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2-D Protein Crystals as an Immobilization Matrix for Producing Reaction Zones in Dipstick-Style Immunoassays

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ABSTRACT

In the present study, the applicability of crystalline bacterial cell-surface layers (S-layers) as novel immobilization matrices and reaction zones for dipstick-style immunoassays was investigated. For this purpose, S-layer-carrying cell-wall fragments from *Bacillus sphaericus* CCM 2120 were deposited on a microporous support, and the S-layer protein was cross-linked with glutaraldehyde. For developing appropriate test systems, either human IgG was directly linked to the carboxylic acid groups from the S-layer protein or it was immobilized using Protein A or, after biotinylation, using streptavidin. A clear correlation was obtained between the amount of anti-human IgG applied and the absorbance values in the immunoassays. S-layers with covalently bound recombinant major birch pollen allergen were used for quantitative and semiquantitative determination of an antibody raised against it. Using S-layers as an immobilization matrix in comparison to amorphous polymers has advantages in that the closed monolayers of functional macromolecules on their outermost surface allows for strong signals in immunoassays, almost completely eliminates background and prevents diffusion.

INTRODUCTION

Crystalline bacterial cell-surface layers (S-layers) represent the outermost cell envelope component of many eubacteria and are a common feature of archaeobacteria (2,13,16). S-layers completely cover the cell surface during all stages of bacterial growth and division, and exhibit oblique (p1 and p2), square (p4) or hexagonal (p3 and p6) lattice symmetry. Depending on the lattice type, one morphological unit is composed of either one, two, three, four or six identical protein or glycoprotein subunits, with molecular weights ranging from 40 000 to 200 000. High-resolution electron microscopic and permeability studies revealed that S-layers from Bacillaceae have a pore size of 4–5 nm (14,18). Because of the presence of pores identical in size and morphology, and the high density and regular arrangement of amino and carboxylic acid groups on the outermost surface, isolated S-layers or S-layer-carrying cell-wall fragments were used for the production of the first type of isoporous ultrafiltration membranes (14,18) and as a matrix for covalent binding of biologically active macromolecules such as enzymes and ligands (15). In comparison to conventional immobilization matrices, S-layer lattices have the advantage that a closed monolayer of biologically active macromolecules can be built up on their surface, thereby avoiding diffusion in the membranes and preventing unspecific adsorption (15).

In the present study, the applicability of S-layer ultrafiltration membranes

(SUMs) as novel immobilization matrices and reaction zones for dipstick-style immunoassays was investigated. S-layer-carrying cell walls from *Bacillus sphaericus* CCM 2120 were selected for SUM production because of the detailed knowledge acquired of their morphological, surface and permeability properties, as well as their great potential for chemical modification (18). Among the SUMs produced using S-layer-carrying cell-wall fragments from *Bacillus* strains, those from *B. sphaericus* CCM 2120 showed the highest storage stability (at least one year at 4°C).

To obtain information on the antibody-binding capacity of SUMs, either human IgG was directly linked to the carboxylic acid groups from the S-layer protein or it was immobilized using Protein A or, after biotinylation, using streptavidin. The three different methods for immobilizing IgG for immunoassays were compared.

For a more specific test system with economic relevance, the recombinant major birch pollen allergen r Bet v Ia (4) was covalently bound to the S-layer lattice. In northern and central Europe, at least 15% of the population is affected by atopic allergy caused by the pollen of birch (4). Whole birch pollen extracts are used for immobilization in all test systems available on the market. This frequently leads to undesired side reactions. In the present study, the immobilization of recombinant birch pollen allergen to SUMs, which was used for quantification of the monoclonal antibody BIP 1 (8), is described for the first time.

MATERIALS AND METHODS

Organism, Growth Conditions, Cell-Wall Preparation, Production of S-Layer Ultrafiltration Membranes (SUMs) and Preparation of S-Layer Microparticles (SMP)

B. sphaericus CCM 2120 was grown as described in a previous article (18). Cell-wall preparation was performed according to the procedure described in Reference 12, in which cells were disrupted by ultrasonication. The purity of the cell-wall fragments was checked on negatively stained and ultrathin-sectioned preparations by electron microscopy (12) and by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The S-layer protein content of the cell-wall fragments was determined by the method of Lowry et al. (11). For production of SUMs, S-layer-carrying cell-wall fragments from *B. sphaericus* CCM 2120 were deposited on nylon microfiltration membranes in a pressure-dependent procedure. After cross-linking the S-layer lattice with glutaraldehyde, Schiff bases were reduced with sodium borohydride (18). For the preparation of SMP, 100 mg of wet pellets of S-layer-carrying cell-wall fragments were suspended in 100 mL 0.1 M phosphate buffer (pH 7.2, 20°C). During stirring the suspension at 500 rpm, 1 mL of glutaraldehyde (50% vol/vol in distilled water) was added to achieve a final concentration of 0.5% vol/vol. After 30 min, the suspension was centrifuged for 20 min at 50 000× g at 4°C, the pellets were repeatedly washed with distilled water and Schiff bases were finally reduced with sodium borohydride (18). S-layer-carrying cell-wall fragments in which the S-layer protein was fixed with glutaraldehyde are referred to as S-layer microparticles (SMP).

Immobilization of Protein A, Human IgG and Recombinant Birch Pollen Allergen r Bet v Ia to SMP

In all immobilization studies, SMPs were used for exact quantification of the amount of covalently bound macromolecules. For immobilization of Protein A from *Staphylococcus aureus* (Pharmacia Biotech, Uppsala, Sweden), human IgG (Sigma-Aldrich Che-

mie, Deisenhofen, Germany) and r Bet v Ia to SMP from *B. sphaericus* CCM 2120, the carboxylic acid groups from the S-layer protein were activated with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) as described in Reference 18. Immobilization of proteins was done by resuspending 100 mg of wet pellets from EDC-activated SMP in 2 mL of distilled water (pH adjusted to 9.0 with 0.1 N NaOH) containing either 4.5 mg human IgG, 1.0 mg r Bet v Ia or 3.5 mg Protein A. After incubation for 18 h at 4°C, unbound protein was removed by centrifugation of the suspensions at 50 000× g for 20 min at 4°C. The amount of r Bet v Ia, human IgG and Protein A covalently bound to SMP was calculated by subtracting the amount of the respective proteins (detected in the collected supernatants according to Reference 17) from the total-ly applied amount. The introduction of covalent bonds between the S-layer lattice and the protein was controlled by SDS-PAGE of SDS extracts from SMP.

Immobilization of Protein A, Human IgG, r Bet v Ia and Streptavidin to SUMs

When SUMs were used as matrices for immobilization of macromolecules, activation of carboxylic acid groups of the S-layer protein and the immobilization procedure were carried out in a Model 202 chamber from Amicon (Beverly, MA, USA), into which SUM disks with a diameter of 62 mm could be inserted. Since only the SUM surface was exposed, diffusion of macromolecules into the microfiltration membrane was prevented. For immobilization of Protein A, human IgG and r Bet v Ia, carboxylic acid groups from the S-layer protein were activated with EDC at pH 4.75 for 60 min at 20°C. Subsequently, the SUMs were washed with ice-cold distilled water and incubated with 10 mL of the respective protein solution (1 mg/62-mm-diameter SUM in distilled water, pH adjusted to 9.0 with 0.1 N NaOH) for 18 h at 4°C. Unbound protein was removed by washing the SUM surface with 50 mM phosphate buffer containing 140 mM NaCl (phosphate-buffered saline [PBS]) and 0.1% Triton® X-100. SUMs with immobilized Protein A were subsequently incubated with hu-

man IgG (1 mg/62-mm-diameter SUM in 10 mL 0.1 M glycine-NaOH buffer, pH 9.0) for 18 h at 4°C.

For immobilization of biotin, free carboxylic acid groups from the acidic amino acids of the S-layer protein were first converted into amino groups. This was done by binding ethylenediamine to EDC-activated carboxylic acid groups under conditions given in Reference 18. Biotinylation of SUMs with free amino groups was carried out according to the procedure given by Pierce (Rockford, IL, USA) by using ImmunoPurc® Sulfo-NHS-Biotin. For the removal of unbound biotin, SUMs were washed at least three times with 50 mM PBS containing 0.1% Triton X-100. Subsequently, 10 mL 0.1 M glycine-NaOH buffer (pH 7.0) containing 1 mg streptavidin (Promega, Madison, WI, USA) were put into the chamber loaded with SUM. After incubation for 18 h at 4°C, streptavidin-SUMs were used for binding of biotinylated human IgG (1.5 mg/62-mm-diameter SUM in 10 mL 0.1 M glycine-NaOH buffer, pH 9.0) for 4 h at 4°C. Biotinylation of human IgG with Sulfo-NHS-Biotin, determination of the protein content (Protein Assay Kit; Bio-Rad, Hercules, CA, USA) and calculation of the molar ratio of biotin to IgG in the conjugate were performed according to the procedures given by the manufacturers and by applying the HABA test (7).

For semiquantitative determination of the amount of streptavidin, of human IgG bound to Protein A-SUMs and of biotinylated human IgG adsorbed to the streptavidin-SUM, disks with a diameter of 14 mm were cut into small pieces (ca. 1 mm) and extracted with 200 µL 2% SDS solution. Subsequently, 5 µL of these extracts were applied to SDS gels. A series of human IgG and streptavidin standards allowed the semiquantitative determination of bound proteins.

Studies on the Applicability of SUMs as Matrices for Immunoassays and Dipsticks

For comparing the three different methods of immobilizing human IgG, disks with a diameter of 14 mm were punched out from the SUMs (62-mm diameter) obtained according to the procedures described above and were fixed

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density of human IgG, either directly bound to carboxylic acid groups from the S-layer protein or to streptavidin-SUM after biotinylation, comparable absorbance values were observed by applying the same concentrations of anti-human IgG (Figure 3). This phenomenon can be explained primarily by the different methods used for IgG immobilization. It can be assumed that, due to the high density of the carboxylic acid groups in the S-layer lattice, multipoint attachment of the directly immobilized IgG molecules had occurred. This is known to lead to structural distortions and to conformational changes, which can therefore influence the biological recognition process (6). In the case of biotinylated IgG, six biotin molecules were attached per molecule, which could anchor the IgG to the streptavidin-SUM (Figure 2). With all three matrices, very low unspecific adsorption could be observed (Figure 3).

Recombinant Bet v 1a was immobilized directly to the EDC-activated carboxylic acid groups on the S-layer

protein of SUMs. The suitability of this matrix for immunoassays and dipsticks was proven by incubation with different concentrations of BIP 1. The resulting curve, when using pNPP as a soluble substrate, demonstrated satisfactory recognition of r Bet v 1a by BIP 1 over a wide concentration range (Figure 4). For visual detection of BIP 1 and semi-quantitative determination relevant to dipstick-style immunoassays, BCIP/NBT was chosen as a substrate, leading to insoluble products. The intensity of the precipitate formed on the SUM surface correlated well with the BIP 1 concentrations applied. The SUMs with immobilized r Bet v 1a showed absolutely no background reactions (Figure 5).

DISCUSSION

The square S-layer lattice from *B. sphaericus* CCM 2120 was used as model system for investigating the applicability of using SUM as an immobilization matrix and a reaction zone for dipstick-style immunoassays. S-layer-carrying cell wall fragments, which revealed a complete outer and inner S-layer (Figure 1b), were used for either the preparation of SMP or for the pro-

duction of SUMs. Since in both cases the S-layer surface was exposed, studies on the binding capacity of SMP could be related directly to the binding capacity of SUMs. Because of the possibility of exact quantification, optimization of the immobilization procedures was done with SMP.

The binding capacity of the square S-layer lattice from *B. sphaericus* CCM 2120 for Protein A correlated well with data obtained in a previous study using the hexagonally ordered S-layer lattice from *Thermoanaerobacter thermohydrosulfuricus* L111-69 (19). On both S-layer lattices, the Protein A molecules were shown to be preferentially arranged with their longitudinal axis perpendicularly to the S-layer lattice. Otherwise, a binding capacity 3-4 times higher than the area theoretically required for a dense Protein A monolayer based on a Stoke's radius of 5 nm would never have been achieved. Assuming that a Protein A monolayer was generated on the S-layer lattice, the actual radius of the elongated molecules was calculated to be in the range of 2.5 to 3 nm.

The binding of human IgG to Protein A-SUM led to the formation of a

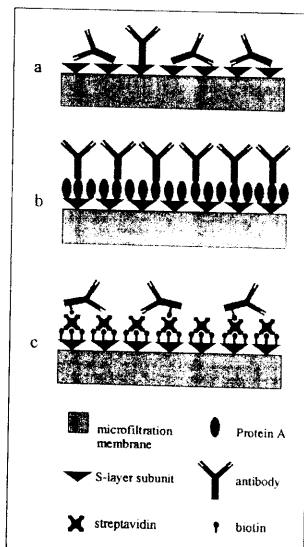


Figure 2. Schematic drawing illustrating immobilization of human IgG covalently bound to carbodiimide-activated carboxylic acid groups from the S-layer lattice (a), to immobilized Protein A (b), after biotinylation to streptavidin (c).

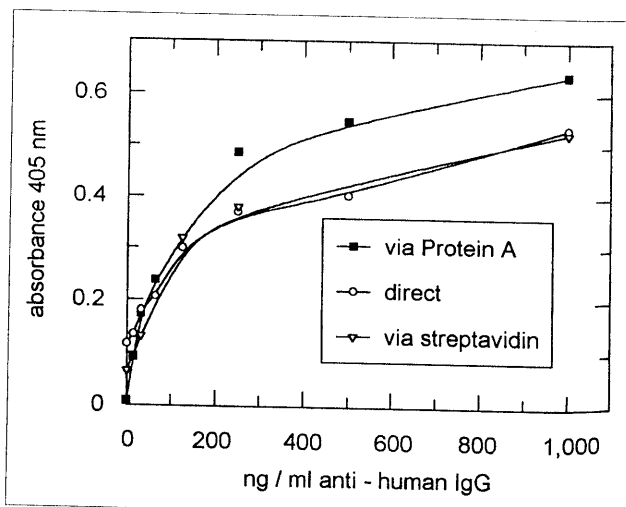


Figure 3. Absorbance values at 405 nm for different concentrations of anti-human IgG applied to SUMs on which human IgG was either covalently bound to the S-layer protein; immobilized via Protein A; or biotinylated and linked to a streptavidin-SUM.

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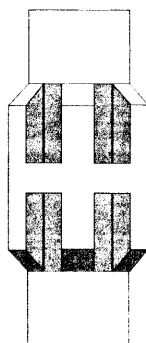
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closed monolayer of densely packed IgG molecules (Figure 2). Because the anti-human IgG used in the present study was raised against the whole molecule, the uniform alignment of the IgG molecules cannot be responsible for the higher absorbance values determined for Protein A-SUM in the immunoassay (Figure 3) but would be advantageous for antigen recognition. Since steric reasons can be excluded, the high absorbance values achieved with Protein A-SUM were attributed to the higher packing density of the IgG molecules in comparison to that of streptavidin-SUM (Figure 2) or the covalently bound human IgG. On the other hand, the existence of a closed monolayer of

uniformly oriented IgG molecules excludes any enhancing effect by the enzyme-antibody conjugate applied in the last step. By immobilizing Protein A to SUMs, a matrix with high affinity to human IgG or rabbit IgG was generated. However, Protein A-SUM cannot be used for the binding of immunoglobulins with low affinity to Protein A such as mouse IgG or goat IgG (10). In this case, direct covalent binding of the antibodies to the S-layer lattice or binding of biotinylated antibodies to a streptavidin-SUM is preferred.

The binding density determined for the covalently bound IgG and for the biotinylated IgG indicated a random orientation of the molecules on the S-

layer lattice (Figure 2, a and c). Although the IgG binding capacity of streptavidin-SUM (150 ng IgG per cm² membrane area) was lower than the amount that could be bound covalently (375 ng per cm² membrane area), the absorbance values measured in the immunoassay were rather similar (Figure 3). These results indicated that, in comparison to the biotinylated IgG, recognition of the covalently bound human IgG by anti-human IgG was clearly reduced. However, all three methods for immobilizing human IgG on S-layer lattices led to reproducible results concerning the binding capacity. The correlation between increasing concentrations of anti-human IgG and increasing absorbance values confirmed that SUMs are well suited as matrix for immunoassays (Figure 3). Nonspecific adsorption and background reactions were generally very low and in most cases negligible.

If SUMs were used as matrices for immobilization and reaction zones for dipstick-style immunoassays, diffusion of antibodies or antibody-enzyme conjugates into the highly adsorptive microfiltration membrane supporting the S-layer-carrying cell-wall fragments had to be prevented. Currently, SUMs used for preparation of dipsticks are either glued together with the two microfiltration membranes facing each other or attached to polymer films (Mader, unpublished observation). Contrary to conventionally used microfiltration membranes, which can adsorb up to milligrams of proteins per cm² membrane area (1,5), the surface of SUMs revealed extremely low nonspecific adsorption and nearly no background without blocking steps in immunological reactions. The main reason for this advantage is seen in the well-defined physicochemical and morphological properties of S-layer lattices composed of identical protein subunits.

In summary, crystalline bacterial cell-surface layers represent unique matrices for a controlled immobilization of monomolecular layers of macromolecules. Because of their crystalline structure, along with the regular arrangement and well-defined orientations of functional groups down to the subnanometer range, S-layer lattices can predetermine the orientation of im-

mobilized macromolecules, as shown for Protein A. Moreover, the presence of surface-located functional groups in high density on the S-layer lattice further allows for the application of a broad spectrum of chemical modification and coupling procedures for generating an immobilization matrix even for very specific demands (18). Since reproducible amounts of functional macromolecules, such as antibodies, enzymes and allergens, can be immobilized, SUMs provide a new type of reaction zone for quantitative and semi-quantitative immunoassays and dipstick test systems with constant signals.

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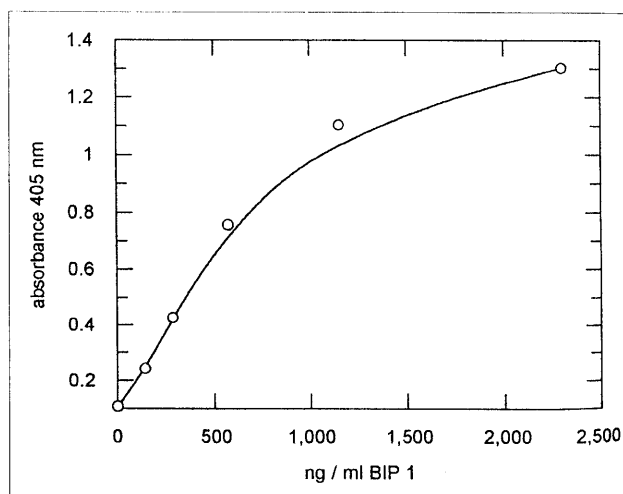


Figure 4. Absorbance values at 405 nm for different concentrations of BIP 1 applied to a SUM on which r Bet v 1a was covalently bound to carboxylic acid groups from the S-layer protein.

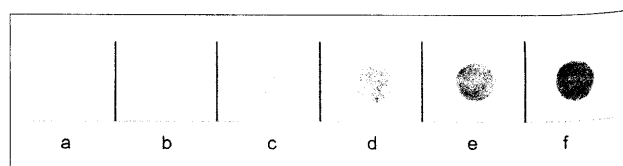


Figure 5. SUM discs with covalently bound r Bet v 1a demonstrating their applicability as reaction zone for dipsticks. By using BCIP/NBT substrate, the intensity of the precipitate formed on the SUM surface correlated well with the different concentrations of BIP 1 applied. (a-f) correspond to the different concentrations of BIP 1 applied in the immunoassay in Figure 4. (a = 0 µg/mL; b = 0.143 µg/mL; c = 0.287 µg/mL; d = 0.575 µg/mL; e = 1.15 µg/mL; f = 2.3 µg/mL).