^3)SLs, both SLs and BS^3SLs; CA, carbonic anhydrase; CALs, CA-containing liposomes; DBB, *p*-diazobenzoyl biocytin; DPPC, dipalmitoyl phosphatidylcholine; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; ELISA, enzyme-linked immunosorbent assay; ferritin-(BS^3)SLs, biotinylated ferritin bound to biotinylated (BS^3)SLs via StA; HDA, hexadecylamine; PBS, phosphate-buffered saline; SCALs, S-layer-coated CALs; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; S-layer, surface layer; SLs, S-layer-coated liposomes; StA, streptavidin

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which assemble into either oblique, square or hexagonal lattices. In bacteria, the subunits interact with each other and with the supporting cell envelope layer by non-covalent forces [1,2,4]. Thus S-layer lattices from Gram-positive bacteria can be disintegrated into their constituent subunits by treatment with high concentrations of hydrogen-bond-breaking agents, such as 5 M guanidine hydrochloride [3]. During removal of the disrupting agents (e.g. by dialysis) the isolated S-layer subunits frequently reassemble into the same regularly structured lattices as observed on whole bacterial cells. Recrystallization of the S-layer subunits into closed lattices was also observed on suitable surfaces such as poly-L-lysine-coated supports, Langmuir (L) lipid films, liposomes or at the air/water interface [4–8]. The stabilizing effect of an S-layer lattice on lipid films (lipid bilayers and tetraetherlipid monolayers) as well as on liposomes has recently been demonstrated [9–11].

Due to the periodic structure of S-layer lattices, functional groups of amino acid side chains are arranged in defined and regularly repeated positions and orientations. Thus S-layers were exploited as matrix for the immobilization of enzymes, antibodies or ligands and most covalently bound macromolecules formed a monolayer on the surface of the S-layer lattice [7,12,13]. In the case of S-layer-coated liposomes (SLs), ferritin, a large marker molecule for electron microscopic investigations, was bound to the S-layer lattice [8].

In most immobilization studies, carboxylic acid groups of the acidic amino acids of the S-layer subunits were activated with carbodiimide. To prevent intra- and intermolecular crosslinking reactions of S-layer subunits during the activation procedure, free amino groups from lysine were first blocked by crosslinking the S-layer lattice with glutaraldehyde. In the present study, the S-layer protein from *Bacillus stearothermophilus* PV72/p2 was used for recrystallization on positively charged unilamellar liposomes composed of dipalmitoyl phosphatidylcholine (DPPC), cholesterol and hexadecylamine (HDA) in a molar ratio of 10:5:4. The S-layer lattice from this organism shows oblique symmetry and is composed of subunits with a molecular weight of 97 000. The lattice constants are $a = 9.7$ nm, $b = 7.4$ nm and $\gamma = 80°$. The gene encoding the S-layer protein (*sbsB*) has been cloned and sequenced [14]. According to the amino acid sequence, the isoelectric point of the mature S-layer protein is 5.0.

The aim of the present work was to establish universal modification and immobilization procedures for binding macromolecules to the S-layer lattice on liposomes without interfering with encapsulated material as required for further biological applications. Thus, reagents had to be identified that did not penetrate the liposomal membrane, but efficiently crosslinked the recrystallized S-layer protein and introduced specific functions without destroying the regular lattice structure.

2. Materials and methods

2.1. Liposome preparation

Liposomes composed of DPPC (Avanti Polar Lipids, Alabaster, AL, USA), cholesterol (Sigma, St. Louis, MO, USA) and HDA (Fluka, Buchs, Switzerland) in a molar ratio of 10:5:4 were prepared by applying the dehydration–rehydration method as previously described [11]. For preparation of carbonic anhydrase (CA) containing liposomes (CALs) rehydration of lyophilized liposome preparations containing 20 μmol DPPC was carried out by adding 250 μl of a CA solution (35 mg/ml in 160 mM KCl), which was followed by repeated extrusion through symmetric polycarbonate membranes with decreasing pore size (400, 200 and 100 nm) using a LiposoFast mini extruder (Avestin, Ottawa, Canada). CALs were separated from non-encapsulated protein by column chromatography on a PD-10 column using Sepharose CL-4B (Pharmacia, Uppsala, Sweden) equilibrated with 160 mM KCl. The concentration of DPPC in liposome containing suspensions was determined by the Steward assay for phospholipids [15]. SLs and S-layer-coated CALs (SCALs) were prepared as recently described [11].

2.2. Crosslinking of the S-layer lattice on liposomes and carbodiimide activation of free carboxylic acid groups

The S-layer lattice on SCALs was crosslinked with glutaraldehyde under conditions given in [8]. Carboxylic acid groups were activated with 1-ethyl-3-

(3-dimethylaminopropyl)carbodiimide (EDC) as described in [8].

For preparing membrane impermeable aldehydes which were capable of reacting with free amino groups of the S-layer protein, vicinal hydroxyl groups from raffinose were oxidized with periodate. For this purpose, 360 mg raffinose pentahydrate (Merck, Darmstadt, Germany) was dissolved in 3 ml of 400 mM sodium metaperiodate (Merck, Darmstadt, Germany) in distilled water and the pH value was adjusted to 5.0. Oxidation was carried out for 3 h whilst stirring in the dark at 22°C. The reaction mixture was passed through a 0.5×15 cm column filled with 1.5 ml strong cation exchanger (AG 50W-X4; H^+-form; Bio-Rad, Hercules, CA, USA) and 1.5 ml strong anion exchanger (AG1-X8; OH^--form; Bio-Rad, Hercules, CA, USA). Fractions of 1 ml were collected and checked for absence of JO_3^- and JO_4^- by non-appearance of precipitation with silver ions in acidic solution. Aldehyde containing fractions as indicated by their UV absorption at 290 nm were pooled. The concentration was adjusted with distilled water in a way that after 1:10 dilution an absorbance of about 1.0 was obtained. Subsequently, 13 mg sodium bicarbonate (Fluka, Buchs, Switzerland) was added per ml. Three ml of this solution were immediately incubated with 1 ml of SCAL suspension (4 μmol DPPC/ml freshly prepared 150 mM sodium bicarbonate solution) for 2 h at 22°C in a Test Tube Rotator, Type 3025 (GFL, Burgwedel, Germany). Subsequently, 250 μl of a freshly prepared sodium borohydride solution (40 mg/ml 0.1 M NaOH) were added. After reduction for 1 h at 22°C, the liposomes were sedimented for 15 min at $18\,000 \times g$ at 4°C.

Crosslinking of the S-layer lattice on SCALs or SLs with bis(sulfosuccinimidyl)suberate (BS^3; Pierce, Rockford, IL, USA) was performed at a liposome concentration corresponding to 5 μmol DPPC/ml 50 mM phosphate buffer, pH 7.5, with 0.75 mg BS^3/ml at 22°C in a Test Tube Rotator. After 1 h reaction time, the suspension was centrifuged at $36\,000 \times g$ for 20 min at 4°C and the liposomes were washed with 50 mM phosphate buffer, pH 7.5.

The effect of several crosslinking or chemical modification reactions on SCALs was investigated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using the Mini Protean-II electrophoresis apparatus (Bio-Rad, Hercules, CA, USA) on 10% T, 3% C gels under non-reducing conditions. Before application to SDS-gels, the differently modified SCALs were boiled in 5% SDS solution for 10 min. Protein bands were visualized by Coomassie staining.

2.3. Isolation of HDA covalently linked to the S-layer protein in SLs treated with BS^3

A suspension of SLs treated with BS^3 (BS^3SLs) containing 1 μmol DPPC was sedimented at $16\,000 \times g$ for 10 min at 22°C. The pellet was extracted twice with 1 ml Triton X-100 solution (2% in distilled water) for 5 min at 100°C and once with 1 ml distilled water and absolute ethanol, respectively, at 22°C. The remaining pellet was subsequently hydrolyzed with 6 N HCl at 110°C for 12 h under nitrogen atmosphere and was then extracted three times with 1 ml chloroform. Extracts were pooled and concentrated to 100 μl in a rotary evaporator. For comparative analysis, liposomes were treated with BS^3 before coating with S-layer protein and were investigated in the same way.

Thin layer chromatography was performed on silica gel 60 TLC aluminium sheets (Merck, Darmstadt) and plates were developed in chloroform:acetone:methanol:acetic acid:water (6:8:2:2:1, v/v) [15]. Spots were visualized with ninhydrin spray reagent.

2.4. Biotinylation of SLs or BS^3SLs

p-Diazobenzoyl biocytin (DBB) was freshly prepared from *p*-aminobenzoyl biocytin (Pierce, Rockford, IL, USA) according to the manufacturer's protocol and diluted with the 3-fold volume of distilled water. Both, SLs and BS^3SLs, together termed (BS^3)SLs, were suspended in 0.2 M borate buffer, pH 8.4, to contain 6 μmol DPPC/ml and were incubated with an equal volume of DBB solution for 2 h at 22°C in a Test Tube Rotator. Suspensions were subsequently dialyzed against phosphate-buffered saline (PBS; 10 mM phosphate, pH 7.2, containing 138 mM NaCl and 2.7 mM KCl). Biotin residues coupled to the S-layer lattice and being accessible for subsequent avidin (Sigma, St. Louis, MO, USA) binding were determined fluorometrically as described in [16].

2.5. Binding of biotinylated ferritin or anti-human IgG to biotinylated (BS^3)SLs via StA

Biotinylated (BS^3)SLs were mixed with the 4-fold volume of streptavidin (StA; Sigma, St. Louis, MO, USA) solution (0.5 mg/ml PBS) and incubated for 30 min at 22°C. After removing unbound StA by centrifugation at $8000 \times g$ for 10 min at 4°C and washing the liposomes once with PBS, the pellet was resuspended in PBS to achieve a concentration corresponding to about 3 μmol DPPC/ml. This suspension was subsequently incubated with an equal volume of a solution containing either 8 mg/ml biotinylated ferritin (F-3652, Sigma, St. Louis, MO, USA) or 0.23 mg/ml anti-human IgG (γ-chain specific) biotin conjugate (goat; Sigma, St. Louis, MO, USA) in PBS for 30 min at 22°C. Liposomes were sedimented at $8000 \times g$ for 10 min at 4°C and washed twice with PBS. In the following, these liposome preparations are referred to as ferritin-(BS^3)SLs and anti-human IgG-(BS^3)SLs, respectively.

2.6. Electron microscopy

Ultrathin-sectioning and negative-staining of liposomes was done as previously described [17]. Specimens were examined in a Philips CM 100 transmission electron microscope (TEM) at 80 kV using a 30-μm objective aperture.

2.7. Testing the biological activity of anti-human IgG-(BS^3)SLs using an enzyme-linked immunosorbent assay (ELISA)

Microtiter plates (96 well; Nunc-Immuno Plate MaxiSorp) were coated with 500 ng human IgG (Sigma, St. Louis, MO, USA) per well (100 μl, 5 μg/ml 0.1 M carbonate buffer, pH 9.5) overnight at 4°C. After washing three times with PBS, excess binding sites were saturated with 200 μl blocking solution (1% hämopearl, bovine serum albumin, Hämosan, Graz, Austria, in PBS) for 1 h at 22°C. The plates were washed again three times with PBS and incubated with 100 μl of dilution series from anti-human IgG-(BS^3)SLs, biotinylated SLs with bound StA (all 200–0.2 nmol DPPC/ml) and human IgG (γ-chain specific) biotin conjugate (1600–1.6 pmol/ml) in blocking solution for 1 h at 22°C. After washing the plates three times with PBS, 100 μl of anti-goat alkaline phosphatase conjugate (Sigma, St. Louis, MO, USA) diluted 1:5000 in blocking solution were added to each well for 1 h at 22°C. The plates were washed as described before and color was developed by adding 150 μl of *p*-nitrophenyl phosphate solution (Sigma, St. Louis, MO, USA; one tablet in 20 ml 0.1 M glycine buffer, pH 10.4, containing 1 mM magnesium chloride and 1 mM zinc chloride). The plates were read after 25 min at 405 nm (with 492 nm reference wavelength) on an Easy Reader EAR 400AT (SLT-Labinstruments, Gröding, Austria).

3. Results

3.1. Effect of crosslinking or carbodiimide activation of S-layer subunits from SCALs on encapsulated CA

To ensure that the applied modification and immobilization procedures are not interfering with subsequent biological applications of SLs, the most commonly used reagents for activating functional groups or for crosslinking the S-layer lattice (EDC and glutaraldehyde) were investigated regarding their penetration through the liposomal membrane composed of DPPC, cholesterol and HDA. For this purpose, SCALs were used as model system and chemical modification or crosslinking of the S-layer protein and enclosed CA was determined by SDS-PAGE. As shown in Fig. 1, in comparison to untreated SCALs (Fig. 1a), application of EDC (Fig. 1e) resulted in the complete disappearance of the monomeric S-layer protein band (molecular mass 97 000) and in a significantly reduced intensity of the CA protein band (molecular mass 31 000) with a slightly reduced molecular mass of the latter. On the other hand, high molecular mass protein bands (molecular mass $\geq 10^6$) became visible on SDS-gels. The disappearance of the monomeric S-layer protein band revealed that EDC-activated carboxylic acid groups had reacted with free amino groups from adjacent S-layer subunits leading to the introduction of peptide bonds between them. An identical pattern of high molecular mass protein bands on SDS-gels was obtained when suspended S-layer self-assembly products were treated with EDC (not shown) which

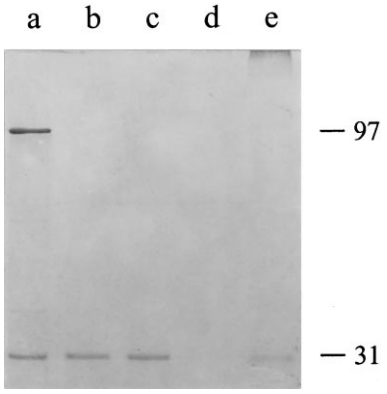

Fig. 1. Effect of different crosslinking and activation procedures on SCALs. Coomassie stained SDS-PAGE gels of SCALs (a) native and after treatment with (b) periodate oxidized raffinose, (c) BS^3, (d) glutaraldehyde and (e) EDC. Molecular weights given are multipliers of 1000.

confirmed that in case of SCALs, encapsulated CA was not involved in the formation of the high molecular mass protein bands. The decreased intensity and slightly reduced molecular mass of the CA protein band further indicated modification and partial polymerization of the enclosed protein which was attributed to the passage of EDC through the liposomal membrane. After treatment with glutaraldehyde (Fig. 1d), neither the S-layer protein nor the CA protein band was visible on SDS-gels which was contributed to complete crosslinking of the S-layer lattice and of CA inside the liposomes. By contrast, the use of periodate oxidized raffinose (Fig. 1b) or BS^3 (Fig. 1c) resulted in complete crosslinking of the S-layer lattice without affecting encapsulated CA. As demonstrated by negative-staining, with all crosslinking reagents, the regular structure of the S-layer lattice on liposomes was well preserved (not shown).

3.2. *Formation of covalent linkages between the S-layer subunits and membrane incorporated HDA during crosslinking of the S-layer lattice on SLs with BS^3*

For investigating whether HDA molecules were covalently linked to the S-layer subunits during crosslinking of the S-layer lattice on SLs with BS^3, unbound lipids were first removed from BS^3SLs by detergent extraction. After acidic hydrolysis, HDA was extracted with chloroform and samples were analyzed by thin layer chromatography. For control experiments, liposomes pretreated with BS^3 and subsequently coated with S-layer protein were used. Since HDA spots were only visible for BS^3SLs (data not shown), heterologous crosslinking between the S-layer protein and membrane incorporated HDA must have occurred during fixation of the S-layer lattice with BS^3.

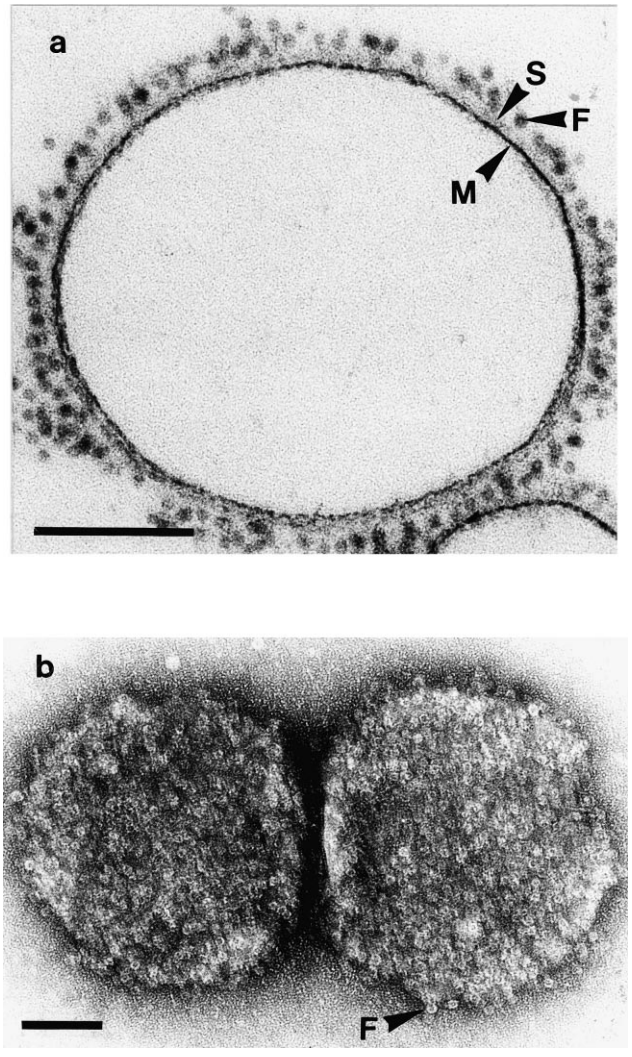

Fig. 2. Electron micrographs of biotinylated SLs with electron dense biotinylated ferritin bound via StA bridges. (a) Ultrathin-sectioned preparation. (b) Negatively stained preparation. S, S-layer; M, liposomal membrane; F, ferritin. Scale bars: 100 nm.

3.3. Biotinylation of (BS^3)SLs

To introduce biotin residues into the S-layer lattice on (BS^3)SLs, diazocoupling of DBB was performed. In general, the arylic diazo group mainly reacts with the phenolic residue of tyrosine and the imidazole residue of histidine [18], but reactions with lysine amines can also occur [19]. Since the native charge distribution is required for maintaining the structural integrity of S-layer lattices, DBB seemed to be well suited for biotinylation of both, SLs and BS^3SLs. In the latter, lysine residues were already blocked due to crosslinking of the S-layer lattice with BS^3. Different molar ratios (e.g. 5:1, 25:1, 60:1) of DBB to S-layer protein were applied, but the molar ratio of about 25:1 was found to be sufficient for diazocoupling to the S-layer lattice with maximal avidin binding capacity. Higher ratios (e.g. 60:1) resulted in precipitation of liposomes during removing excess reagent by dialysis against PBS. The amount of biotin residues which was introduced into the S-layer lattice and was accessible for avidin binding was determined to be about 11 nmol/μmol DPPC for both, biotinylated SLs and biotinylated BS^3SLs. Since SLs contained 5.7 nmol S-layer protein/μmol DPPC [11], about two biotin residues per S-layer subunit were accessible for avidin binding. Lowering the DBB to S-layer protein molar ratio during diazocoupling resulted in a decrease of accessible biotin residues (e.g. 5 nmol/μmol DPPC). The integrity of the S-layer lattice on both, biotinylated SLs and biotinylated BS^3SLs, was demonstrated by electron microscopy of negatively-stained samples. In both preparations, the oblique S-layer lattice was clearly visible (not shown).

3.4. Attachment of biotinylated macromolecules via StA to biotinylated (BS^3)SLs

To demonstrate that biotinylated (BS^3)SLs are capable of binding biotinylated molecules via StA bridges, biotinylated ferritin, a marker molecule for electron microscopy, was chosen. Electron microscopic investigations of ultrathin-sectioned preparations (Fig. 2a) revealed that incubation of biotinylated (BS^3)SLs with StA and biotinylated ferritin resulted in the formation of a monolayer of densely arranged ferritin molecules on the liposome surface. Negative-staining (Fig. 2b) further demonstrated that the bound ferritin molecules roughly resembled the oblique lattice structure. Since the molecular size of ferritin is 12 nm, a single molecule occupies up to two subunits of the oblique S-layer lattice.

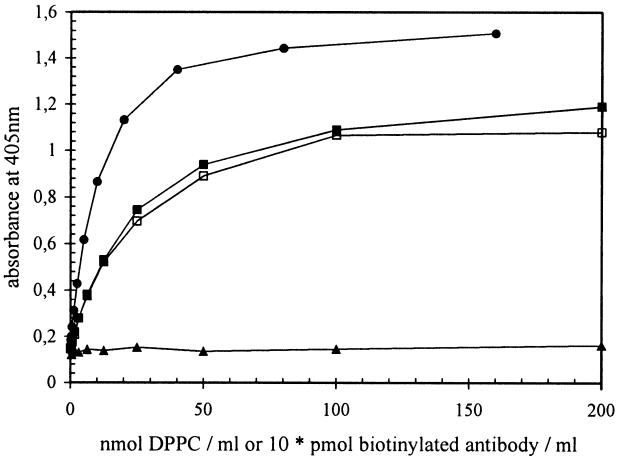

Fig. 3. Influence of attaching biotinylated anti-human IgG via StA to biotinylated (BS^3)SLs on their binding to ELISA plates precoated with human IgG. Absorbance values at 405 nm are plotted versus different concentrations of (■) anti-human IgG-SLs, (□) anti-human IgG-BS^3SLs, (▲) biotinylated SLs with bound StA (given in nmol DPPC/ml) and (●) biotinylated anti-human IgG (given in 10×pmol antibody/ml).

3.5. Specific recognition and binding of anti-human IgG-(BS^3)SLs to human IgG

One of the most effective ways for specific ligand-mediated targeting of liposomes to cells is through high affinity binding of antibodies to their antigens [20]. For this purpose, anti-human IgG was used as a model molecule for the attachment of a targeting ligand to (BS^3)SLs via the avidin–biotin system. The biological activity of attached anti-human IgG was evaluated through the functional competence to specifically recognize and bind to human IgG which was used for coating ELISA plates. In Fig. 3, the concentrations of anti-human IgG-(BS^3)SLs, biotinylated SLs with bound StA and free biotinylated anti-human IgG is plotted versus the absorption values at 405 nm. For both anti-human IgG-SLs and anti-human IgG-BS^3SLs clear correlations were obtained between the liposome concentration and the ELISA signal indicating the suitability of the liposomes to recognize and to bind to the antigen. The shape of the absorption curves resembled that of free biotiny-

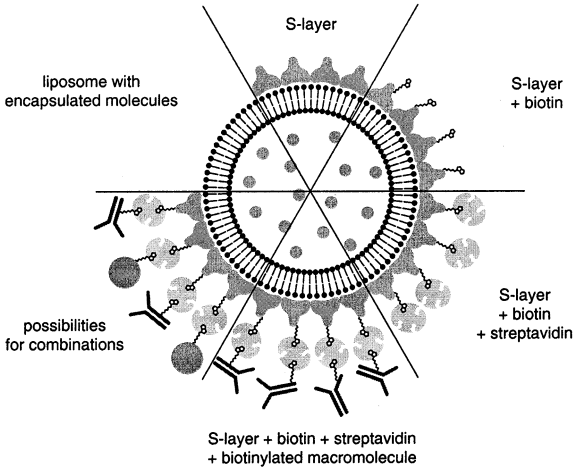

Fig. 4. Schematic drawing clockwise illustrating the setup of biotinylated SLs with bound StA and their use for the attachment of biotinylated macromolecules.

lated anti-human IgG. In comparison to anti-human IgG-BS^{3}SLs, slightly higher absorption values were obtained for anti-human IgG-SLs, but the effect of crosslinking the S-layer lattice with BS^3 before biotinylation on the ELISA signal was very modest. The control samples in which biotinylated SLs with bound StA were used were not recognized in this assay. These data confirmed that all steps involved in the preparation of anti-human IgG-(BS^3)SLs could be successfully combined to achieve the desired functionality.

4. Discussion

For preparing SLs for biological applications, such as drug delivery or immunodiagnostic assays, the question had to be answered which crosslinking and modification reagents (glutaraldehyde and EDC) penetrate through the liposomal membrane and react with encapsulated substances. Since the CA concentration inside the liposomes was kept very high, it could be expected that even small amounts of reagents passing through the lipid membrane will cause crosslinking or at least chemical modification of this protein which was evaluated by SDS-PAGE. From the disappearance or weakening of the CA protein bands on SDS-gels after applying glutaraldehyde or EDC to SCALs, it was concluded that both reagents must have passed through the liposomal membrane to some extent. Thus, alternative methods for crosslinking of the S-layer lattice without affecting encapsulated molecules had to be established. For this purpose dialdehydes were prepared by limited periodate oxidation of raffinose. This non-reducing trisaccharide was chosen to generate highly hydrophilic and more bulky dialdehyde derivates for achieving membrane impermeability and for preventing formation of formaldehyde or degradation from the reducing end leading to only monoaldehyde derivates. From the selected raffinose to periodate molar ratio of 1:2, preferred cleavage of the terminal galactose residue between the *cis* hydroxyl groups and further degradation of one carbon unit was expected to occur. Upon reaction of the generated aldehyde groups with SCALs and reduction of formed Schiff bases, the constituent S-layer subunits were crosslinked while the enclosed CA was not affected. From these results it was concluded that membrane passage of the aldehyde was successfully prevented and that at least two amine groups on adjacent protomers in the S-layer lattice must be present within the crosslinking span which is similar to that of monomeric glutaraldehyde. Due to the introduction of the short sugar moiety, a more hydrophilic surface of SLs is obtained by applying this crosslinking reaction. Moreover, the sugar residue will be susceptible for further activation reactions and may thus be exploited for binding of additional molecules. From a previous study it was evident that the *N*-hydroxysuccinimide ester of suberic acid with a crosslinking span of 1.1 nm completely crosslinked S-layer lattices [21]. Thus, another approach was the use of the analogon BS^3 as crosslinking agent. Because of the presence of sulfonic acid residues, BS^3 was membrane impermeable and did not crosslink encapsulated CA. Due to the six methylene groups introduced by this crosslinker, a more hydrophobic surface of SLs was most probably created. After both crosslinking procedures, the integrity of the S-layer lattice was well preserved.

In previous studies, it was suggested that crosslinking of composite structures from S-layer lattices and lipid membranes will also result in heterologous crosslinking between the S-layer protein and membrane components bearing free amino groups, like HDA or phosphatidylethanolamine [6,8,11]. In contrast to glutaraldehyde which can span various dis-

tances due to different degrees of polymerization, BS^3 possesses a defined crosslinking span. Nevertheless, heterologous crosslinking between membrane incorporated HDA and the S-layer protein had occurred during treatment of SLs with BS^3, as it could be qualitatively demonstrated by the presence of HDA non-extractable from the S-layer lattice. Thus, at least a part of the membrane lipids was covalently linked to the S-layer lattice, which implies that lateral diffusion of free lipid molecules and consequently the fluidity of the whole membrane will be modulated, as it was predicted for the semifluid membrane model [6].

To generate a single well-characterized liposomal system which can bind a great variety of molecules in defined manner, the immobilization potential of S-layer lattices recrystallized on liposomes was combined with the versatile avidin–biotin system as schematically shown in Fig. 4. For this purpose, a sandwich protocol was established which utilizes the high affinity binding of StA for biotin [22], its four binding sites [23] and its lower non-specific interactions in comparison to avidin [24] due to the slightly acidic isoelectric point [23] and the absence of carbohydrate moieties [23]. Since biotinylation of bioactive molecules, such as antibodies, does not significantly influence their binding properties [25], the attachment of StA to SLs can be expected to generate an extremely versatile liposomal matrix applicable for binding of numerous ligands of interest. This was the reason why biotin residues were linked to the S-layer lattice of SLs by diazocoupling of DBB. Both SLs and BS^3SLs could be directly biotinylated by this procedure since free amino groups are not necessary for the coupling reaction. The integrity of the S-layer lattice was preserved even without previous crosslinking. This was attributed to coupling of DBB to tyrosine and histidine residues [18] which does not influence the native charge distribution in the S-layer lattice. Direct electrostatic interactions between carboxylic acid and amine groups of adjacent S-layer subunits were demonstrated to be important for the integrity of native S-layer lattices [4]. Under identical reaction conditions, about two biotin residues being accessible for subsequent avidin binding were introduced per S-layer subunit in both SLs and BS^3SLs, clearly indicating that free amino groups of the S-layer lattice were not involved in the coupling reaction. Considering a molecular diameter of about 6 nm for the roughly spherical avidin or StA [26,27], this nearly corresponded to the theoretical saturation capacity of the S-layer surface, which was calculated to be 2.2 avidin or StA molecules per S-layer subunit assuming a planar surface. Thus, a dense monomolecular StA layer on the (BS^3)SLs was built up upon incubation with StA. Due to the 2-fold symmetry of StA after binding to surfaces many biotin sites remain exposed which can be saturated with a second biotinylated ligand [28]. By using biotinylated ferritin, which can easily be visualized by electron microscopic investigation, the formation of a monomolecular StA layer on the surface of biotinylated (BS^3)SLs was confirmed (Fig. 2a). Since antibodies are one of the most commonly used biologically active molecules conjugated to liposomes [29], biotinylated anti-human IgG was linked as a second model ligand to biotinylated (BS^3)SLs via the StA bridge. The functionality of this liposomal system to recognize and specifically bind to human IgG was determined by ELISA (Fig. 3).

Biotinylated (BS^3)SLs with bound StA provide the possibility for attaching biotinylated molecules to the outermost surface generating a well-defined liposomal system. Anchoring of various ligands is not achieved via lipids incorporated into the liposome membrane, but occurs via an S-layer lattice which completely cages the liposomes. Thus, the properties of the lipid membrane become absolutely independent of the quantity and kind of attached ligands which is in contrast to liposomes employing biotinylated lipids [30]. In the same way, avidin/StA binding to the liposome will not be influenced by the phase behavior of the lipid membrane as it was reported for the interaction with biotinylated lipids [31]. Moreover, no leakage of substances encapsulated into liposomes was observed, as it was reported to be induced by coupling of Fab′ fragments to liposomes containing an already encapsulated solute [32]. Furthermore, a combination of different biotinylated molecules may be attached to the surface of (BS^3)SLs, which is especially of interest in the field of immunodiagnostics.

In summary, the combination of SLs with the versatile avidin–biotin system provides a single, powerful and well-defined liposomal system for a dense and ordered attachment of different biotinylated mole-

cules to the surface without affecting the membrane properties and encapsulated molecules. The specific properties of SLs will be particularly interesting for administration of liposomes as aerosols with nebulizers, for injectable formulations to withstand fluid shear forces in the mammalian vasculature or for liposome application as microreactors, in which the vesicles have to resist process steps like stirring, pumping or re-suspension [33]. Thus, a high application potential of (BS^3)SLs in context with drug targeting or immunodiagnostic assays can be expected.

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