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Magnetic beads functionalized with recombinant S-layer protein exhibit high human IgG-binding and anti-fouling properties

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ABSTRACT

Magnetic beads are routinely used for separation steps in many downstream processes in biotechnology. Functionalization of magnetic beads that will specifically bind to analytes with high capacity and specificity supported by low unspecific binding is a desired feature in bio-separation, *in vitro* diagnostics and immune precipitation. A novel approach to functionalize magnetic beads is the usage of recombinant S-layer fusion proteins. Here, $rSbpA_{31\text{-}1068}ZZ$ exploiting the fragment crystallisable (Fc) antibody binding region of Protein A was used as a model system. The intrinsic property of S-layer proteins to self-assemble in a crystalline monomolecular array on solid supports is combined with the functionality of the genetically introduced moiety. The highly ordered $rSbpA_{31\text{-}1068}ZZ$ S-layer lattice also presents the binding sites for IgG in an oriented, exposed and reproducible way down to the nanometer range. The coating procedure, overall handling properties and the resulting IgG recoveries of various magnetic beads having different diameters ranging from 0.271-1.48 μm and different surface properties (-NH_2, -COOH, epoxy groups) were investigated and compared. The $rSbpA_{31\text{-}1068}ZZ$-coated beads could specifically bind and recover IgG in high purity within 30 minutes. No unspecific binding of serum components was observed on the bead surface confirming the expected anti-fouling properties of S-layer-coated solid phases.

KEYWORDS: functionalization of magnetic beads, S-layer fusion proteins, downstream process, IgG purification, anti-fouling properties

ABBREVIATION

IgG : Immunoglobulin G

1. INTRODUCTION

Magnetic separation is an easy and fast-handling procedure that avoids columns and centrifugation steps. Due to uniformity of the bead size, shape and surface area, optimal accessibility and rapid liquid-phase reaction kinetics can be achieved. The magnetization of magnetic beads made of superparamagnetic materials increase with the applied field allowing fast separation or washing steps and are therefore perfectly suited for separation in a large range of applications such as solid phase immunoassays, high throughput screening, cell sorting, biosensors and immune precipitation [1-6]. Generally the magnetic core of such particles is encapsulated with polymers providing chemical and colloidal stability further introducing functional groups for additional surface modification [7]. Superparamagnetic particles exhibit magnetic properties only when an external magnetic field is applied. In the absence of a magnetic field these particles show high dispersibility in solution and avoid the formation of aggregates [7, 8].

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A key feature for functionalization of magnetic beads is the coating with a ligand that specifically binds to target molecules. The binding capacity of magnetic beads is not only dependent on the diameter of the beads, but also on the conformation and orientation of the bound ligands. Additional unspecific binding should be avoided to obtain high binding rates, specificity, high signal-to-noise ratios and purity.

A broad range of beads at various sizes with manifold surface properties, functional groups (amino, carboxylic acid or hydroxyl groups) or in pre-activated states (epoxy groups, tosyl and chloromethyl groups) is commercially available. This allows covalent attachment of ligands by chemical coupling or further functionalization with proteins (streptavidin, biotin, Protein A or Protein G) or antibodies for non-covalent binding of ligands. In many cases functionalization of magnetic beads follows standard chemical surface modification and crosslinking protocols resulting in randomly bound and oriented proteins with lower binding capacity or even protein denaturation [9]. Alternative approaches to bind IgG use ligands mimicking Protein A on dextran-coated magnetic beads [10] or gum Arabic-coated magnetic particles to purify monoclonal antibodies out of cell animal culture [11].

Another approach to provide a highly ordered binding matrix is the usage of S-layer proteins. Crystalline surface layers (S-layers) are the outermost part of the cell wall of many archaea and bacteria forming a uniform protein sheet fully covering the bacterial cell at all stages of growth [12, 13]. Their construction principle is based on a single type of protein or glycoprotein assembling into a highly ordered, porous array. An important property of isolated S-layer proteins is their intrinsic ability to re-assemble into crystalline lattices on various materials with the same repetitive physicochemical properties found originally on the cell, thus forming a stable uniform crystalline monolayer [14-18].

Finally, S-layer proteins by nature carry functional domains in defined position and orientation that enable them to interact with other biomolecules in a highly controlled and well-organized way so that S-layers can be used as carriers for those biomolecules [19, 20]. *Via* genetic engineering bioactive coatings based on fusion proteins consisting of an S-layer and an introduced moiety with specific biological activity, such as a streptavidin-, Protein A, an antibody- or antigen domain can be created [21-26]. Such crystalline S-layer fusion protein coatings allow for the reproducible, dense, oriented, and uniform presentation of binding sites while at the same time improving signal-to-noise ratios because of the intrinsic anti-fouling properties of the S-layer [13, 27-30].

The S-layer protein SbpA from *Lysinibacillus sphaericus* CCM 2177 [31] is an extraordinarily easy-to-handle coating system as the recrystallization can be induced by the addition of $CaCl_2$ to a monomeric protein solution. Also previous studies demonstrated that domains of the S-layer at the C-terminus could be replaced by other moieties without interfering with the lattice structure. As the S-layer attaches *via* the N-terminus to the solid phase the genetically introduced fusion domains remain exposed on the outermost surface of the protein lattice [31].

Based on the above-described application potential of recombinant S-layer proteins a comparative study was conducted herein with the aim of establishing whether S-layer coatings could be used for functionalization of magnetic beads. The recombinant S-layer protein rSbpA$_{31\text{-}1068}$ZZ comprising two IgG binding moieties from Protein A [23] was used as a model protein in this study to functionalize the magnetic beads. IgGs from distinct species can be bound to the S-layer fusion protein *via* the Fc region at neutral or basic pH and subsequently eluted at acidic pH, which corresponds to the intrinsic property of Protein A.

The procedure and stabilization of the S-layer coating onto the magnetic bead surface by cross-linking was optimized and investigated. The amount of rSbpA$_{31\text{-}1068}$ZZ that could be bound, the stability of the coating and the tendency of the beads to stay in a mono-disperse way were compared with beads having different sizes and surface chemistry. Additionally a test protocol was established for the binding of antibodies to rSbpA$_{31\text{-}1068}$ZZ functionalized beads and their recovery. Human IgG, which was chosen as the model system, was applied to the beads at basic pH and subsequently eluted from the beads by

applying a pH shift. The purity of the eluate and possible S-layer leakage was investigated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS Page) [32] and different coated beads types were compared.

Anti-fouling properties of all $rSbpA_{31\text{-}1068}ZZ$ functionalized magnetic beads were investigated by incubating the beads with human serum. The loaded beads and the respective eluates were tested for detectable serum protein bands using SDS Page.

2. MATERIALS AND METHODS

2.1. Production of S-layer protein

The chimeric gene encoding a C-terminally-truncated form of the S-layer protein SbpA from *Lysinibacillus sphaericus* CCM 2177 and two copies of the Fc-binding Z-domain was constructed, cloned, and heterologously expressed in *Escherichia coli* HMS174(DE3) as described in [23, 33]. The recombinant S-layer protein was over-expressed in *E. coli* and accumulated in inclusion body-like structures which were stored after a downstream processing that includes a homogenization step at -20 °C [25, 31].

2.2. Production of a monomeric S-layer protein solution

The starting point for the production of a monomeric protein solution of the S-layer fusion protein $rSbpA_{31\text{-}1068}ZZ$ was lyophilizates obtained after purification of inclusion bodies' extracts by gel chromatography as described previously [25]. The pooled fraction containing the S-layer protein was dialysed (membrane Biomol cut-off: 12-16 kD; pore size 25A) over night against Milli-Q water at 4 °C. After the dialyses step the protein solution was filtrated through a 0.2 µm syringe filter and lyophilized. Protein lyophilizates were completely dissolved in a chaotropic agent solution comprising 1 mg/mL 5 M GHCl (Gerbu Nr. 1057) in 50 mM Tris/HCl, pH 7.2 and dialyzed against Milli-Q to remove the chaotropic agent at RT. The retrieved protein solution was centrifuged at 14000 rpm (20,000 g) for 5 min to remove precipitations (e.g. self-assemblies). To determine the protein concentration of the supernatant UV measurements were performed at 280 nm using a spectrometer and a quartz cuvette. Subsequently the protein concentration was adjusted to 1 mg/ml with ice-cold Milli-Q water applying the absorbance coefficient for $rSbpA_{31\text{-}1068}ZZ$ (absorbance at 280 nm x 1,6529 = concentration in mg/ml). The recrystallization property of the monomeric protein solution was controlled using AFM (atomic force microscopy) as described previously [34] (Figure 1). The protein solution was stored at 4 °C for a maximum of 2 weeks.

2.3. Coating of magnetic beads with $rSbpA_{31\text{-}1068}ZZ$

Although there are many different magnetic beads available on the market, it was chosen to use beads from one supplier only (ESTAPOR, France). In this case the variability of solid content and magnetic pigments are negligible, allowing a fair comparison of the coating success of the recombinant S-layer $rSbpA_{31\text{-}1068}ZZ$ and the functionality to bind IgG that depends only on size and surface chemistry. All beads are characterized by a core-shell structure having an iron oxide material encapsulated by a film of polymer made of polystyrene and were obtained in a liquid state having a solid content of 10% (Table 1).

All washing steps were carried out using the SepMag A system (SepMag, Spain) allowing extremely fast separation and up scaling of the used processes.

First, M1 070/40 and M2 070/40 beads that have identical bead properties except for the -COOH or $-NH_2$ surface functionality were used to establish a coating and crosslinking protocol and to develop a human IgG binding test to decide whether beads exhibiting amino or carboxylic acid groups should be used in further studies. Here 140 µg $rSbpA_{31\text{-}1068}ZZ$ per mg beads were applied in recrystallization buffer (10 mM $CaCl_2$ in 5 mM Tris, pH = 9.0) and bound recombinant S-layer protein was cross-linked with 10 mM DMP (Dimethyl-pimelimidate-dihydrochloride; Fluka 80490) in 0.1 M Hepes buffer (N-(2hydroxyethyl) piperazine-2-ethanesulfonic acid; GERBU 1009) pH 8.0 containing 10 mM $CaCl_2$ for 90 min. The reaction was stopped by the addition of Tris buffer and beads were stabilized and stored at a concentration of 5 mg/ml in StabilGuard (SurModics; 1:2 with PBS) added with azide (15 mM).

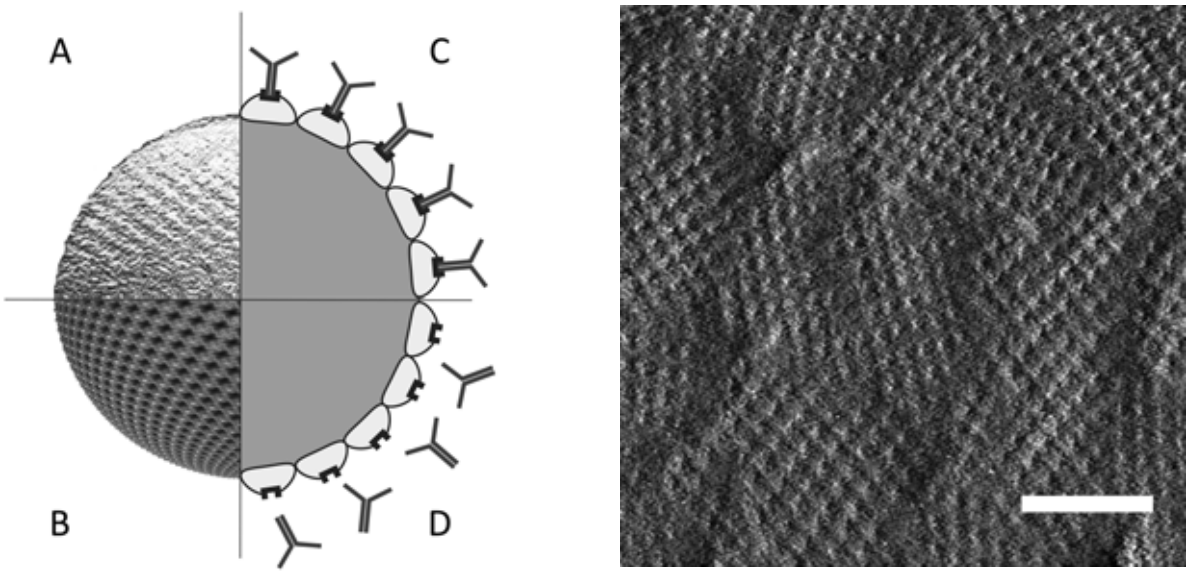

Figure 1. Left: Tem image of the surface of a freeze-etched and metal-shadowed S-layer-coated liposome **(A)** [35, 36]; computer model of magnetic beads coated with a crystalline S-layer **(B)**; schematic drawing of rSbpA$_{31\text{-}1068}$ZZ functionalized magnetic beads allowing the oriented binding of IgGs at basic or neutral pH **(C)** and elution and recovery of IgGs at acidic pH **(D)**. Right: AFM image of rSbpA$_{31\text{-}1068}$ZZ recrystallized on silicon wafer. The crystalline S-layer showing square (p4) lattice symmetry is clearly visible. Bar 100 nm.

Table 1. The surface properties and sizes of bead types from Estapor are shown.

Product No.	Diameter (µm)	Surface group	Magnetic pigment %	Abbreviation of the S-layer-coated beads
M1 070/40	1.48	-COOH	35-45	M1ZZ
M2 070/40	1.48	-NH_2	35-45	M2ZZ
EM1 100/40	0.89	-COOH	56	EM1 ZZ
EM2 100/40	1.30	-NH_2	40	EM2A ZZ
EM2 100/40	1.05	-NH_2	48	EM2B ZZ
R 04-11	0.271	-NH_2	50	R ZZ
R 06-19	1.23	Epoxy groups	40.3	R Ep ZZ

Beads with smaller diameter were coated overnight with higher amounts of S-layer proteins (300-500 µg/mg). Also up-scaling experiments, where up to 250 mg beads were coated in one approach, were performed.

In the final coating procedure beads were washed two times with crystallization buffer using the SepMag A magnetic device allowing at least 5 min separation time for each washing step. The recombinant protein solution was applied at a concentration of 200 µg/ml crystallization buffer. After mixing the beads with the prepared protein solution, recrystallization was allowed to take place overnight by gentle rotation using an overhead rotator (Heidolph, Reax 2) at 4 °C. Subsequently unbound rSbpA$_{31\text{-}1068}$ZZ was removed from the beads using the magnetic device, followed by washing the beads two times with crystallization buffer. All supernatants were collected, combined and after the volume was measured by weight the concentration was determined by reading the UV value of an aliquot at 280 nm using the Spectronic

Genesys® 5 photometer after a 10 min centrifugation step (Hermle Z233 MK centrifuge) to guarantee that no beads interfere with the reading. The amount of bound $rSbpA_{31-1068}ZZ$ per mg beads was calculated by subtracting the concentration of applied protein from the amount of protein that were not bound.

Subsequently the $rSbpA_{31-1068}ZZ$-coated beads were washed once in 100 mM HEPES buffer pH 8.0 containing 10 mM $CaCl_2$. To crosslink the S-layer protein the beads were re-suspended with 10 mM DMP in 100 mM HEPES buffer pH 8.0 containing 10 mM $CaCl_2$. The beads were incubated using a Heidolph Reax 2000 vortex shaker at RT for 90 min. To stop the cross-linking process the entire solution was transferred into Tris buffer (50 mM; pH 7.2) and incubated for 15 min. Finally, the beads were washed two times with PBS buffer pH 7.2 and diluted with StabilGuard to the desired concentration (5 mg coated beads/ml and 1 mg coated beads/ml for the smaller R ZZ beads) to determine the volume that had to be added by weight, using the empty container as tare weight. All separation steps were performed using the magnetic device.

Beads having epoxy groups available for covalent binding (R06-19) followed the identical coating protocol as described above with the exception that $rSbpA_{31-1068}ZZ$ was applied directly in 100 mM HEPES buffer pH 8.0 containing 10 mM $CaCl_2$ at a concentration of 400 µg/ml without any further cross-linking step.

2.4. Studies on the binding of human IgG to $rSbpA_{31-1068}ZZ$-coated beads

A human IgG stock solution was prepared (SIGMA; I4506: reagent grade > 95%) (10 mg/ml in 0.9% NaCl) and 100 µl aliquots were stored at -20 °C. After thawing at RT, 900 µl glycine buffer pH 9.0 was added resulting in a final concentration of 1 mg/ml. The IgG solution was further diluted to the desired concentration (200-500 µg/ml in 0.1 M glycine buffer pH = 9.0).

5 mg magnetic beads (or 1 mg for the smaller R ZZ beads) coated with $rSbpA_{31-1068}ZZ$ were transferred into Eppendorf tubes and washed two times with 1-2 ml 0.1 M glycine pH = 9.0 using the magnetic device from SepMag. During the washing steps the beads were separated using a vortex. Subsequently the beads were incubated with 500 µl of a solution containing 200-500 µg IgG on a Heidolph Reax 2000 overhead shaker at RT for 10 min.

Afterwards the beads were washed with 1 ml 1 M NaCl. The bound IgG was eluted by applying a pH shift by incubating the beads in 1 ml 0.1 M glycine buffer (pH 2.5) on a shaker for 10 min. Then the tubes were put into the magnetic device and the eluate containing IgG was collected followed by a washing step with 0.1 M glycine pH 2.5. All supernatants were collected and further investigated. Used beads were discarded or stored at 4 °C in StabilGuard with 0.02% NaN_3 (Sigma, S2002) for reusability studies.

The amount of adsorbed and eluted IgG was determined by $UV_{280\ nm}$ measurements of the supernatants, washing waters and elution buffers and calculated using an extinction coefficient of 1.4 for human IgG (datasheet, SIGMA Aldrich). For all tests a centrifugation step was crucial as traces from beads influenced the UV values.

2.5. Purity of the eluates and stability of the $rSbpA_{31-1068}ZZ$ coating

The eluates were investigated with SDS Page to obtain information about the IgG concentration, purity and leakage of S-layer protein. For this, the eluates were diluted with 4-time concentrated Laemmli buffer containing ß-mercapto-ethanol and applied to a 10% SDS Page gel. A dilution series of human IgG was used, allowing for a rough estimation of the IgG concentration in the eluates. The purity of the eluates and the presence of S-layer protein in the case of protein leakage were investigated using Coomassie and silver staining.

2.6. Investigations of anti-fouling properties of $rSbpA_{31-1068}ZZ$-coated beads

To investigate the anti-fouling properties especially the unspecific binding of serum components onto magnetic beads all $rSbpA_{31-1068}ZZ$-coated beads were incubated with a known human IgG concentration (190 µg) spiked to IgG depleted serum (Sigma S5143) for 10 min. Subsequently the IgG binding assay was performed as described above and the eluates were diluted in 4-time concentrated Laemmli solution without β-mercapto-ethanol. Subsequently, a SDS Page was performed.

Depleted serum and human IgG were used as standard.

3. RESULTS

3.1. Coating of magnetic beads with rSbpA$_{31\text{-}1068}$ZZ

Optimization steps were carried out to determine the success of the rSbpA$_{31\text{-}1068}$ZZ coating using the beads M1 070/40 and M2 070/40. If 140 µg rSbpA$_{31\text{-}1068}$ZZ was applied per mg magnetic beads, M1 070/40 could be coated with 91 µg rSbpA$_{31\text{-}1068}$ZZ/mg and M2 070/40 beads could be coated with 81 µg rSbpA$_{31\text{-}1068}$ZZ/mg. The stability of the S-layer could be improved by cross-linking the protein since SDS-Page of rSbpA$_{31\text{-}1068}$ZZ-coated beads exhibit no S-layer protein band if the beads are cooked in Laemmli buffer (data not shown). Separation of beads using the SepMag A device could be performed within 5 minutes, even for larger volumes. A minimum of 3 min per separation step using the magnets was performed.

First results indicated that raising the amount of rSbpA$_{31\text{-}1068}$ZZ applied could increase the amount of rSbpA$_{31\text{-}1068}$ZZ attached onto the bead surface. In almost all approaches more than 80% of S-layer protein applied could be bound onto the magnetic beads. Therefore, in all further approaches beads were coated with 300 µg/mg, and the smaller beads having a diameter of 200 nm were incubated overnight with 500 µg S-layer protein per mg beads. If 300 µg rSbpA$_{31\text{-}1068}$ZZ was applied per mg magnetic beads, EM1 100/40 could be coated with 288 +/- 41 µg rSbpA$_{31\text{-}1068}$ ZZ/mg, EM2 100/40 having a diameter around 1.3 µm with 217 +/- 11.2 µg/mg and EM2 100/40 beads (d = 1.05 µm) could be coated with 253 +/- 12.8 µg rSbpA$_{31\text{-}1068}$ZZ/mg. The smallest beads R 04-11 used in this study could be coated with 437 +/- 12 µg/mg if 500 µg rSbpA$_{31\text{-}1068}$ZZ/mg beads was applied.

Magnetic beads R06-19 with epoxy surface chemistry were investigated for their ability to covalently bind to rSbpA$_{31\text{-}1068}$ZZ. If 400 µg/mg was applied, approximately 240 +/- 90 µg/mg rSbpA$_{31\text{-}1068}$ZZ could be bound per mg beads. After coating the solutions became turbid and the amounts that could be coated were not reproducible. The results of all the studies on the binding of rSbpA$_{31\text{-}1068}$ZZ S-layer onto magnetic beads from ESTAPOR are summarized in table 2.

3.2. Studies on the binding of human IgG to rSbpA$_{31\text{-}1068}$ZZ-coated beads

The amount of bound and eluted IgG was determined using $UV_{280\ nm}$ measurements (Table 2), and confirmed by protein assays and with SDS Page using Coomassie and Silver Staining. Human IgG having a high affinity towards Protein A was chosen as a model system, thus allowing to investigate the binding capacities of recombinant rSbpA$_{31\text{-}1068}$ZZ-coated magnetic beads. Human IgG was applied in glycine buffer at pH 9.0 and after washing with 1 M NaCl, bound IgG was eluted by applying a pH shift to pH 2.5. All separation steps were performed using magnets from SepMag. Although the magnetic separation was extremely fast (within 30 seconds, detected visually) for $UV_{280\ nm}$ measurements of the supernatants, a centrifugation step was performed to guarantee that no beads affected the results. As no S-layer leakage was seen in SDS Pages of the eluates (Figure 2) the $UV_{280\ nm}$ values could be used to quickly determine the amount of eluted IgG and bound IgG, after subtraction of the bound from applied concentration. SDS Page of the eluates and protein tests (Lowry) roughly confirms the human IgG concentration in the eluates. The entire procedure could be carried out within 30 minutes.

Both bead types (M1ZZ, M2ZZ) allowed similar concentrations of S-layer proteins that could be bound and IgG that could be recovered. Standard human IgG test showed that if 170 µg IgG was applied to 5 mg each of M1ZZ and M2ZZ beads, 124 +/- 2 µg IgG could be recovered using the M1ZZ beads and 126 +/- 6 µg IgG could be recovered using the M2ZZ beads by applying a pH shift. But diminished handling properties including aggregation of the beads, binding of beads to the container walls and less reproducibility were observed for the magnetic beads exhibiting carboxylic acid groups on the surface. This could be explained by the crosslinking step with DMP necessary to avoid S-layer leakage. Here, intra-molecular covalent bonds will occur not only between the S-layer proteins but also between the

Table 2. Results of S-layer coating and IgG recovery concentrations are displayed (mean +/- standard deviation). Concentrations of applied and unbound rSbpA$_{31\text{-}1068}$ZZ were determined using $UV_{280\ nm}$ measurements and the amount of bound rSbpA$_{31\text{-}1068}$ZZ was calculated. The concentration of applied and recovered human IgG is presented for various beads types ($UV_{280\ nm}$). For the IgG recovery assay the amount of 5 mg beads was used with the exception for R ZZ beads where 1 mg was used. Both, coating and IgG recovery studies were repeated at least for 6 times.

Bead type	rSbpA$_{31\text{-}1068}$ZZ applied µg/mg	rSbpA$_{31\text{-}1068}$ZZ bound µg/mg	h IgG applied µg/5mg beads	h IgG eluted µg/5mg beads
M1ZZ	140	91	170	124 +/- 2
M2ZZ	140	81	170	126 +/- 6
EM1 ZZ	300	288 +/- 41	170	128 +/- 32 µg
EM2A ZZ	300	217 +/- 11	170	146 +/- 12 µg
EM2B ZZ	300	253 +/- 13	375	215 +/- 5
R Ep ZZ	400	240 +/- 90	170	110 +/- 10
R ZZ	500	437 +/- 12	375 µg/mg	174 +/- 7 µg/mg

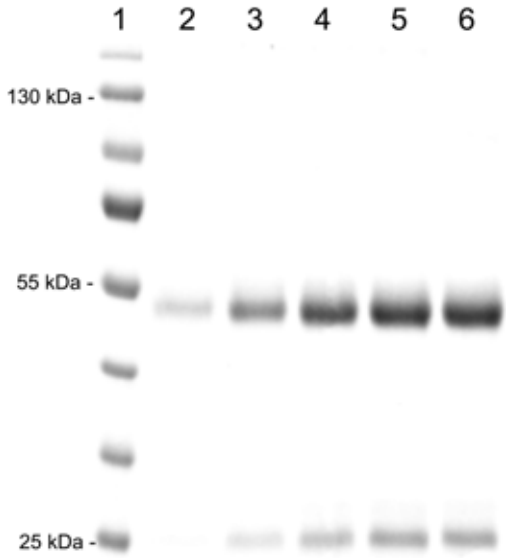

Figure 2. SDS Page. 1) Marker; 2-5) human IgG dilution series 50, 100, 150 and 200 µg/lane; 6) human IgG in the eluate of 5 mg EM2B ZZ beads.

amino groups of the S-layer proteins and the amino groups of the beads, resulting in the stabilization of the coating and a change in the surface charge. Crosslinking of the rSbpA$_{31\text{-}1068}$ZZ after recrystallization was necessary to increase the stability of the S-layer protein coating under harsh conditions (pH 2.5) in the test used for IgG recovery.

EM1 ZZ beads could bind to 137 +/- 42 µg human IgG whereof 128 +/- 31 µg could be recovered. Again beads exhibiting carboxylic acid groups on the surface showed a high degree of irreproducibility. EM2A ZZ beads could bind to 168 +/- 12 µg IgG/5 mg beads. Here 146.3 +/- 12.0 µg could be recovered by lowering the pH. This result could be confirmed by performing a standard Lowry protein assay (158 µg/5 mg beads).

Though for the beads described so far the amount of IgG recovered could not be raised by applying higher IgG concentration, EM2B ZZ beads having a diameter of 1 µm could bind to 270 +/- 5 µg IgG, and 215 +/- 5 µg IgG could be detected in the eluate if 375 µg IgG was applied to 5 mg beads.

Pre-activated epoxy beads exhibited a high variation in the IgG-binding capacity. Also S-layer leakage was observed in the eluate after applying a pH shift. Therefore the results obtained with UV measurements were not reliable (data not shown).

The smallest beads in this study R ZZ could bind to 203 +/- 3.7 µg human IgG per mg rSbpA$_{31\text{-}1068}$ ZZ-coated beads, whereof 174 +/- 6.6 µg could be recovered per mg if a pH shift was applied. But not all beads could be separated using the magnetic device as traces of them could always be found in the supernatants.

3.3. Purity of the eluates

The $UV_{280\ nm}$ data are reliable only if IgG is present in the eluted samples. Therefore, a SDS Page of the eluates was performed. Coomassie staining roughly confirmed the measured IgG concentration by comparing the band intensity to a known IgG concentration series for all the used bead types.

Compared to other beads the results of the SDS Page for eluates of the EM2B ZZ shown in figure 2 confirmed the findings of $UV_{280\ nm}$ measurements. For EM2B ZZ beads the recovered IgG concentration could be estimated using 200 µg/5 mg beads, which is in good accordance with the UV measurements where 215 +/- 5 µg/5 mg beads could be detected. As only IgG, but no S-layer protein bands, could be detected in the eluate the stability of the S-layer coating within the elution procedure could be shown.

With EM2A ZZ rSbpA$_{31\text{-}1068}$ZZ-coated beads an IgG concentration in the range of 100 to 150 µg (IgG that corresponds to the data measured with UV (146.3 +/- 12.0 µg)) could be detected in the eluate. The eluate of the ZZ-coated epoxy beads showed high molecular protein bands, which could be related to S-layer protein (data not shown).

Additionally with SDS Page and subsequently using the more sensitive silver staining method the purity of the eluates could be confirmed as no S-layer leakage could be detected, further confirming the usability of $UV_{280\ nm}$ measurements to determine the human IgG concentration as no other proteins were detectable. In all eluates of the bead types EM1 ZZ, EM2A ZZ and EM2B ZZ no leakage of rSbpA$_{31\text{-}1068}$ZZ could be detected confirming the stability of the S-layer coating and the purity of the eluates.

3.4. Anti-fouling properties of rSbpA$_{31\text{-}1068}$ZZ-coated beads

To investigate unspecific binding and anti-fouling properties of rSbpA$_{31\text{-}1068}$ZZ-functionalized magnetic beads, human IgG was spiked into IgG depleted serum and the binding of serum components onto the bead surface was visualized with SDS Page (Figure 3).

The human protein components, which could be clearly seen in sample 2 and 3, could not be detected in the rSbpA$_{31\text{-}1068}$ZZ-coated magnetic bead samples indicating no unspecific binding of the major human serum proteins to the coated bead surface. Additionally, in a second approach bound IgG was eluted with a pH shift. The eluates showed a satisfying purity (Figure 3) as again no serum components could be seen.

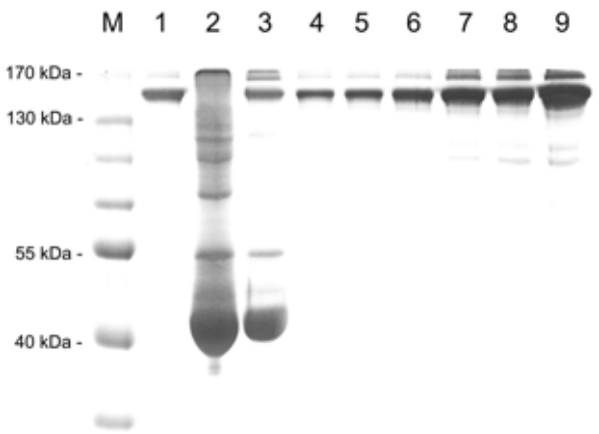

Figure 3. SDS Page of eluates checking the purity of human IgG obtained from serum. To avoid overlapping protein bands no reducing agent was used in the Laemmli buffer. 1) human IgG standard; 2) human serum IgG depleted; 3) human serum IgG depleted plus IgG spike; 4-6) beads incubated with serum and investigated with SDS Page to release and detect bound proteins; 4) EM1 ZZ beads; 5) EM2A ZZ beads; 6) EM2B ZZ beads; 7) eluate of EM1 ZZ beads incubated with serum; 8) eluate of EM2A ZZ beads incubated with serum; 9) eluate of EM2B ZZ beads incubated with serum.

Further, the S-layer-coated beads showed excellent reusability. The beads could be used 5 times without decrease of the binding properties (data not shown). Also stability studies were conducted showing the maintenance of the binding properties even after one week storage at 37 °C.

4. DISCUSSION

Crystalline bacterial cell surface layers (S-layers) are monomolecular arrays of protein or glycoproteins that are found as the outermost cell envelope component of many bacteria and archea. After isolation from the cell wall or in the case of recombinant S-layer proteins after extraction out of inclusion bodies, many S-layer proteins maintain the ability to self-assemble in suspension

or to recrystallize on solid supports, opening a broad potential for application in biotechnology, molecular nanotechnology and biomimetics [13]. S-layer fusion proteins represent the most accurate way of controlled patterning and presenting recombinant-introduced functionalities in a highly ordered crystalline monolayer at the nano-meter scale.

The S-layer fusion protein rSbpA$_{31-1068}$ZZ comprising two IgG-binding domains of Protein A was used as the model system to investigate the potential of recombinant S-layer proteins to functionalize magnetic beads. Comparative studies using different bead types with regard to size and surface properties showed that beads with amino groups have a better reproducibility and colloidal behaviour after the S-layer coating and within the IgG recovery assay compared to beads presenting carboxylic acid groups on the surface. Depending on the bead size, up to 437 µg rSbpA$_{31-1068}$ZZ/mg could be recrystallized on the bead surface (diameter of 270 nm). Beads with diameters ranging from 890 nm, 1050 nm and 1300 nm could be coated with 288 +/- 41 µg, 253 +/- 13 µg and 217 +/- 11 µg per mg, respectively, indicating that the coating success depends on the size and surface area of the beads. Preactivated magnetic beads with epoxy groups exhibited a large deviation in the amount of covalently bound rSbpA$_{31-1068}$ZZ.

The most robust system with regard to magnetic separation, reproducibility and excellent IgG binding and recovery properties was found to be rSbpA$_{31-1068}$ZZ-coated EM2 100/40 beads with a diameter of 1 µm and amino groups on the surface.

IgG binding studies showed that at basic pH 270 +/- 5 µg IgG could bind, and 215 +/- 5 µg IgG could be recovered in the eluate by applying a pH shift per 5 mg beads. The entire assay (binding of IgG at neutral or basic pH and recovery of IgG by applying a pH shift to pH 2.5) could be performed within 30 min confirming the easy handling properties of magnetic beads for separation and purification purposes.

By investigating the purity of the eluates with SDS Page no S-layer protein leakage could be detected in the eluates, confirming sufficient stability of the cross-linked rSbpA$_{31-1068}$ZZ layer even under the harsh condition of a pH shift. The anti-fouling properties of rSbpA$_{31-1068}$ZZ-coated magnetic beads serum samples were confirmed as IgG could selectively be purified out of serum. The rSbpA$_{31-1068}$ZZ-coated beads showed no binding of serum components [27].

To compare the capacity of S-layer-functionalized beads to bind IgG with commercially available magnetic beads Protein A-functionalized beads from Dynal (INVITROGEN) were investigated following the IgG recovering protocol of the producer. The amount of IgG that could be recovered per mg beads was about 3 times lower compared to rSbpA$_{31-1068}$ZZ-coated beads. But, it must be taken into consideration that the Dynal beads exhibit a smaller surface area (2.8 µm diameter) compared to the EM2B ZZ beads (1.05 µm diameter). Moreover, the exact amount of Dynal beads used could only be estimated, as the exact concentration of the beads in the solution was not given by the producer (supplied at a concentration of 30-40 mg/ml, personal communication).

5. CONCLUSION

The use of S-layer fusion proteins is a novel approach to functionalize magnetic beads. In this study, the S-layer protein rSbpA$_{31-1068}$ZZ comprising two IgG-binding domains of Protein A was used as a model system. The S-layer coating resulted in a highly ordered crystalline monolayer introducing densely packed IgG binding sites. In addition to the intrinsic anti-fouling properties of S-layer proteins a highly effective system to purify IgG from human serum could be generated.

Magnetic beads with diameters in the range of 1 µm presenting amino groups on the surface fulfilled the requirements with regard to stability, fast separation and mono-dispersity in combination with the S-layer coating and the subsequently performed IgG purification assay.

S-layer fusion protein coatings may represent an alternative protein immobilization mechanism on magnetic beads. The high versatility of the S-layer coating system and the availability of other S-layer fusion proteins allow the development of bioactive coatings with various different binding molecules such as peptides, antibody domains and enzymes.

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CONFLICT OF INTEREST STATEMENT

The authors declare no financial or commercial conflict of interest.

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