Two-dimensional paracrystalline glycoprotein S-layers as a novel matrix for the immobilization of human IgG and their use as microparticles in immunoassays

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Abstract

In the present study, cup-shaped 1–3 μm large cell wall fragments from *Thermoanaerobacter thermohydrosulfuricus* L111-69 covered with a hexagonal S-layer lattice composed of glycoprotein subunits were shown to act as a matrix for the immobilization of human IgG. After cross-linking the S-layer glycoprotein lattice with glutaraldehyde (S-layer microparticles), IgG was either bound to carbodiimide activated carboxyl groups from acidic amino acids from the protein moiety or to the carbohydrate chains activated with cyanogen bromide or oxidized with periodate. After determining the binding capacity of the S-layer lattice for human IgG, the orientation of the immobilized antibody molecules was investigated using anti-human IgG peroxidase conjugates with different specificity. Attachment of S-layer microparticles with covalently bound human IgG to microplates precoated with anti-human IgG of different specificity led to clear correlations between the amount of applied human IgG and the absorption values in the immunoassays. The steepest absorption curves were obtained when human IgG was bound to the carbohydrate chains exposed on the surface of the S-layer lattice. This confirmed that the location and the accessibility of the immobilized antibodies on S-layer microparticles is of major importance for the response in immunoassays. In addition to the high reproducibility of the amount of IgG which could be bound to the S-layer lattice and the high reproducibility of the absorption curves in the immunoassays, one major advantage of using cup-shaped S-layer microparticles can be seen in the considerable increase of the actual surface available for binding processes and immunological reactions.

Keywords: IgG, human; Paracrystalline bacterial cell surface layer; S-layer; Immunoassay; Immobilization; Microparticle

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1. Introduction

Paracrystalline bacterial cell surface layers (S-layers) represent the outermost cell envelope component of many eubacteria and archaeobacteria (for reviews see Beveridge and Koval, 1993; Messner and Sleytr, 1992; Sleytr et al., 1993, 1996). High-resolution electron microscopic studies and computer image reconstruction analyses revealed that S-layers exhibit oblique (p1, p2), square (p4) or hexagonal (p3, p6) lattice symmetry. The lattice type, the center-to-center spacing of the morphological units and the molecular weights of the S-layer subunits are strain specific features. Depending on the lattice type, one morphological unit consists of one (p1), two (p2), three (p3), four (p4) or six (p6) identical protein or glycoprotein subunits with molecular weights ranging from 40000 to 200000 (for recent compilation see Sleytr et al., 1996). Since S-layers are composed of identical subunits, functional groups such as amino and carboxyl groups (and in the case of S-layer glycoproteins covalently bound carbohydrate chains) display identical positions and orientations on each S-layer subunit. According to their regular structure, S-layer lattices possess pores identical in size and morphology, which endow these paracrystalline arrays with very sharp exclusion limits (Sára and Sleytr, 1987; Sára et al., 1992). Compared to conventional polymers with amorphous structure, S-layers represent a unique biomaterial with repetitive surface properties down to the sub-nanometer range. This permits S-layers to be exploited for various biotechnological, biomimetic and nanotechnological applications. Nowadays, S-layers particularly from Bacillaceae are used for the production of isoporous ultrafiltration membranes, as a matrix for the controlled immobilization of macromolecules, for functionalization of solid surfaces, and for stabilizing liposomes and Langmuir-Blodgett films (for recent review see Sleytr et al., 1996).

S-layer proteins from Gram-positive eubacteria can be extracted from cell wall fragments with hydrogen bond breaking agents (Nermut and Murray, 1967). Upon removal of the disrupting agent by dialysis many S-layer subunits have shown the ability to reassemble into lattices identical to those observed on intact cells. These S-layer self-assembly products may have the shape of flat sheets or open ended cylinders, and can be built up of mono- or double layers (Sleytr and Messner, 1989). For many S-layer based technologies it was of great importance that instead of S-layer self-assembly products, S-layers carrying cell wall fragments could be used (for review see Sára et al., 1996). Most important, electron microscopic preparations of S-layers carrying cell wall fragments from Bacillaceae revealed that the rigid, peptidoglycan-containing layer was completely covered on the outside and the inside with an S-layer. The inner S-layer was formed from an S-layer protein pool entrapped in the peptidoglycan-containing layer of intact cells. During the cell wall preparation procedure involving the removal of the plasma membrane the S-layer subunits are liberated and assemble on the inner face of the rigid cell wall layer (Breitwieser et al., 1992). After cross-linking the S-layer protein with glutaraldehyde, S-layer carrying cell wall fragments revealed a remarkable stability towards mechanical and shear forces. This characteristic feature has already been exploited by using them as 'escort' particles in affinity cross-flow filtration processes (Weiner et al., 1994).

The S-layer lattice from *Thermoanaerobacter thermohydrosulfuricus* L111-69 (formerly *Clostridium thermohydrosulfuricum* L111-69 (Lee et al., 1993) shows hexagonal symmetry, a center-to-center spacing of the morphological units of 14.2 nm (Crowther and Sleytr, 1977) and is composed of glycoprotein subunits with a molecular weight of 120000. Chemical analyses indicated a total carbohydrate content of 15%. NMR analysis has shown that the linear carbohydrate chains are composed of disaccharide repeating units of α-mannosyl and α-rhamnosyl residues (Bock et al., 1994). In numerous studies, the paracrystalline S-layer lattice from this organism proved to be well suited as a matrix for the immobilization of enzymes or ligands. Biologically active macromolecules could either be linked to the carbodiimide activated carboxyl groups from the acidic amino acids of the S-layer protein or to the carbohydrate chains. Most macromolecules formed a monomolecular layer on the surface of the S-layer lattice (Küpcü et al., 1995; Jahn-Schmid et al., 1996; Messner et al., 1992; Sára et al., 1989, 1993, 1996).

In the present study, human IgG was immobilized to the protein moiety and to the carbohydrate chains of the S-layer glycoprotein using cup-shaped S-layer

carrying cell wall fragments of *Th. thermohydrosulfuricus* L111-69. After determination of the binding capacity of the S-layer lattice and the orientation of the immobilized human IgG, the applicability of S-layer carrying cell wall fragments as 'microparticles' for immunoassays was investigated.

2. Material and methods

2.1. Organism, growth conditions and preparation of S-layer carrying cell wall fragments

Th. thermohydrosulfuricus L111-69, a strictly anaerobic organism, was grown in continuous culture in a 10 l Braun bioreactor (Braun, Melsungen, Germany) under conditions described previously (Sára and Sleytr, 1989). S-layer carrying cell wall fragments were prepared according to the procedure of Sleytr and Glauert (1975) except that whole cells were broken in half using ultrasound. The purity of S-layer carrying cell wall fragments was controlled by SDS-PAGE and negative staining performed as described by Messner et al. (1984). The presence of a complete outer and inner S-layer on cell wall fragments was examined on ultrathin-sectioned preparations. The S-layer protein content of S-layer carrying cell wall fragments was determined by the method of Lowry et al. (1951) using purified S-layer protein for calibration.

2.2. Preparation of S-layer microparticles (SMP)

For the preparation of SMP, the S-layer protein in S-layer carrying cell wall fragments was cross-linked with glutaraldehyde (0.5% in 0.1 M phosphate buffer, pH 7.0) for 20 min at 20°C as described (Sára and Sleytr, 1989). The water content of pellets of SMPs obtained after centrifugation at $40000 \times g$ for 20 min at 4°C was determined by drying at 120°C for 2 h.

2.3. Immobilization of human IgG to the carbohydrate residue of the S-layer glycoprotein after activation of vicinal hydroxyl groups with cyanogen bromide or oxidation with periodate

One hundred milligram of SMP (sedimented at $40000 \times g$ for 20 min at 4°C) were suspended in 2 ml 0.1 M sodium carbonate buffer, pH 8.5. Subse-

quently, 100 mg cyanogen bromide (BrCN) was dissolved in 2 ml of the same buffer and added to the suspension of the SMP (Axen and Ernback, 1971). During the activation period of 8 min, 5 M NaOH was added to keep the pH value at 11.0. Finally the suspensions were centrifuged at $40000 \times g$ for 15 min at 4°C. Cyanogen bromide activated SMP was resuspended in 3 ml 0.1 M sodium carbonate buffer, pH 8.5, which contained 4.5 mg human IgG (Sigma I-4506). The suspensions were incubated for 14 h at 4°C in a Test Tube Rotator, Type 3025 (GFL, Burgwedel, Germany) with a rotation speed of 12 min^{-1}. After centrifugation at $40000 \times g$ for 15 min at 4°C, the pellets were washed four times with 4 ml 0.1 M phosphate buffer, pH 7.0, at 4°C. The amount of covalently bound human IgG was quantified as described below.

For the cleavage of vicinal hydroxyl groups on the carbohydrate chains of the S-layer glycoprotein, 100 mg wet pellets of SMP were suspended in 1 ml 20 mM sodium periodate ($NaIO_4$) in 1 mM sodium acetate, pH 4.4. The activation reaction was carried out for 1 h whilst stirring in the dark at 20°C (Hsiao and Royer, 1979). After centrifugation at $40000 \times g$ for 20 min at 4°C the pellets were resuspended in 3 ml 0.1 M sodium carbonate buffer, pH 9.0, which contained 4.5 mg human IgG. The suspensions were incubated and washed as described above.

2.4. Activation of free carboxyl groups from the S-layer protein with carbodiimide

Activation of free carboxyl groups from the S-layer protein in SMP with 1-ethyl-3,3'-(dimethyl-amino)propylcarbodiimide (EDC) was performed as described previously (Sára and Sleytr, 1989). EDC activated SMP were resuspended in 3 ml distilled water containing 4.5 mg human IgG. The pH value of this solution was adjusted to 9.0 by adding 10 mM NaOH. The suspensions were incubated at 4°C and the pellets were washed as previously described.

2.5. Quantification of the amount of human IgG immobilized on the S-layer lattice of Th. thermohydrosulfuricus L111-69

The standard curve for human IgG was obtained using 2–10 μg of human IgG (100 μg/ml) in 0.1 M

phosphate buffer, pH 7.0, for the bicinchoninic acid (BCA) protein assay (Smith et al., 1985). The amount of human IgG bound to the S-layer lattice was calculated after subtraction of the amount of human IgG determined in the collected supernatants from all wash cycles from the total amount applied during the immobilization procedure.

2.6. Use of SMP with immobilized human IgG as microparticles for immunoassays

The use of SMP with immobilized human IgG as microparticles for immunoassays was investigated by

adsorbing them to microplates precoated with anti-human IgG and applying different anti-human IgG peroxidase conjugates (Fig. 1). In order to obtain information about possible differences in orientations of human IgG molecules immobilized either to the protein moiety or to the carbohydrate chains of the S-layer glycoprotein, the immunoplates were precoated with Fab specific (goat; Sigma, I-9010), Fc specific (goat; Sigma, I-8885) or γ-chain specific (goat; Sigma, I-1136) anti-human IgG. Coating of microplates was carried out with 100 μl of each anti-human IgG diluted 1/3000 in 0.1 M sodium
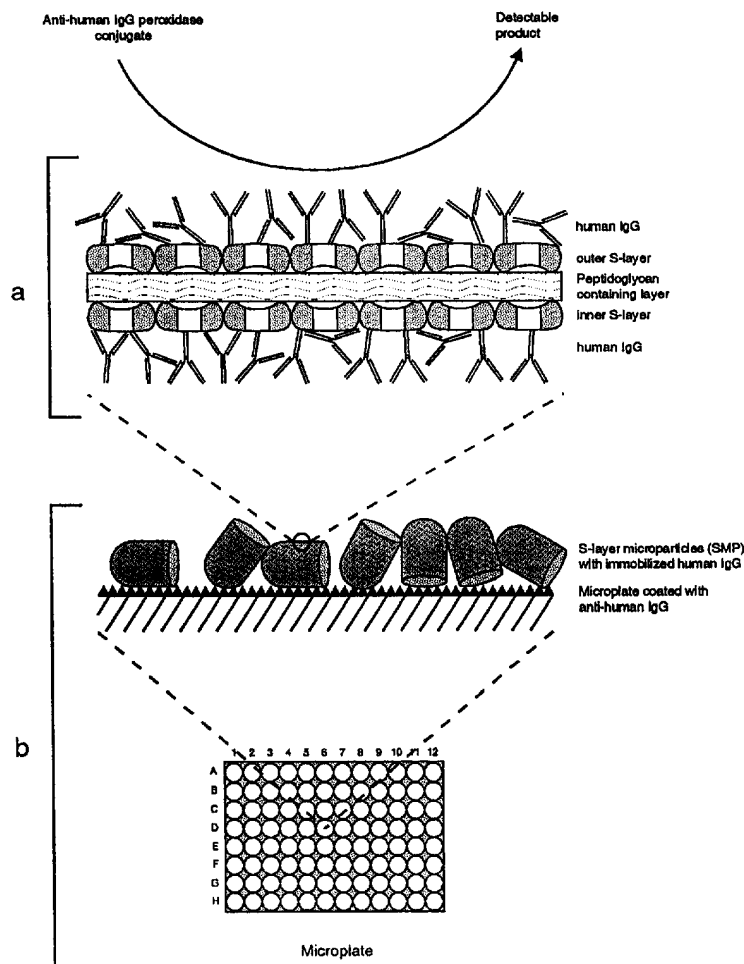

Fig. 1. Schematic drawing illustrating the principles of immunoassays based on S-layer microparticles (SMP) with human IgG. *a*: both the outer and the inner S-layer lattice which completely covered the peptidoglycan-containing layer of SMP are available for immobilization of human IgG. SMP are bound to microplates precoated with anti-human IgG. *b*: adsorbed SMP were detected with an anti-human IgG peroxidase conjugate.

carbonate buffer, pH 9.5, overnight at 4°C. Subsequently, the plates were emptied and washed three times with phosphate buffered saline (PBS) (10 mM sodium and potassium phosphate, 0.1 M sodium and potassium chloride and 0.1% Triton X-100, pH 7.2).

After extensive washing, the wells were incubated with 200 μl blocking solution (1% hämopearl, BSA, Hämosan, Austria, in PBS buffer) for 1 h at 37°C. Then the plates were filled with 100 μl of a suspension of SMP in blocking solution and incubated for 1
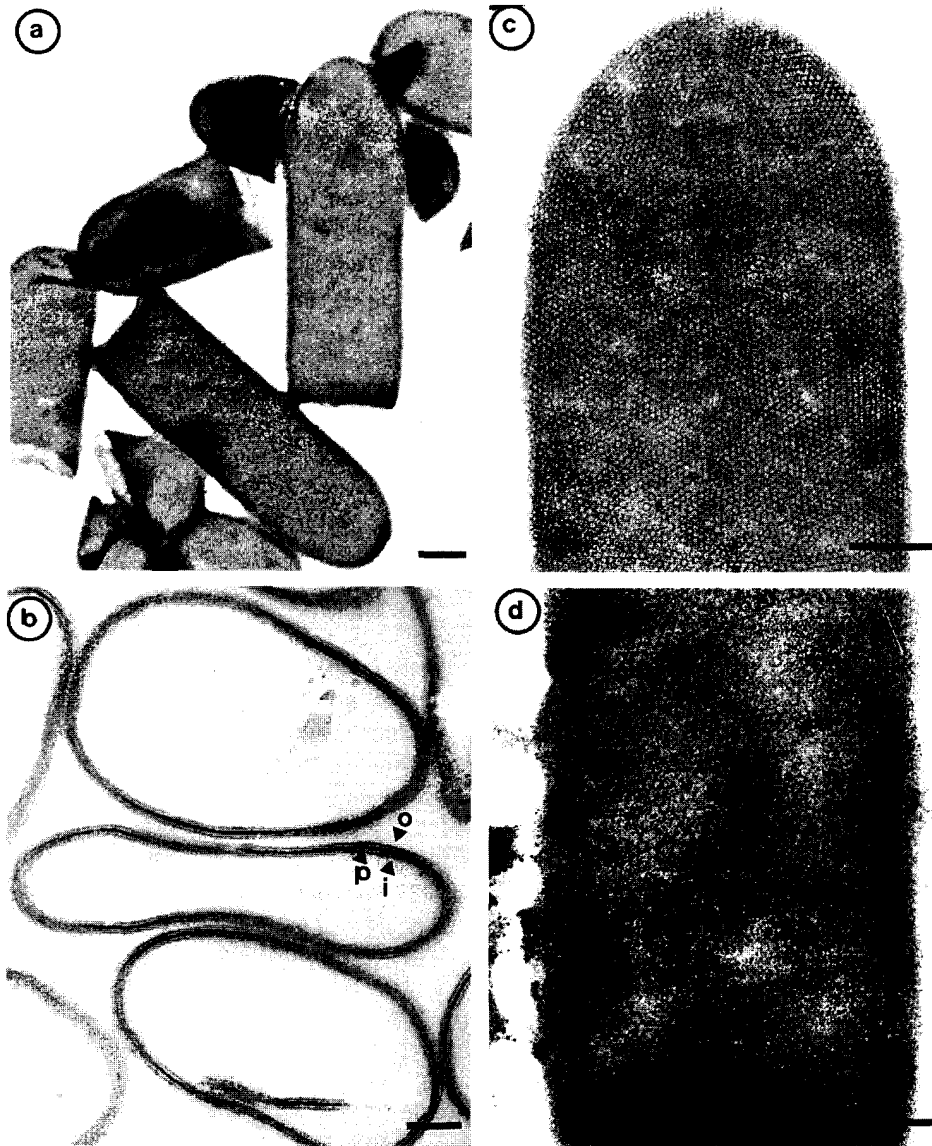

Fig. 2. Electron micrographs of (a) a negatively stained preparation of S-layer microparticles (SMP) of *Th. thermohydrosulfuricus* L111-69 demonstrating the cup-shaped structure of cell wall fragments obtained by disrupting whole cells using ultrasound; (b) ultrathin cross-sectioned SMP. Both faces of the peptidoglycan-containing layer (p) are covered with a complete outer (o) and inner (i) S-layer; (c) negatively stained preparation of SMP of *Th. thermohydrosulfuricus* L111-69 showing the hexagonally ordered S-layer lattice; (d) negatively stained preparation after immobilization of human IgG to SMP and labeling with a γ-chain specific anti-human IgG gold conjugate. Bars, 250 nm in panel a, 100 nm in panels b, c, d.

h at 37°C. The amount of SMP suspended (150–500 μg wet pellet) corresponded to 300 ng–10 μg human IgG per ml blocking solution. After washing the plates three times with PBS buffer, the wells were incubated with 100 μl of a Fab specific (goat; Sigma, A-0293), Fc specific (goat; Sigma, A-0170) or γ-chain specific (goat; Sigma, A-6029) anti-human IgG peroxidase conjugate diluted 1/800 in blocking solution for 1 h at 37°C. The plates were then washed again three times with PBS buffer and the wells were filled with 150 μl substrate solution which contained 6 mg 3,3',5,5'-tetramethylbenzidine hydrochloride (TMB; Sigma, T-8768) and 10 μl 30% hydrogen peroxide in 10 ml 0.1 M citric acid, pH 4.9 (slightly modified procedure given by Sigma). The reaction was allowed to continue for 3 min at 20°C and was stopped by the addition of 50 μl 1 M HCl. The intensity of the color generated was determined at 405 nm in an Easy Reader EAR 400AT (SLT-Labinstruments, Austria). The standard curve was established using human IgG over the range of 3–214 ng per 100 μl blocking solution. Microplates were precoated as described above.

2.7. Determination of the binding capacity of suspended SMP for Fab, Fc and γ-chain specific anti-human IgG conjugates

To investigate the orientation of the human IgG immobilized to the S-layer glycoprotein by the three different methods, 10 mg wet pellets of SMP were suspended with 100 μl blocking solution in Eppendorf tubes. Subsequently, a twofold excess of Fab, Fc or γ-chain specific anti-human IgG peroxidase conjugate was added and the suspensions were incubated for 1 h at 37°C. After sedimentation at 40 000 × g and washing three times with PBS, the wet pellets were suspended in blocking solution. The amount of anti-human IgG peroxidase conjugate that had bound to the suspended SMP was determined via the peroxidase activity.

In all conjugates, (Fab, Fc and γ-chain specific) the total protein content was measured by the BCA protein assay. The concentration of peroxidase was separately determined by the absorption of the heme group at 403 nm. The molar ratio of anti-human IgG to peroxidase was calculated.

2.8. Labeling of immobilized human IgG with anti-human IgG gold conjugate

After immobilization of human IgG to the S-layer protein or to the carbohydrate chains of SMP the immobilized human IgG was labeled with an anti-human IgG (γ-chain specific) gold conjugate, 5 nm (Sigma, G-9520). Labeling experiments were performed on 300 mesh copper grids which were floated on a drop of suspension containing the SMP for 5 min. Subsequently, the grids were extensively washed with distilled water and transferred to a drop of the anti-human IgG gold conjugate which was allowed to adsorb for 1 h. Excess fluid was removed using a filter paper and the grids were negatively stained with uranyl acetate (0.5% in distilled water, pH 4.5) for 30 s. The preparations were examined with a Philips model CM 100 electron microscope (Philips, Eindhoven, The Netherlands) operated at 80 kV using a 30 μm objective aperture.

3. Results

3.1. SMP from Th. thermohydrosulfuricus L111-69

Electron microscopic examination of negatively stained preparations of S-layer carrying cell wall fragments from Th. thermohydrosulfuricus L111-69 showed that they were completely covered with a hexagonally ordered S-layer lattice (Fig. 2a,c). Ultrathin sections revealed a three-layered cell envelope profile consisting of the outer (o) S-layer, the peptidoglycan-containing layer (p) and the inner (i) S-layer (Fig. 2b). The yield of S-layer carrying cell wall fragments from biomass was in the range of 20–30%.

The S-layer protein content of S-layer carrying cell wall fragments was 54%. SMP were prepared by cross-linking the S-layer protein from S-layer carrying cell wall fragments with glutaraldehyde. One milligram of SMP was estimated to contain 37.8 μg S-layer protein. As derived from the center-to-center spacing of the morphological units in the S-layer lattice (14.2 nm), the area occupied by one hexameric unit cell was calculated to be 175 nm^2. For determining the binding density of human IgG in ng/cm^2 S-layer lattice, the molecular weight of the

S-layer glycoprotein subunits (120 000), the protein content of SMP and the area occupied by one hexameric unit cell need to be taken into consideration.

3.2. Immobilization of human IgG to the protein moiety and to the carbohydrate chains of the S-layer glycoprotein

When human IgG was immobilized to EDC-activated carboxyl groups of the S-layer lattice, 580 μg of the antibody could be bound per mg S-layer protein. Since the molecular weight of human IgG is 150 000 and that of the S-layer subunits 120 000, the molar ratio between human IgG and the S-layer subunits was 0.46. Consequently, each hexameric unit cell with an area of 175 nm² was occupied by 2.7 covalently bound IgG molecules. As shown in Table 1, the Stokes' radius of the IgG molecules depends on the state of the Fab regions and lies between 3.5 and 5.8 nm (Kim et al., 1991). Therefore, the theoretical saturation capacity of a planar surface for a monomolecular layer of IgG can range from 650 ng/cm² for the more compact state of the Fab regions to 240 ng/cm² for the more expanded form. Assuming a random state of the Fab regions of the immobilized human IgG, the 580 ng bound per

cm² would correspond to approx. 80% coverage of the S-layer surface (Table 1).

As determined by protein and periodate-Schiff staining reactions the S-layer glycoprotein of Th. thermohydrosulfuricus L111-69 migrated as a single band on SDS gels. This indicated that carbohydrate chains with identical length are available for immobilization of IgG on each S-layer subunit (Sleytr and Thorne, 1976). The amount of human IgG that could be linked to the carbohydrate chains after periodate oxidation was 660 μg per mg S-layer glycoprotein which corresponded to three IgG molecules per hexameric unit cell. On the other hand, after cyanogen bromide activation of vicinal hydroxyl groups, only 290 μg human IgG could be immobilized per mg S-layer glycoprotein. This resembles approximately half the amount of IgG bound by the periodate oxidation method. NMR analyses revealed that the linear carbohydrate chains are composed of 27 disaccharide repeating units with a total length of about 60 nm (Bock et al., 1994). Although on such long carbohydrate chains a large number of vicinal hydroxyl groups are available, it can be assumed that particularly activated groups at the distal end of the carbohydrate chains represent the first contact site and binding region for the IgG molecules. Due to
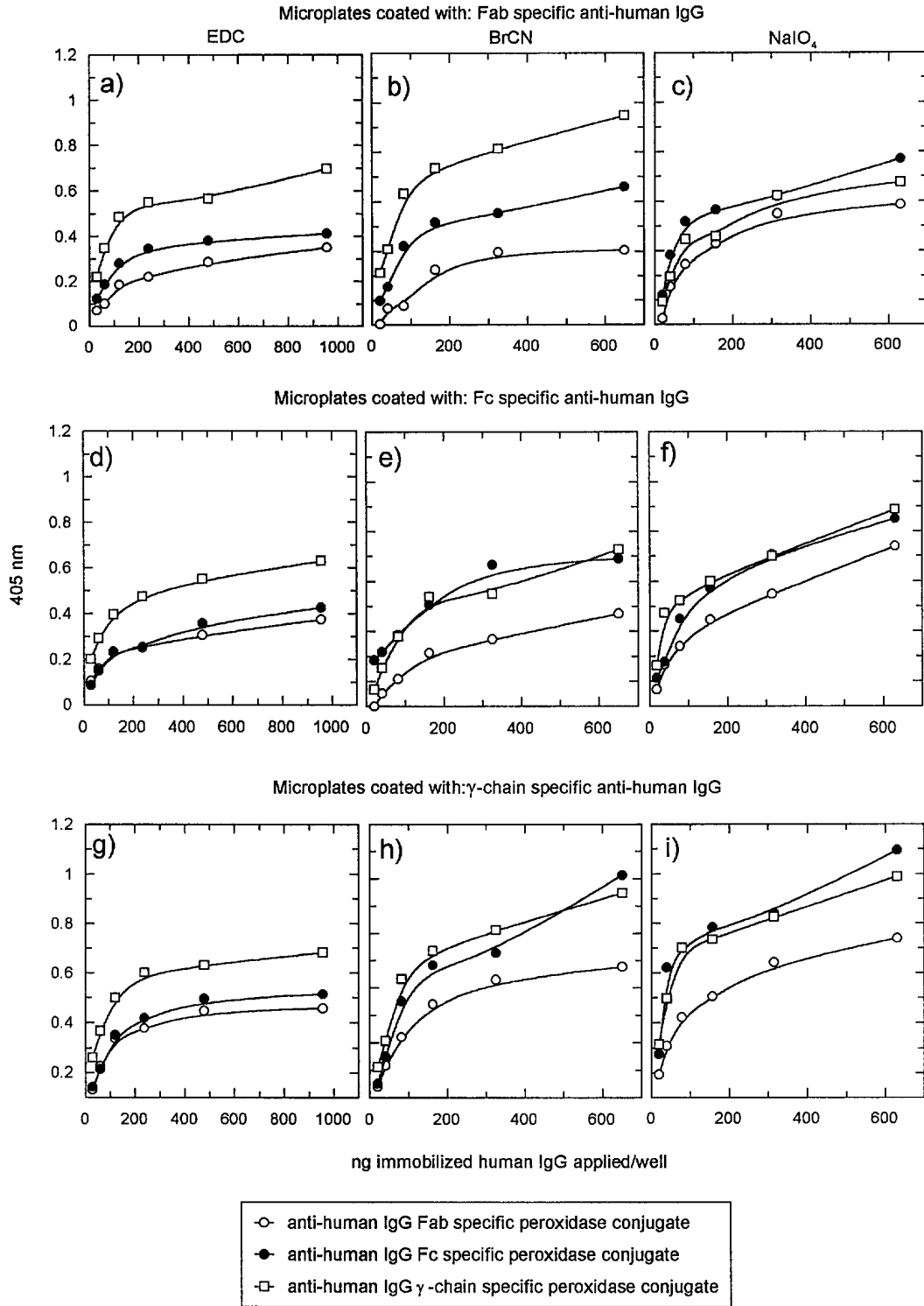
Table 1

Immobilization capacity of S-layer microparticles (SMP) from Th. thermohydrosulfurics L111-69 for human IgG and determination of the orientation of the immobilized human IgG using Fab, Fc and γ-chain specific anti-human IgG peroxidase conjugates

		EDC	BrCN	NaIO$_4$
μg human IgG bound/mg SMP		314	157	357
μg human IgG bound/mg S-layer protein		580	290	660
Molar ratio of human IgG to S-layer subunits		0.46	0.21	0.50
Human IgG molecules bound per morphological unit cell (six S-layer subunits)		2.7	1.3	3.0
Theoritical saturation of a planar monomolecular layer of human IgG [a] in ng/cm²		650–240		
Determined number of human IgG molecules bound/cm² S-layer lattice		1.5×10^{12}	7.2×10^{11}	1.7×10^{12}
Determined binding capacity of the S-layer lattice in ng/cm²		280	140	310
% of theoretical saturation capacity of human IgG(mean with range in brackets)		79 (42–115)	39 (21–57)	89 (48–130)
% immobilized human IgG molecules recognized by the anti-human IgG peroxidase conjugates with differing specificty	Fab	31	37	41
	Fc	15	9	25
	γ-chain	54	54	34

Carboxyl groups from the acidic amino acids of the S-layer glycoprotein were activated with 1-ethyl-3,3'-(dimethylamino)propylcarbodiimide (EDC). Hydroxyl groups from the carbohydrate chains were activated with cyanogen bromide (BrCN) or oxidized with sodium periodate (NaIO$_4$).

[a] Stokes' radius of human IgG = 3.5–5.8 nm (Kim et al., 1991).

Microplates coated with: Fab specific anti-human IgG

EDC   BrCN   NaIO$_4$

Microplates coated with: Fc specific anti-human IgG

405 nm

Microplates coated with:γ-chain specific anti-human IgG

ng immobilized human IgG applied/well

-O- anti-human IgG Fab specific peroxidase conjugate
-●- anti-human IgG Fc specific peroxidase conjugate
-□- anti-human IgG γ-chain specific peroxidase conjugate

steric hindrance, human IgG molecules bound to the end of the carbohydrate chains will inhibit or prevent the passage of further IgG molecules to the inner region of the carbohydrate chains. Assuming a random state for the Fab regions, about 90% of the theoretical saturation capacity was achieved when human IgG was linked to the periodate oxidized carbohydrate chains (Table 1). The lower binding capacity of the cyanogen bromide activated carbohydrate chains for IgG (Table 1) is explained by the instability and hydrolysis of the intermediate products (Hermanson et al., 1992).

Covalent attachment of human IgG to the S-layer glycoprotein was checked by extraction of SMP with SDS, and examining the SDS extracts by SDS-PAGE. Since no protein bands could be observed on SDS gels, it was confirmed that human IgG was covalently bound to the S-layer glycoprotein and that no non-specific adsorption had occurred (not shown). Furthermore, no leakage of human IgG or S-layer protein could be observed after storing the SMP in 0.1 M phosphate buffer, pH 7.0, for 1 month at 4°C.

3.3. Determination of the orientation of human IgG immobilized to SMP

In order to obtain information on the orientation of the immobilized human IgG, defined amounts of SMP were incubated with an excess of anti-human IgG-peroxidase conjugate of different specificity. The molar ratio of peroxidase to human IgG was 1.3 for the Fab specific, 1.0 for the Fc specific and 1.1 for the γ-chain specific conjugates, respectively. After sedimentation of the SMP, the adsorbed anti-human IgG was determined via the peroxidase activity. As summarized in Table 1, the human IgG which was immobilized to the periodate oxidized carbohydrate chains was recognized by all the three different conjugates to a comparable extent, indicating a random orientation for the IgG molecules. Covalent attachment of human IgG to EDC activated carboxyl

groups of the acidic amino acids of the S-layer protein or to the cyanogen bromide activated carbohydrate chains led to comparable results. In both cases, more than 50% of the immobilized IgG was recognized by the γ-chain specific conjugate and approximately one third of the IgG molecules was recognized by the Fab specific conjugates. Thus, only a relatively small proportion of the IgG molecules was immobilized via the Fab region (Table 1).

3.4. The use of SMP with covalently bound human IgG as microparticles for immunoassays

In order to evaluate the use of SMP with immobilized human IgG as microparticles for immunoassays, they were adsorbed to microplates precoated with Fab, Fc or γ-chain specific anti-human IgG. Fab, Fc and γ-chain specific anti-human IgG peroxidase conjugates were used in the detection step (Fig. 3b). As shown in Fig. 3, for all the three methods of immobilizing human IgG, a clear correlation was obtained between the amount of applied SMP with differently immobilized human IgG and the absorption values at 405 nm. In addition, the absorption curves revealed a shape typical of ELISA curves. Since SMP has a cup-shaped structure and immobilization of human IgG occurred on the outer and inner S-layer lattice, positive results were also obtained when an anti-human IgG with identical specificity was used for the precoating and the detection step. This was in contrast to the results with free human IgG which in this case gave only very flat absorption curves (not shown).

When human IgG was linked to the EDC activated carboxyl groups from the S-layer protein or to the periodate oxidized carbohydrate chains, an approximately monomolecular layer of the antibody was generated (Table 1). Although the binding capacity of the EDC activated S-layer lattice was comparable to that of the periodate oxidized carbo-

Fig. 3. Absorption curves obtained after applying S-layer microparticles (SMP) with immobilized human IgG to microplates coated with Fab, Fc or γ-chain specific anti-human IgG and detected using Fab, Fc and γ-chain specific peroxidase conjugate. Human IgG was immobilized to carbodiimide (EDC) activated carboxyl groups from acidic amino acids of the S-layer protein (a, d, g) or to cyanogen bromide (BrCN) activated (b, e, h) or periodate (NaIO₄) oxidized (c, f, i) carbohydrate chains.

hydrate chains, the latter led to higher absorption values in the immunoassay (Fig. 3c,f,i). This is explained by the different location of the immobilized human IgG on the S-layer lattice. The IgG molecules bound to the acidic amino acids of the S-layer protein can be expected to be in intimate contact with the rigid protein moiety of the cross-linked S-layer lattice. It is also possible that IgG molecules linked to the flexible carbohydrate chains exposed on the outermost surface of SMP could favor the adsorption of the SMP to the precoated microplates. Because of this improved local accessibility, the human IgG bound to the carbohydrate chains may also be more accessible to the anti-human IgG peroxidase conjugate applied in the detection procedure. The reduced interactions between the human IgG linked to the carboxyl groups from the S-layer protein and the precoated microplates led to relatively low adsorption of SMP and less steep absorption curves in the immunoassays (Fig. 3a,d,g). Independent of the specificity of the anti-human IgG used in the precoating step, the use of a γ-chain specific anti-human IgG peroxidase conjugate gave the highest absorption values. When SMP with human IgG linked to the periodate oxidized carbohydrate chains were used in the immunoassays, the highest absorption values were obtained when the microplates were precoated with the γ-chain specific anti-human IgG and when an Fc specific or γ-chain specific anti-human IgG peroxidase conjugate was used in the detection procedure. As determined with suspended SMP, the human IgG molecules linked to the periodate oxidized carbohydrate chains were characterized by a nearly random orientation (Table 1).

The binding capacity of the cyanogen bromide activated carbohydrate chains resembled an approx. 40% coverage of the S-layer surface with human IgG. Because of this significantly lower binding density, a higher concentration of SMP had to be applied to the microplates to achieve an IgG concentration comparable to that of EDC activated and periodate oxidized SMP. With cyanogen bromide activated SMP, the highest absorption values were obtained when the microplates were precoated with a Fab specific anti-human IgG and when a γ-chain specific anti-human IgG peroxidase conjugate was used in the detection step. Precoating with a γ-chain

specific anti-human IgG was also shown to be advantageous (Fig. 3h). The lowest absorption values were measured for microplates precoated with an Fc specific anti-human IgG (Fig. 3e). These results were in accordance with data obtained on the orientation of the immobilized human IgG using suspended SMP (Table 1).

By immobilizing human IgG either to the protein moiety or to the carbohydrate chains which are exposed to the ambient environment (Sára et al., 1989), it could be demonstrated that the accessibility of the human IgG is of major importance for the adsorption of the SMP to the microplates, the steepness of the absorption curves and the maximum absorption values obtained in the immunoassays. Even within one method of immobilizing human IgG to SMP, the specificity of the anti-human IgG selected for precoating the microplates had a clear influence on the absorption curves obtained.

3.5. Electron microscopic examination of the immobilized human IgG by immunogold labeling

SMP with human IgG immobilized to the carbohydrate chains were labeled with a γ-chain specific anti-human IgG gold conjugate. As shown in Fig. 2d, the S-layer lattice was uniformly covered with gold particles indicating the presence of human IgG molecules on which the γ-chain was exposed. The binding density determined by this method was of the order of 20%.

4. Discussion

In the present study, human IgG was selected as a model system for evaluating the use of SMP as 'microparticles' for immunoassays. Depending on the immobilization procedure, the amount of covalently bound human IgG corresponded to 40–90% of the theoretical saturation capacity of a planar surface. Previous studies with S-layer ultrafiltration membranes as a matrix for immobilization of protein A or streptavidin for dipstick development have shown that immobilization of macromolecules as a monomolecular layer is advantageous because it prevents non-specific adsorption of components applied in the assay (Breitwieser et al., 1996). In contrast to

S-layer lattices, when amorphous polymers are used for the production of microplates (Kurosawa et al., 1995) or microparticles (Nilsson, 1989) closed monolayers of antibodies cannot be achieved. The maximum binding capacity for IgG of amorphous polymers such as preactivated agarose gels has been reported to be 150 mg/g dry gel (Füglistaller, 1989; Pharmacia and Pierce Data Sheets).

Immobilization of human IgG to the protein moiety or the carbohydrate chains of the S-layer glycoprotein lattice provided differing levels of accessibility of the antibodies. In general, covalent binding of human IgG to the flexible carbohydrate chains of the S-layer glycoprotein led to a higher response in the immunoassays. Since in this case the IgG molecules were located on the outermost surface of the S-layer lattice, adsorption of the SMP to the precoated microplates was favored. In addition to the location, the orientation of the immobilized human IgG had a clear influence on the adsorption of SMP to the precoated microplates.

Compared to polymeric matrices, one major advantage of SMP as an immobilization matrix for immunoassays can be seen in the high reproducibility in the number of macromolecules that could be covalently bound to the S-layer lattice. This was also reflected by the absolutely reproducible absorption curves and the constant signals in the immunoassays. Another specific feature of SMP is their cup-shaped structure. Since SMP possess an outer and an inner S-layer lattice, the surface available for immobilization of macromolecules, for binding processes and chemical reactions is significantly enhanced in comparison to materials with a flat surface. Moreover, it could be shown that SMP with immobilized human IgG revealed no non-specific adsorption to blank microplates.

Acknowledgements

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