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Development of phage-based antibody fragment reagents for affinity enrichment of bacterial IgG binding proteins

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ABSTRACT

Disease and death caused by bacterial infections is a global health problem. Effective bacterial strategies are required to promote survival and proliferation within a human host and it is important to explore how this adaption occurs. However, the detection and quantification of bacterial virulence factors in complex biological samples are technically demanding challenges. These can be addressed by combining targeted affinity enrichment of antibodies with the sensitivity of LC-SRM MS. However, many virulence factors have evolved properties that make specific detection by conventional antibodies difficult. We here present an antibody format that is particularly well suited for detection and analysis of IgG-binding virulence factors. As proof of concept we have generated scFv antibodies that specifically targets the IgG-binding surface proteins M1 and H of *Streptococcus pyogenes*. The binding ability of the developed scFv is demonstrated against both recombinant soluble protein M1 and H as well as the intact surface proteins on a wild-type *S. pyogenes* strain. Additionally, the capacity of the developed scFv antibodies to enrich their target proteins from both simple and complex backgrounds, thereby allowing for detection and quantification with LC-SRM MS, was demonstrated. We have established a workflow that allows for affinity enrichment of bacterial virulence factors.

KEYWORDS

selected reaction monitoring (SRM), mass spectrometry, virulence factors, Streptoccous pyogenes, scFv antibody fragments, in vivo biotinylation, affinity proteomics

INTRODUCTION

Bacterial infectious diseases are a major cause of death worldwide. The survival and proliferation of a bacterial pathogen inside the human host requires effective bacterial defense strategies to evade host innate and adaptive immune system. These evasion strategies are orchestrated by a large number of virulence factors expressed by the bacteria responsible for degrading or binding to specific host proteins to enhance bacterial survival. The complex and sometimes redundant function of the virulence factor repertoire requires quantitative measurement of both virulence factors and host proteins to reveal the molecular mechanisms behind virulence and antimicrobial resistance, which is an increasingly serious threat to global public health [1]. The rapid development of mass spectrometry based proteomics techniques has resulted in several studies outlining close to complete relative quantitative analysis of bacterial proteomes, showing how bacterial pathogens can adapt to host-like environments *in vitro* [2,3]. Via the use of heavy labeled stable isotope reference peptides, absolute protein quantification of a substantial part of the bacterial proteome is possible [4,5,6]. However, to accurately determine how bacterial pathogens adapt to the host *in vivo*, absolute measurement of important virulence factors during the disease process directly in samples obtained from *in vivo* is required.

Selected reaction monitoring (SRM) mass spectrometry has increasingly been applied for more quantitatively accurate measurements of predetermined sets of proteins [7]. In SRM, *a priori* defined tryptic peptides generated from whole proteome digests are specifically and exclusively targeted with developed SRM assays using triple quadrupole mass spectrometers, providing identification and quantification of proteins with high sensitivity and specificity [7]. Yet, mass spectrometric measurements of specific bacterial proteins from *in vivo* isolated samples, such as swabs or body fluids like blood plasma, serum and cerebrospinal fluid, is challenging as abundant host proteins can

prevent identification of the target proteins. A promising strategy to enable quantitative analysis of minute amounts of target proteins in complex biological samples is to combine the sensitive detection and quantification of SRM with targeted immunoaffinity enrichment using antibodies. Several different platforms combining the sensitivity of antibody enrichment with the specificity of mass spectrometry analysis have been established for quantitative measurements of proteins in plasma and serum [8-11]. In addition, the use of antibodies has also become increasingly important for tracking specific microorganisms in various infectious diseases [12,13].

Developing specific antibodies for microorganisms can be challenging for several reasons. Firstly, the efficient capture of a specific protein from a microorganism requires that the targeted protein is available in the extracellular space. This means that the protein needs to be surface exposed or secreted and constitutively expressed under *in vivo* conditions, preferably at high concentrations. Secondly, the targeted epitope needs to be accessible. Many microorganisms have developed strategies to avoid detection by antibodies, by for example, binding to host proteins to mask the immunogenic parts of the bacterial proteins [14], whereas other parts of the protein can be hypervariable [14]. For example, several pathogenic bacteria produce proteins that can immobilize immunoglobulin G (IgG) by binding the fragment crystable (Fc) part of IgG [15-18]. The presence of IgGFc-binding proteins can protect the bacteria against phagocytosis and the bacteria is thereby protected in its natural environment [19]. These IgGFc binding surface proteins makes it difficult to specifically detect and capture the bacteria in a complex mixture using affinity probes such as antibodies. Lastly, the development of antibodies against bacterial proteins with methods relying on immunization can potentially be problematic due to the toxicity of these.

Immunoaffinity enrichment or immunological detection requires a suitable antibody or capture molecule. The availability of appropriate antibodies still remains a major bottleneck in the establishment of immunoassays [8]. Phage display-technology [20,21] is the most commonly used *in vitro* system for the development of specific antibodies. In phage display the antibody is displayed on the surface of the phage particle while the genes encoding the antibody is kept within the phage particle [22]. By cloning millions of genes encoding different antibodies into the phage genome, large libraries can be created and used for isolation of antibodies with desired specificities and characteristics [22]. Antibody phage display libraries are often constructed with either single chain variable (scFv) or Fab fragments [23]. The scFv antibody format consists of the variable regions of the antibody connected by a linker thereby retaining its antigen binding ability but having no Fc region. Consequently, the use of this format diminishes the risk of IgGFc-binding between the target protein and the antibody.

The major aim of this study was to develop antibodies suitable for immunoaffinity enrichment through phage-display technology to enable bacterial detection with SRM mass spectrometry in complex samples, using the major human pathogen *Streptococcus pyogenes* as a model system. One of the most abundant surface proteins expressed by *S. pyogenes* is the M protein, which is regarded as a major virulence factor due to its anti-phagocytic ability [24]. The M protein is expressed on the bacterial surface, but local considerable concentrations of secreted M protein can be reached due to self-cleavage from the bacterial surface [25]. There are more than 100 different serotypes of the M proteins [26], all consisting of a hypervariable N-terminal, a less variable central domain and a more well conserved C-terminal [27]. The M1 protein consists of repeat regions, starting with the 91 amino acid long A domain at the N-terminus, followed by two repeat sequences of 28 residues each (domain B1 and B2) and then a 35 amino acid, non-repeat

sequence (domain S). Following the S domain is three repeat regions of respectively 42, 42 and 31 amino acids (domains C1, C2 and C3) and then the rest of the protein consists of the D domain [28]. The binding sites of plasma proteins albumin, fibrinogen and IgG are mapped to the C domains, B domains and S domain respectively [28 29]. Some *S. pyogenes* strains also produce M-like proteins such as protein H [28 30]. The function of protein H is less well characterized than that of the M1 protein. Importantly however, protein H just like M1 is unique to *S. pyogenes,* and is therefore a suitable target for immunological detection of this bacterium. Both M1 and protein H possess features typical to virulent bacterial proteins, by for example their ability to bind the IgGFc part [18,24,28,30]. Injection of protein M1 into mice has been shown to cause vascular leakage and lung lesions [25,31]. Rabbits injected with protein H have been shown to develop anuria and died within 48 h [32]. An *in vitro* system, such as phage display, that would allow us to by-pass immunization, is therefore particularly desirable when generating antibodies against these types of bacterial proteins.

In this article, we show the isolation of scFv fragments against protein H and M1 of *S. pyogenes* using phage display. A particular vector construct was used that allows for site-specific *in vivo* biotinylation whilst expressing the scFv [33,34]. This format allows us to overcome the typical shortcomings of using full-length antibodies to analyze Fc binding antigens, which potentially could lead to a non-specific assay. We show that the developed scFv antibodies specifically bind their target proteins both as soluble proteins as well as a part of the intact bacteria. The developed scFvs were further shown to have the ability to enrich their target proteins and successfully enable detection in complex samples by SRM-MS. To conclude, a workflow has been established that allows immunoaffinity enrichment of bacterial target proteins.

EXPERIMENTAL PROCEDURES

Materials

Streptococcal proteins M1 and H were produced and purified as previously described [28,32,35]. The bacterial strains AP1, BM27.6, MC25 and BMJ71 were cultured as previously described [36,37]. The M1 peptide fragments ABS, AB, DCS and DC were kindly provided by Prof. Lars Björk (Division of Infection Medicine, Lund University). Antibody fragments were selected against the target proteins from the Hell-11 phage display library. In short, HELL-11 is a human synthetic scFv library based on heavy chain (IGHV3-23) combined with either kappa light chain (IGKV1-39) or lambda light chain (IGLV1-47). Diversity was mainly restricted to surface-exposed residues in four of the six complementarity determining (CDR) loops; CDR-H1, CDR-H2, CDR-H3 and CDR-L3, using trinucleotide primers and an optimized Kunkel mutagenesis methodology similar to previously described by Fellouse and Sidhu [38]. Library size was estimated to >2 x 10^{10} unique members, as measured by the number of bacterial colonies obtained after transformation.

Selection of specific scFv antibodies by phage display

Phage display selections were performed on proteins M1 and H, 1 ml of target proteins at a concentration of 5 µg/ml in phosphate buffered saline (PBS) was immobilized in immunotubes (Nunc A/S, Roskilde, Denmark) over night at 4 °C. Unbound protein was removed by washing once with 4 ml 0.05% Tween20 in PBS (PT) and subsequently blocked at room temperature for 1 hour with 0.5% BSA and 0.05% Tween20 in PBS (PBT). In order to remove potential non-specific binders, the phage stock was prior the selection incubated with immunotubes coated over night at 4 °C with 2 ml streptavidin at a concentration of 5 mg/ml in PBS. Subsequently, the phage stock was transferred to the antigen-coated immunotube and incubated for 1.5-2 hours with slow rotation at room

temperature. The immunotubes were washed 6-11 times with PT followed by one wash with PBS. Bound phages were eluted with 1 ml trypsin at a concentration of 0.5 mg/ml and incubated for 30 minutes at room temperature. The enzymatic activity of trypsin was inhibited by the addition of 0.5 ml aprotinin (0,2 mg/ml). The eluted phages were propagated by infection of *E.coli* XL1-blue (Stratagene, La Jolla, CA, USA), infected with M13K07 (New England Biolabs, Ipswich, MA, USA) for phage production and amplified in solution over night at 30 °C. Harvested phage stocks were precipitated with PEG8000/NaCl and re-suspended in PBT. The precipitated phage stock were used for subsequent selection rounds as described above with the exception that the protein target concentration was decreased to 2.5 µg/ml in the third round and to 1.0 µg/ml in the fourth round.

Re-cloning of specific scFv antibodies and determination of specific binding

DNA from the third and fourth round of selection was isolated and recloned to allow production of scFv antibodies in a soluble format. The DNA was digested with *SfiI* and *AvrII* (Fermentas, Thermo Fisher Scientific Inc., Waltham, MA, USA) and ligated into a novel developed vector (Figure 1b). The vector system provides the soluble scFv with a biotin acceptor domain (BAD) and a hexahistidine tag (HIS) at their C-termini. The gene encoding the scFv is connected to the BAD gene by a gene encoding the IgA-hinge region (Hin). The constructs were transformed into AVB101 (Avidity LLC, Aurora, Colorado, USA), an *E. coli* B strain containing pBirAcm, encoding biotin ligase and chloramphenicol resistance (Avidity LLC, Aurora, Colorado, USA). Soluble scFvs were produced from single colonies in 96-well plate format, using media supplemented with 0.25 mM isopropyl thiogalactioside (IPTG) and 50 µM biotin by incubation over night at 37 °C and vigorous shaking (800 rpm). The soluble scFvs were screened for specific binding to target proteins in ELISA. Target antigens as well as BSA were immobilized at a concentration of 1 µg/ml

in 384-well plates (Corning Inc., New York, NY, USA) and incubated over night at 4 °C. Unbound protein was removed by washing once with 154 mM NaCl and 0.05% Tween20 (wash buffer) and the surface was subsequently blocked with PBT for 2 hours at room temperature with shake. Soluble scFvs were evaluated for binding by incubation with the target protein for 1 hour at room temperature with shake. Horesradish peroxidase (HRP)-labelled streaptavidin (Thermo Scientific Pierce, Rockford, IL, USA) was added and incubated for 1 hour at room temperature with shake. The plates were washed four times with wash buffer between the different steps. The amount of scFv bound was determined with the usage of TMB-ELISA (Thermo Scientific Pierce) as chromogen. Sulfuric acid (1M) was used to stop the reaction and absorbance was measured at 450 nm. Clones that bound their target protein but not BSA were DNA sequenced.

Production and Purification of soluble antibody fragments

Four unique clones (B-M1-K8, B-M1-L7, B-ProH-K5 and B-ProH-K13), two each for the two target antigens, were chosen for further characterization. The clones were grown over night and used to inoculated 15 ml TB medium (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA) supplemented with 25 µg/ml kanamycin (Saveen & Werner AB, Sweden), 10 µg/ml chloramphenicol (Saveen & Werner AB) and 0.2M sucrose. The expression of both the scFv fragments and the biotin ligase were induced by addition of 1.5 mM IPTG and 50 µM biotin and incubated over night at 30 °C at 220 rpm. The bacteria were harvested by centrifugation and the scFv fragments were purified from the periplasmic space with the use of MagneHis™ Protein Purification System (Promega Corporation, Madison, WI, USA) and a KingFisher Flex robot (Thermo Fisher Scientific Inc., Waltham, MA, USA). The purity of the purified scFvs was confirmed by SDS-PAGE and an ELISA assay, as described above, was performed to confirm the binding specificity.

Bacteria ELISA

In order to evaluate the binding capacity of the generated scFv on whole bacteria, the soluble scFv clones were analyzed in an in-house developed bacteria ELISA. The bacterial strain (AP1, MC25 or BMJ71) was immobilized at a concentration of 10^6 cfu in 384-well plates (Corning Inc.) and incubated over night at 37 °C. After one washing step and blocking with PBT, the scFvs were added and the ELISA performed as describe above.

Epitope mapping ELISA

The binding profile for the two scFv antibodies developed against protein M1 were further investigated by including peptide fragments of the M1 protein. The peptide fragments and full-length M1 were immobilized at a concentration of 5 µg/ml and 1 µg/ml in 384-well plates (Corning Inc.), respectively, and incubated over night at 4 °C. After one washing step and blocking with PBT, the scFvs were added and the ELISA performed as describe above.

Selection of target peptides and transitions

SRM assay were constructed by testing at least the ten most abundant peptide fragments for selected proteotypic peptides. Proteins that were selected for detection in addition to protein M and H were the human proteins Apolipoprotein E, Immunoglobulin γ (1-4) and Fibrinogen (α, β, γ). The scFv antibodies as well as streptavidin, constituting the proteins on the bead surface, were also selected along with retention time normalization peptides. The streptococcal proteins M1 and H were measured and absolute quantified using heavy isotope labelled peptides (HeavyPeptide AQUA, Thermo Fisher Scientific Inc. and JPT, Berlin, Germany). Only top-performing peptides for each protein was selected (data not shown), one to three peptides were selected for final SRM analysis for each protein. The four to five transitions with highest intensity was selected for each peptide with the

exception of the retention time peptides that have two transitions each. A full list of the SRM assay can be found in the supplement table S1.

Capture of target proteins in PBS or a serum background

Soluble biotin-tagged scFv (2.5 µg) were coupled to magnetic Dynabeads M-270 streptavidin (Invitrogen Dynal AS, Oslo, Norway) for 1 hour at room temperature with shake according to manufacturers instructions. 100 µg of coupled beads were used for each capture reaction. Recombinant target proteins were spiked in PBS or 10% human serum (Sigma-Aldrich Corp, St. Louis, MO USA) diluted in PBS at a concentration of 0, 20, 100 and 500 ng/ml. A magnetic bead processor, KingFisher Flex (Thermo Fisher Scientific Inc.), was used for the automated capture procedure. The procedure included two washes of the scFv-coupled beads in 1 ml 0,05% Tween20 in PBS, followed by 1-hour incubation with the target protein spiked samples. The beads were, after sample incubation, washed 3 times in 0.1% CHAPS in PBS, once in 0.03% CHAPS in PBS and once with 0.03% CHAPS in 50 mM ammonium bicarbonate and afterwards transferred to Eppendorf tubes (Maximum recovery tubes, Axygen). Subsequently, the wash solution was removed and 15 µl of 12 ng/ml trypsin (Sequencing grade modified, Promega Corp.) was added. The trypsin digestion was carried out for 5 h at 37 °C with shake, followed by addition of 1,5 µl of 5% formic acid. The samples were dried and reconstituted in 0.1% formic acid, 3% acetonitirle and transferred to HPLC vials for LC-SRM MS.

LC-SRM analysis

The SRM measurements were performed on a TSQ Vantage triple quadrupole mass spectrometer (Thermo Fisher Scientific Inc, San Jose, CA, USA) equipped with a nano electrospray ion source (New Objective, Woburn, MA, USA), mass spectrometric settings were previously described [19]. Chromatographic separations of peptides were preformed

on an Easy-nLC II system (Thermo Fisher Scientific Inc.), the non-linear gradient ranging from 3-10% acetonitrile over 3 minutes and from 10-32% acetonitrile over 34 minutes with a flow-rate of 300nl/min. PicoChip (New Objective) columns packed with Reprosil-PUR C18, a length of 105 mm, inner diameter of 75 μm and a tip size of 15 μm were used.

Data Analysis

The data was analyzed in the SRM management software Skyline (MacCoss Lab Software, University of Washington). All data is published on a Panorama server, se supporting information.

RESULTS

Development of scFv antibodies

Streptococcus pyogenes express a wide range of surface associated and secreted virulence factors. Screening for highly abundant virulence factors using stable isotope labeled reference peptides and SRM revealed that proteins H and M1 are among the most abundant virulence factors present on the *S. pyogenes* AP1 strain surface (data not shown). These two virulence factors were produced in recombinant full length form excluding the N-terminal signal sequence and the C-terminal cell-wall anchor region. Importantly, these recombinant formats have been proven active and biologically relevant mimics of their native counterparts [25,28,39,40]. Phage display was then used to isolate scFvs, binding protein H and M1 using an in-house generated human synthetic scFv phage display library consisting of more than 1×10^{10} unique clones. The antigen binding capacity of an scFv is kept intact by linking the antibody variable regions by a peptide linker while the Fc-region of the antibody is removed (Figure 1a) thereby avoiding the binding between the developed antibody and the IgGFc binding domain of the target proteins. In addition, we used a production system that allows site-specific biotinylation using a biotin acceptor domain (BAD) and biotin ligase BirA for site-specific *in vivo* biotinylation of the developed scFvs (Figure 1b). The biotinylated scFv format enables the use of streptavidin as secondary reagent rather than antibodies traditionally used in immunoassays, such as ELISA and FACS. Importantly, we observe no binding between protein M1, protein H or the *S pyogenes* wild-type strain, AP1, expressing both M1 protein and protein H, when using streptavidin as a secondary reagent in the ELISA analysis (data not shown). By making use of scFv in combination with streptavidin, the Fc region is omitted in both the primary and secondary interaction steps, ensuring that the observed binding is not

contributed by the Fc domain, thereby increasing the specificity of the immunoassay and reducing the risk of potentially identifying false positive binders.

After the phage display selection, 92 clones for each target were screened for binding by ELISA. 30 out of 92 clones were shown to bind specifically to protein M1, whereas 24 out of 92 clones showed specific binding to protein H (data not shown). These initial hits were sent for sequencing. In total, 15 and 16 sequence unique clones were identified for protein M1 and H, respectively. Based on these results we selected two scFvs per target protein to characterize further, namely B-M1-K8, B-M1-L7, B-ProH-K5 and B-ProH-K13. The amino acid sequence of the four clones can be viewed in figure S1. The binding specificity of the selected scFv antibodies were confirmed in ELISA against soluble surface proteins M1 and H (Figure 2). The four clones bind exclusively to their expected target proteins with no cross-reactivity although the two target proteins have been proposed to have structural similarities [28]. The M1 protein is divided into four parts referred to as A, B, S, C and D, all associated with binding to specific human host proteins (Figure 2b). We localized the binding of the M1 specific scFv antibodies by ELISA against peptide fragments of the full M1 protein. Both scFv antibodies bind the ABS as well as the AB peptide fragment of the M1 protein (Figure 2c). We observed higher intensity in the binding assay to the ABS fragment than AB fragment, indicating that the presence of the S part is beneficial for binding. In contrast there was no binding to the DCS or DC peptide fragments (Figure 2b). Collectively these results show the isolation of two scFv clones each for the two target proteins. The two unique M1 scFv antibodies bind to similar regions in the M1 proteins, most likely somewhere in the AB part.

scFv antibodies recognize protein H and the M protein on the surface of S pyogenes

The binding abilities of the scFvs were also investigated on whole *S. pyogenes* bacteria. We used the wild-type AP1 (wt) strain expressing high levels of both the M1 protein and

protein H and two isogenic mutants, MC25 and BMJ71. BMJ71 is an mga- negative strain (*H-, M-*), which do not express the proteins M1 and H [36], whereas MC25 (*H+, M-*) has no cell wall anchored domain of the M1 protein and can only produce M1 protein in secreted form [37]. The binding of the scFvs to the bacterial strains was assessed in a developed bacteria ELISA (Figure 3). All the developed scFv antibodies were found to bind the intact native surface proteins M1 and H present on the bacterial surface of the wild-type strain (Figure 3a). As expected, none of scFv antibodies bound to the *H-M-* strain (Figure 3b). The scFv antibodies directed against protein H did bind to the H+, M- strain (Figure 3b). However, there is also a signal for one of the scFv antibodies directed against the M1 protein. This observation can possibly be explained by a low amount of secreted M1 protein associated with the surface. These results correlate with measured SRM data where M1 protein and protein H was not detected on the *H-, M-* negative strain and high levels of protein H on the *H+, M-* strain (data not shown). The SRM data also revealed small amounts of M1 protein in the bacterial pellet after PBS washing of the *H+, M-* strain. In conclusion, these results indicate that the generated scFv antibodies can bind intact native surface proteins and not only the secreted part, which is essential for potential detection of whole bacteria.

Immunoaffinity enrichment of target proteins

The scFv antibodies were further investigated for their capacity to enrich the target proteins from PBS as well as a complex serum background to enable quantification using LC-SRM MS. The biotin-tag on the scFv antibodies was used to couple the scFv antibodies to magnetic streptavidin beads. The target proteins were spiked in PBS in a concentration range of 0-500 ng/ml and enriched using the antibody coupled magnetic streptavidin beads followed by on-bead trypsin digestion, separated using liquid chromatography (LC) and analyzed by LC-SRM. MS. Peptides selected for analysis is presented in table S1. By

selecting the five most intense fragments for each peptide the set of transitions analyzed by LC-SRM MS is generated. Prior to LC-SRM MS heavy labeled aqua reference peptides were added for absolute protein quantification. The SRM data reveals a high linearity and the respective target protein was at least detectable in samples with an initial concentration down to 4 ng/ml (80 fmol/ml and 100 fmol/ml for M1 and H respectively) (Figure 4a & 4b). Importantly, no target proteins are detected in samples with non-coupled magnetic beads or in samples spiked with 500 ng/ml of a non-target protein (Figure 6a). This indicates that the enrichment is specific, mediated by the scFv antibodies, and that there is no cross-reactivity for the scFv antibodies against respective non-target protein.

The efficacy of the enrichment in PBS is expressed as the ratio between the theoretical maximum amount and the actual detected amount of the target protein, determined via the heavy labeled aqua reference peptides. A high efficacy was attained, especially for the low concentration samples, as can be seen in figure 4c and 4d. Although an efficacy close to 1.0 is desirable it is unlikely due to possible sample loss during the various sample processing steps. Still, the efficacy of the enrichment of M1 protein was above 0.5 for all samples analyzed and was relatively stable over the whole concentration range with the exception for the highest concentration sample (Figure 4c). A lower efficacy can be seen for the enrichment of protein H compared to protein M1. There was also a larger decrease in efficacy for the higher concentrations of protein H (Figure 4d). The decrease in efficacy for both protein M1 and H capture at the highest target protein concentration (500 ng/ml) can potentially be explained by a limited amount of available scFv. There is also a possibility for crowding effects on the beads, meaning that even though scFvs are available on the beads, the high antigen concentration surrounding the beads and steric hindrance hinders the scFv from binding target protein. By increasing

the bead amount and thereby also the amount of available scFv the enrichment efficacy for the higher target protein concentrations could likely be increased.

Local infections caused by *S. pyogenes* can give rise to vascular leakage. To determine if the scFv antibodies can enrich the target proteins from complex protein mixtures we spiked the target proteins in concentrations ranging from 0-500 ng/ml in PBS containing 10% human serum. The proteins were enriched as above and analyzed in LC–SRM MS together with spiked in heavy labeled reference peptides (Figure 5). M1 protein was successfully enriched from the samples (Figure 5a) although at lower levels compared to samples where M1 protein was spiked in PBS without serum (Figure 4a). The enrichment was possible despite the presence of several serum proteins that bind the M1 protein with high-affinity [28,29]. In contrast, only a very low amount of protein H was detected after enrichment (Figure 5b). Protein H has previously been shown to bind the complement protein C4bp [39] and the Fc part of IgG, but it cannot be excluded that protein H can bind additional serum proteins. The results indicate that competitive binding of serum proteins probably interrupts the enrichment of protein H. The enrichment from human serum samples was assessed with the use of non-coupled beads in the same way as for the PBS samples (Figure 6b). In these experiments, M1 protein was below detection limit, indicating that the enrichment is dependent on specific enrichment mediated by the scFv antibodies.

DISCUSSION

In this work we have used phage display to develop scFv antibodies against the *S. pyogenes* surface proteins M1 and H. The generated scFv antibodies showed sensitive and specific binding against their respective target proteins. Importantly, the antibodies were demonstrated to bind both the soluble, recombinantly produced target proteins as well as the intact native surface protein M1 and H on the bacterial surface of wild-type strain AP1. In addition, we showed that the antibodies had the ability to enrich its target antigens from both PBS and a complex serum background to enable detection and quantification with LC-SRM MS.

Many bacterial pathogens express surface proteins capable of binding IgGFc to avoid detection of the host immune system, an ability that both target proteins, M1 and H, used in this study have [18,28,30]. The IgGFc binding ability complicates the specific detection of the target proteins, mediated by the antigen binding site, due to potential binding via the Fc region both from the developed antibodies as well as from secondary detection antibodies used in different immunoassays. To address these challenges we used a specific format of the developed antibodies associated with several key advantages. Firstly, the scFvs have no Fc-part, as illustrated in Figure 1. Secondly, we make use of site-specific biotinylation, which allows us to use streptavidin as a secondary agent. Several detection assays such as ELISA and FACS rely on secondary antibodies. Combining the scFv format with biotin omits the Fc region from both the primary and secondary interaction steps, ensuring that the observed binding is not contributed by the Fc domain. Proteins can be chemically labeled with biotin through incorporation of biotin at primary amines (e.g. side chains of lysine) [41]. However, the uncontrollable inclusion of biotin with chemical labeling is unspecific and the incorporation at certain positions can affect the proteins activity as well as their conformations [41,42]. Therefore, the use of site-specific

labeling is favorable. Different approaches have been taken to imitate the biotinylation process performed in nature by taking advantage of enzymatic biotinylation sites [33,34,43,44] to control the site where the biotin is attached. Here we have used the biotin ligase enzyme BirA from *E. coli* and a 16 amino acid long biotin acceptor domain (BAD) for *in vivo* site-specific biotinylation of the selected scFvs (Figure 1b). The biotinylation of the developed scFvs are thereby controlled and the use of full-length antibodies as secondary reagent can be avoided. The site-specific biotinylation of our scFv antibodies also offer an advantage when coupling the scFv antibodies to magnetic beads, as in the developed protocol for immunoaffinity enrichment. The site-specific biotinylation ensures that the scFv antibodies are coupled in an oriented manner exposing the antigen-binding site outwards. Thus, increasing the likelihood of the coupled antibodies to be available for binding.

The developed biotinylated scFv with no IgGFc binding properties and the use of streptavidin as secondary reagent in detection assays offers several opportunities for application. In this article, we demonstrate the detection of the targeted surface proteins on intact bacteria. Our developed scFv antibodies specifically bind native intact surface proteins on the bacteria and no evidence of cross-reactivity could be seen. The ability of binding the intact surface protein is needed for the potential detection of samples containing whole bacteria and not only the secreted part of the protein. The generated bacteria ELISA in combination with our scFv antibodies enable the evaluation of different expression levels of proteins present on the bacterial surface. The second application entails enrichment of the target proteins from complex biological samples for identification and quantification with LC-SRM MS. Human plasma and serum are known to contain proteins spanning a high dynamic range. Serum contains proteins ranging over 9 orders of magnitude in concentration [45] and without sample fractionation, low abundant

proteins will not be detected due to the complex background. SRM has an exceptional sensitivity and selectivity, making the technique well suited for analysis of samples with high complexity and dynamic range. One of the initial aims of this study was to evaluate the capability to use the developed scFv antibodies for enrichment of the target proteins from a serum background followed by detection and quantification with the use of LC-SRM MS. Combining antibody enrichment with mass spectrometric readout has previously been demonstrated to enable detection of target proteins in complex sample formats such as serum or plasma [8-10]. More specifically and highly relevant to this study, it has been shown that recombinant scFv antibodies can be used in combination with LC-SRM MS for detection of proteins in a human serum background [11]. Following antibody enrichment in this developed set-up, soluble target proteins M1 and H were detectable in samples down to 4 ng/ml in PBS (Figure 4a & 4b) and for the M1 setup soluble protein were detectable at a concentration of 500 ng/ml in 10% human serum (Figure 5a). Targeted antibody enrichment, diminishes the amounts of high abundant proteins to facilitate the detection and quantification of the target proteins. As an example, albumin, the most abundant serum protein, constitute about 60% of the total protein content of serum [45]. Assuming a total protein concentration of serum of 60 mg/ml [45], the albumin concentration would be more than 5000 times the concentration of M1 protein in our corresponding serum sample and thereby near challenging the dynamic range of the mass spectrometer. We demonstrate the detection of 20 fmol per injection of M1 protein in 10% human serum sample initially containing 500 ng/ml of protein M1 with a theoretical maximum of 417 fmol per injection. This indicates sample loss probably due to the interaction between the M1 protein and different serum proteins as well as an incomplete trypsin digestion. To increase the sensitivity and efficiency of the developed platform the conditions for the different workflow steps, such as capture and wash, could be further

evaluated and optimized. Altered pH, buffer choice or detergent concentration as well as incubation timecould potentially improve the binding between the antibodies and the target proteins to increase the efficiency of the platform. An evaluation of the trypsination process could also possible help to diminish the eventual sample loss in the digestion step. Increased digestion time or extra addition of trypsin could possibly improve the digestion and thereby also the sensitivity of the assay. Yet, protein M1 is still captured and this allows us to detect M1 protein in the samples without the interference from background proteins such as albumin. Both protein H and M1 have the ability of binding albumin while M1 in addition also binds the serum protein fibrinogen [28]. However, the albumin content was measured after enrichment and no albumin was detectable. This highly supports the ability of the developed M1-specific scFv antibodies to make the M1 protein available for detection.

In conclusions, we have generated scFv antibodies with the use of phage display that specifically bind the virulence factors and major surface proteins M1 and H of the *S. pyogenes* strain AP1, both as soluble proteins and on the intact bacteria. In addition, the scFv antibodies have been shown to have the capability to enrich their target proteins from both PBS as well as complex serum samples to enable identification and detection with the use of LC-SRM MS. A workflow has been developed, particularly well suited for the analysis of Fc binding proteins. However, the workflow can of course be adapted to other types of bacterial target proteins and thereby also enable detection of other bacterial species.

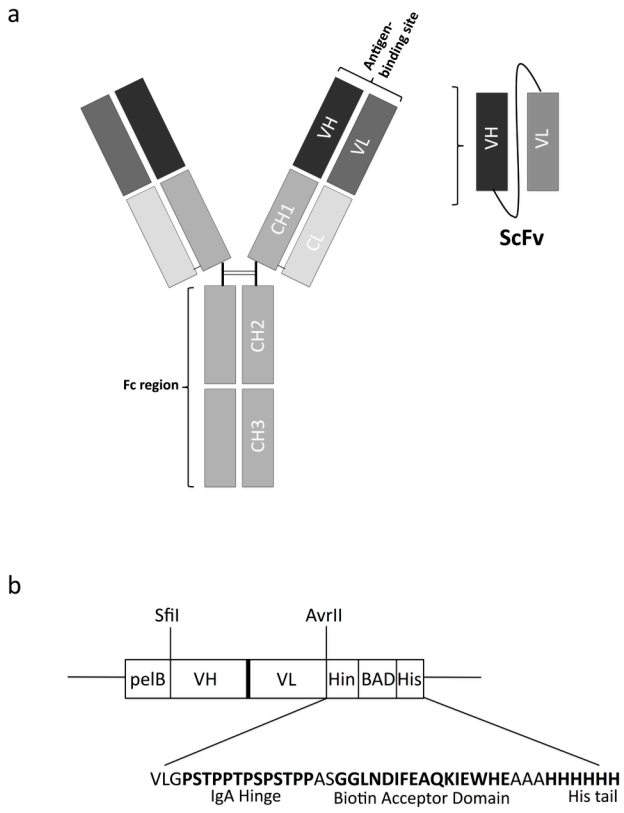

Figure 1. (a) Schematic overview of a full-length antibody and a single chain variable fragment (scFv). An scFv consists of the variable regions of an antibody's heavy and light chains connected with a peptide linker. (b) Schematic outline of the vector construct used for *in vivo* biotinylation of soluble scFv antibody fragments. The scFv gene is connected to a gene encoding part of the IgA hinge (Hin) followed by a biotin acceptor domain (BAD), also known as an avi-tag and a His-tail (His). By co-expressing this vector with pBirAcm, a vector encoding the BirA biotin ligase, the expressed enzyme covalently attaches a biotin molecule to the lysine residue of BAD.

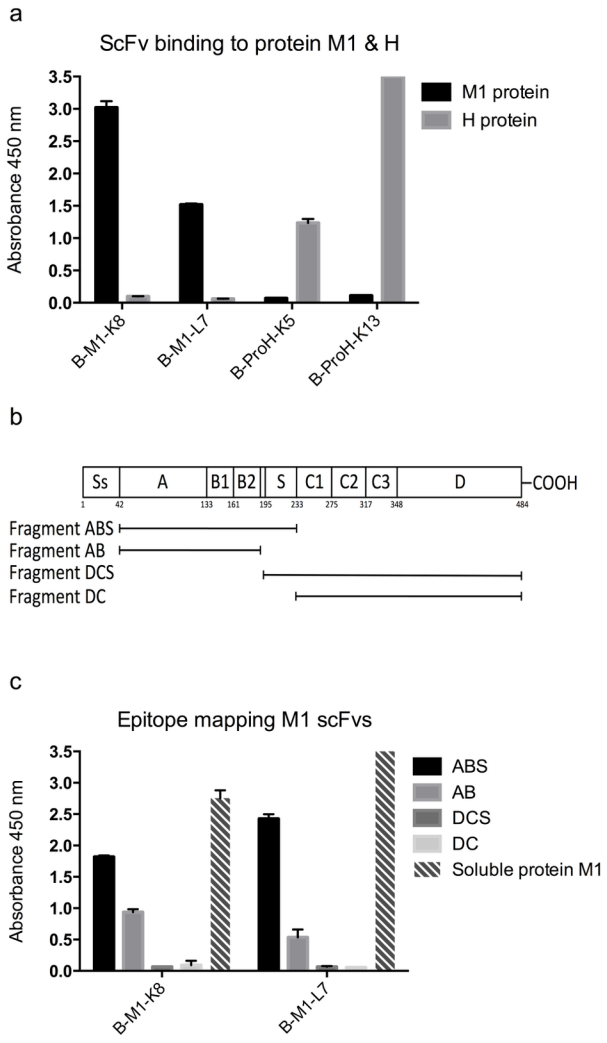

Figure 2. (a) Binding characteristics of developed scFvs determined by ELISA. ScFvs B-M1-K8 and B-M1-L7 were generated by selection on M1, while B-ProH-K5 and B-ProH-K13 were obtained by selecting on protein H. The developed scFvs were analyzed on recombinant protein M1 and H. (b) The different M1 protein fragments and the full-length M1 protein used for determining which part of the protein the developed scFv bind. (c) The M1 specific scFvs (B-M1-K8 and B-M1-L7) evaluated in an ELISA epitope assay using peptide fragments of M1 and full-length M1 protein.

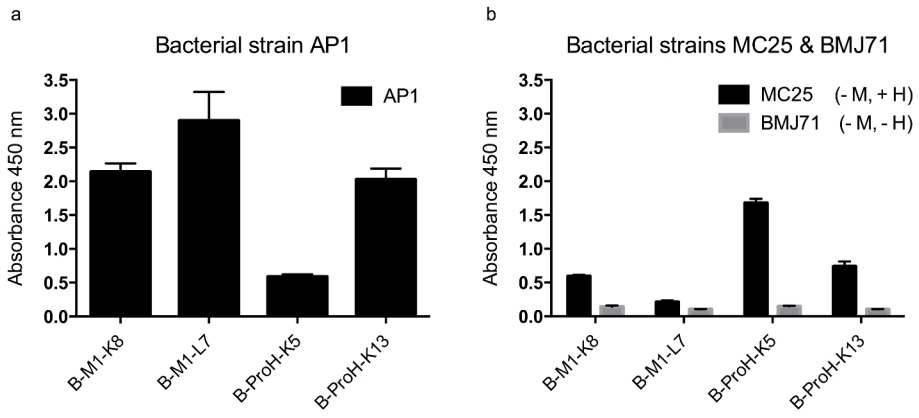

Figure 3. Binding characteristics of the developed scFvs as determined by bacterial ELISA. The four scFvs (B-M1-K8, B-M1-L7, B-ProH-K5 and B-ProH-K13) were analyzed on wild-type bacterial strain AP1 (a) as well as bacterial strains MC25 and BMJ71 (b). The results confirm the binding to the native proteins present on the surface of wild-type bacterial strain AP1.

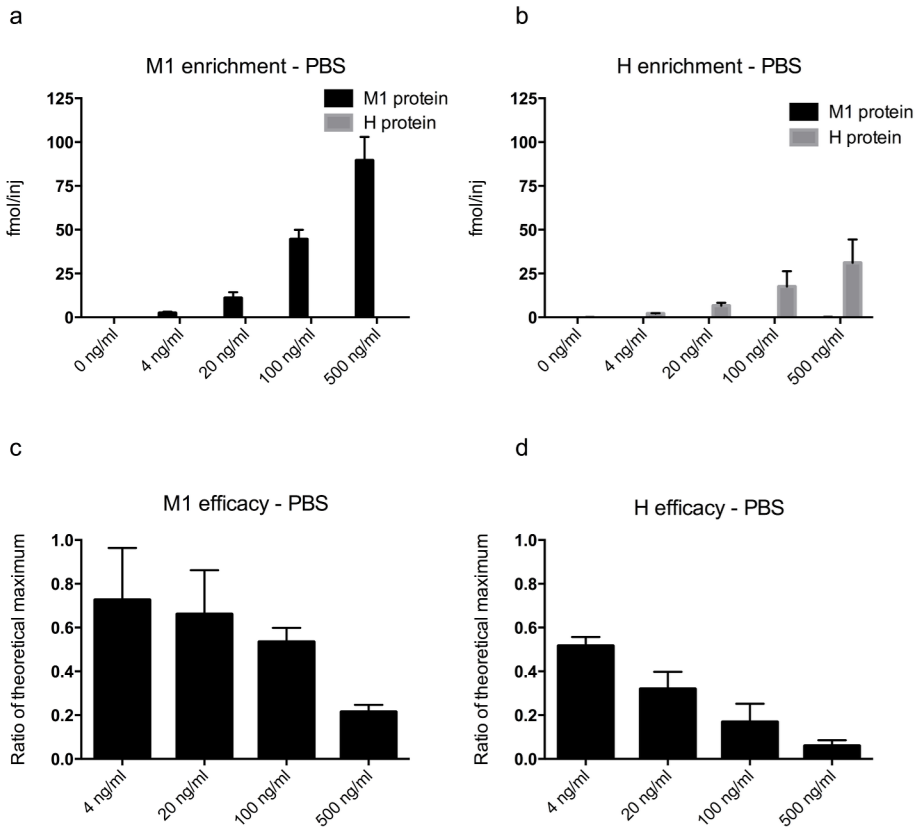

Figure 4. Detection of target proteins M1 and H in PBS at different concentrations after capture with the generated scFv antibodies coupled to magnetic streptavidin beads and LC-SRM MS readout. The enrichment efficacy is expressed as the ratio between the theoretical maximum amount and the actual detected amount of the target protein. Capture of protein M1 in PBS (a), capture of protein H in PBS (b), M1 efficacy in PBS (c), H efficacy in PBS (d).

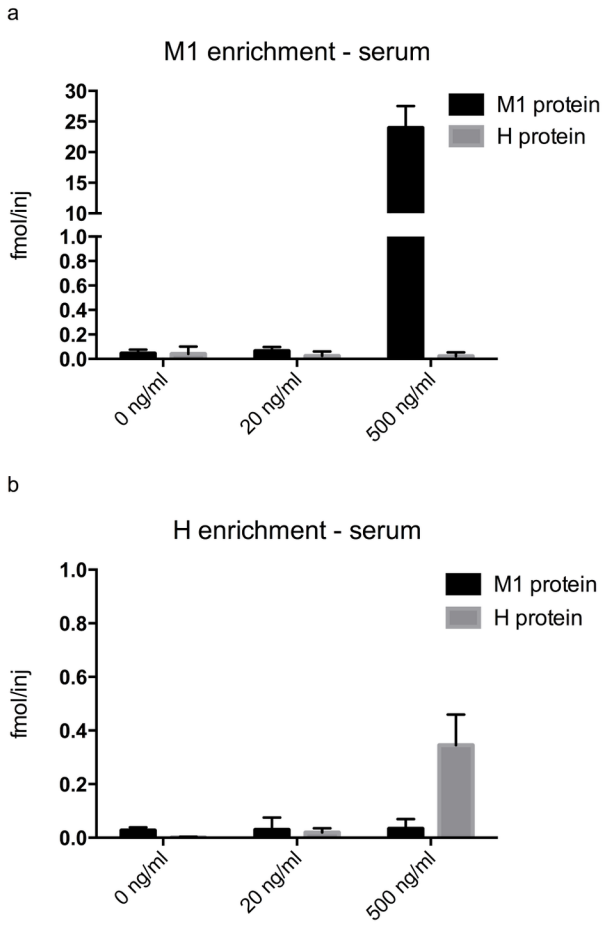

Figure 5. Detection of target proteins M1 and H in 10% serum diluted in PBS at different concentrations after capture with the generated scFv antibodies coupled to streptavidin magnetic beads and LC-SRM readout. Capture of protein M1 in 10% serum (a), protein H in 10% serum (b).

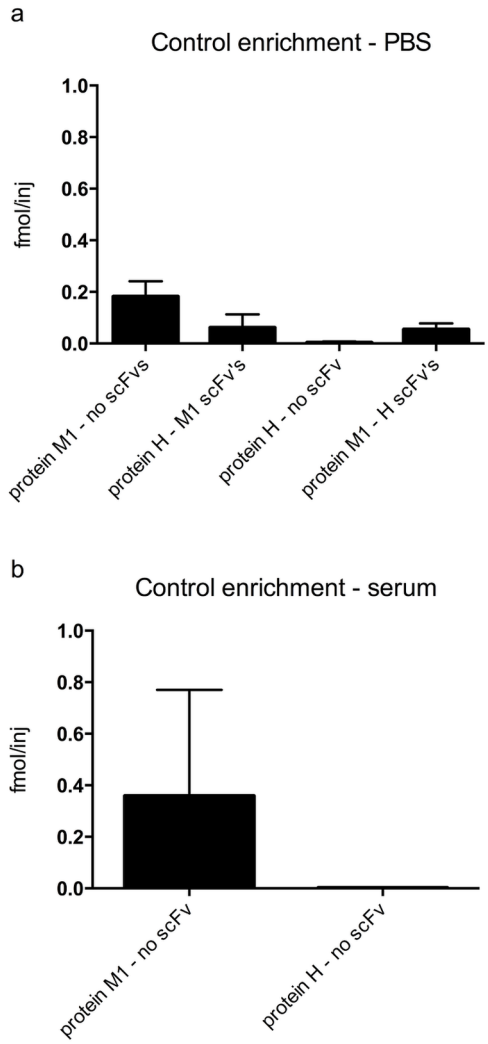

Figure 6. Control captures experiments in PBS (a), in 10% serum (b). Detection of target proteins after capture with non-coupled magnetic streptavidin beads or after capture with the generated scFv antibodies coupled to magnetic streptavidin beads from samples spiked with 500 ng/ml non-target protein and LC-SRM MS readout. No cross reactivity can be seen for the developed scFv antibodies in the capture format in a PBS background. Also, no target proteins are enriched with the use of non-coated beads neither in a PBS background nor in a 10% serum background.

ASSOCIATED CONTENT

Supporting Information

The amino acid sequences of the unique generated scFv antibodies are provided as supplementary figure S1. Information for all measured peptides is provided as supplementary table S1, including peptide sequence, protein name, isotope label type, precursor Mz, precursor charge, collision energy and transition(s). This material is available free of charge via the Internet at http://pubs.acs.org. All mass spectrometry data is published on a Panorama server: https://panoramaweb.org/labkey/project/Lund%20University%20IMP/Captures/SkylineData_M-H_protein/begin.view?

The data will be open to the public when the paper is accepted to be published.

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Notes

The authors declare that there are no conflicts of interests.

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