A novel affinity protein selection system based on staphylococcal cell surface display and flow cytometry

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Here we describe the first reported use of a Gram-positive bacterial system for the selection of affinity proteins from large combinatorial libraries displayed on the surface of Staphylococcus carnosus. An affibody library of 3 × 10^9 variants, based on a 58 residue domain from staphylococcal protein A, was pre-enriched for binding to human tumor necrosis factor-alpha (TNF-alpha) using one cycle of phage display and thereafter transferred to the staphylococcal host (~10^8 variants). The staphylococcal-displayed library was subjected to three rounds of flow-cytometric sorting, and the selected clones were screened and ranked by on-cell analysis for binding to TNF-alpha and further characterized using biosensor analysis and circular dichroism spectroscopy. The successful sorting yielded three different high-affinity binders (ranging from 95 pM to 2.2 nM) and constitutes the first selection of a novel affinity protein using Gram-positive bacterial display. The method combines the simplicity of working with a bacterial host with the advantages of displaying recombinant proteins on robust Gram-positive bacteria as well as using powerful flow cytometry in the selection and characterization process.

Keywords: affibody/cell surface display/combinatorial protein engineering/Grampositive bacteria/Staphylococcus carnosus

Introduction

The recent and successful introduction of specific affinity proteins, e.g. antibodies, antibody derivatives and scaffold proteins, in the fields of biotherapy (Adams and Weiner, 2005; Gill and Damle, 2006; Tolmachev et al., 2007) and molecular imaging (Weissleder, 2006; Orlova et al., 2007) has rapidly increased the attention to all areas involving their generation and characterization. The traditional method for obtaining specific antibodies has been immunization of laboratory animals together with hybridoma technology if monoclonal molecules were desired. However, mainly due to immunogenicity issues, several techniques to generate human antibodies have been developed, among others display of human antibody repertoires on the M13 bacteriophage, the so-called phage display technology (Smith, 1985; Rader and Barbas, 1997). Over the last 20 years, phage display has been, and is still today, the most widely used method and has been employed with great success. Nevertheless, more recently, several alternative in vitro techniques for the generation of affinity proteins have emerged, such as different cell-free systems (Amstutz et al., 2001) [e.g. ribosome display (Hanes and Plückthun, 1997)], protein complementation assays (Koch et al., 2006) and various formats of cell display (Daugherty et al., 2007; Gai and Wittrup, 2007).

The use of cells instead of phages for the display of combinatorial peptide or protein libraries offers a number of advantages. Most importantly, cells are large enough to be screened and sorted using high-speed flow cytometers. Together with the high polyvalency on the cell surface, it enables a real-time quantitative screening of the affinity of all individual library members, on which the sorting can be based. The enrichment after each sorting cycle is easily monitored in each new round, and optimizing sorting parameters and conditions is hence straightforward. After selection, the affinity of the candidate clones can rapidly be determined on-cell using flow cytometry without any need for laborious subcloning and protein purification (Feldhaus et al., 2003; Löfblom et al., 2007b). So far, cell display systems for protein engineering applications have only been developed using the yeast strain Saccharomyces cerevisiae (Feldhaus et al., 2003) and the Gram-negative bacteria Escherichia coli (Chen et al., 2001; Bessette et al., 2004; Harvey et al., 2004). As in most areas of biotechnology, working with bacterial hosts is attractive due to, among other things, easy handling and manipulation. In contrast to Gram-negative bacteria, Gram-positive bacteria contain only one cell membrane, which potentially facilitate translocation of recombinant proteins to the cell surface. Moreover, surface proteins are not membrane spanning, but covalently anchored to the peptidoglycan cell wall via the C-terminus, resulting in a high tolerance to recombinant fusions and a potential advantage over display on Gram-negative bacteria. Gram-positive bacteria are hence excellent hosts for cell display systems, and the high viability upon high-speed flow-cytometric sorting, resulting from a thick cell wall, provides for efficient isolation of candidate clones from combinatorial cell-displayed libraries. Nevertheless, attempts to utilize them for large library applications have so far been hampered by the low transformation efficiency.

We have previously described the use of the Gram-positive bacteria Staphylococcus carnosus for the display of recombinant proteins in a number of different studies (Wernérus and Ståhl, 2004), and recently, the method was improved to allow for protein engineering applications (Wernérus and Ståhl, 2002; Wernérus et al., 2003; Löfblom et al., 2005; Löfblom et al., 2007a). In this paper, we describe the first reported selection of a novel affinity protein using Gram-positive bacterial display. We have subcloned a pre-enriched protein library of 1.1 × 10^6 staphylococcal protein A (SpA)-derived affibody molecules (Nord et al., 1997; Orlova et al., 2006), displayed the library on the surface of staphylococci and successfully isolated binders
with subnanomolar apparent affinity for the rheumatoid arthritis-associated human tumor necrosis factor-alpha (TNF-alpha).

Materials and methods

Pre-enrichment using phage display

Preparation of phage stocks from the library [a portion of Zlib2002 (Grönwall et al., 2007)] was performed according to previously described procedures (Nord et al., 1997) using the helper phage M13KO7 (New England Biolabs, Beverly, MA, USA). Polyethylene glycol with NaCl was used for phage precipitation, routinely yielding phage titers of about $10^{13}$ plaque-forming units (pfu) per milliliter. Biotinylated human TNF-alpha was used as target protein during the selection, and streptavidin (SA)-coated paramagnetic beads (Dynabeads® M-280 SA; Dynal A.S., Oslo, Norway) were used as solid support. Unspecific binders were removed by one round of negative selection against the SA-coated beads supplemented with biotinylated bungarotoxin before the biopanning. The phage stock was incubated for 1 h at room temperature, and the supernatant was used as input for the selection. The biopanning was performed as follows. All tubes and beads were blocked with phosphate-buffered saline (PBS) containing 0.05% Tween 20, 0.02% Na-azid and 5% bovine serum albumin (TPBSB) to avoid unspecific binding. The phage solution was incubated for 10 min at 37°C followed by a short centrifugation at maximum speed in a micro centrifuge (Biofuge Pico, Heraeus Instruments, Langenselbold, Germany) prior to the panning procedure. The selection was carried out in solution by mixing phages $(1 \times 10^{13}$ phage particles per milliliter) and target protein in a total volume of 2.4 ml with TPBSB (as described earlier but with 3% bovine serum albumin). The concentration was 33 nM biotinylated TNF-alpha (trimer concentration). The selection mix was incubated at 4°C overnight followed by 30 min at room temperature with rotation. The samples were thereafter incubated with blocked SA-coated beads for 15 min to capture phages bound to biotinylated TNF-alpha. The beads were washed once with TPBSB and once with PBS. The bound phages were eluted with 500 µl of 0.1 M glycine–HCl, pH 3.0, for 10 min at room temperature, followed by immediate neutralization with 50 µl of 1 M Tris–HCl, pH 8.0, and 450 µl of PBS, pH 7.2. Eluted phages were used to infect TG1 E. coli cells in logarithmic growth phase. After 1 h of incubation at 37°C, the cells were centrifuged and the pellet was dissolved and spread on tryptic soy broth supplemented with yeast extract (TSB+Y; Merck, Darmstadt, Germany) agar plates and incubated overnight at 37°C.

Cloning of affibody library to the staphylococcal display vector

The E. coli strain RR1ΔM15 (Rüther, 1982) was used as host strain for all plasmid constructions. The new staphylococcal vector, pSCZ1, was created by ligating a PCR-amplified gene fragment from the phage display vector [pAffi1 (Grönwall et al., 2007)], containing a dummy fragment followed by the non-randomized helix three of the affibody and flanked by two new restriction sites (XhoI and NheI), to the previously described staphylococcal vector pSCXm (Wernérus and Ståhl, 2002) digested with BamHI and SalI (Fermentas, Vilnius, Lithuania). The phage display plasmid, containing the pre-enriched affibody library, was prepared with the JETSTAR Kit (Genomed, Bad Oeynhausen, Germany) according to the supplier’s recommendations. The plasmid was digested with restriction enzymes XhoI and NheI (Fermentas), and the DNA encoding helices one and two of the randomized affibody library was purified using preparative agarose gel electrophoresis. The randomized affibody library was thereafter ligated using T4 DNA Ligase (Invitrogen, Carlsbad, CA, USA) according to the supplier’s recommendations into the staphylococcal display vector, pSCZ1, digested with the same enzymes, XhoI and NheI (Fermentas). The library was transformed to electrocompotent Staphylococcus carnosus TM300 (Götz, 1990) as described previously (Löfblom et al., 2007a) and stored in 15% glycerol at −80°C (the staphylococcal library is hereinafter denoted Sc:Z_{TNF-alpha,LIB}).

Labeling of TNF-alpha and human serum albumin

Human TNF-alpha (ProSpec, Rehovot, Israel) was biotinylated and purified using the Fluoreporter® Biotin-XX Labeling Kit (Invitrogen) according to the supplier’s recommendations. Human serum albumin (HSA) was labeled with fluorophore using Alexa Fluor® 647 succinimidyl ester (Invitrogen) according to the supplier’s recommendations. The concentration of labeled proteins was determined using amino acid analysis.

Cell labeling and fluorescence-activated cell sorting

An aliquot of Sc:Z_{TNF-alpha,LIB} (at least 10 times the library size) was inoculated to 100 ml TSB+Y with 20 µg ml$^{-1}$ chloramphenicol and grown overnight at 37°C and 150 rpm. After 16 h, 105 cells (103 cells in rounds 2 and 3) were washed with 1 ml PBS (pH 7.4) with 0.1% Pluronic® F108 NF surfactant (PBSP; BASF Corporation, Mount Olive, NJ, USA). Cells were pelleted by centrifugation (3500×g, 4°C, 6 min) and resuspended in 1.5 ml PBSP containing biotinylated TNF-alpha and incubated at room temperature with gentle mixing for 1 h to reach equilibrium binding. Cells were thereafter washed with 1 ml of ice-cold PBSP followed by incubation in 1 ml PBSP containing 1.25 µg ml$^{-1}$ SA, R-phycocerythrin conjugate (Invitrogen) and 225 nM Alexa Fluor® 647, HSA conjugate for 1 h on ice in the dark (4 h and with the addition of 100 nM unlabeled TNF-alpha in rounds 2 and 3). After a final washing step in 1 ml of ice-cold PBSP, cells were resuspended in 400 µl of ice-cold PBSP before sorting. Cells were sorted using a FACSVantage SE (BD Biosciences, San Jose, CA, USA) flow cytometer. The sort gate was set to sort out the top fraction of affibody-displaying cells (typically 0.1%) showing the highest R-phycocerythrin to Alexa Fluor® 647 fluorescence intensity ratio. The cells were sorted directly into 0.5 ml B2 medium (Löfblom et al., 2007a) and spread onto blood agar base (Merck) plates containing 10 µg ml$^{-1}$ chloramphenicol and incubated at 37°C for 24 h. In the last round, cells were sorted into individual wells in 96-well plates, containing semi-solid medium, to form colonies.

DNA sequencing

Colonies from the flow-cytometric sorting were screened using PCR with primers SAPA-23 ([5’-GGCTCCTAA
AGAAAATACACGGC-3') and SAPA-24 (5'-TGTGTA ATTCCTTAAAGGACATCTGC-3'). The PCR products were sequenced with BigDye thermo cycle sequencing reactions and an ABI Prism 3700 instrument (Applied Biosystems, Foster City, CA, USA).

Whole-cell ELISA

Staphylococcal cells, isolated from the flow-cytometric sortings, displaying each of the 15 unique affibody molecules respectively, were grown and labeled with biotinylated TNF-alpha as described earlier but in a 96-well filter plate and in triplicate. After labeling, cells were incubated in 200 ml SA, alkaline phosphatase conjugate (Invitrogen) at 0.5 units per milliliter for 30 min at room temperature. After incubation, cells were washed in substrate buffer (1 M diethanolamine, 0.5 mM MgCl₂, pH 9.8), and 100 μl of the cell suspensions was transferred to an ELISA microtiter plate. To perform the enzymatic reaction, 100 μl of p-nitrophenylphosphate (Sigma, St Louis, MO, USA) was added to each well and the change in A₄₀₅ₙₘ was measured during 20 min using an ELISA plate reader (SUNRISE, Tecan, Grödingen, Austria).

Flow-cytometric Kᵩ determination

To determine the equilibrium dissociation constants on-cell using flow cytometry, cells were prepared as described earlier and pellets of Sc:Z₉TNF-alpha1, Sc:Z₉TNF-alpha2 and Sc:Z₉TNF-alpha3 (10⁶ cells) were resuspended in 1.5 ml PBSP containing different concentrations of biotinylated TNF-alpha (0.005–20 nM for Z₉TNF-alpha1 and Z₉TNF-alpha2 and 0.03–70 nM for Z₉TNF-alpha3). Cells were incubated at room temperature for 1 h with gentle mixing to reach equilibrium binding and then washed in 1 ml ice-cold PBSP. Cells were then resuspended in 200 μl of ice-cold PBSP containing 1.25 μg ml⁻¹ SA, R-phycocerythrin conjugate (Invitrogen) and incubated for 30 min on ice in the dark. After a last washing step in 1 ml ice-cold PBSP, cells were resuspended in 300 μl ice-cold PBSP and kept on ice until flow-cytometric analysis. The mean fluorescence intensity (MFI) was measured using a FACSVantage SE (BD Biosciences) flow cytometer. The experiment was carried out in triplicate on different days using freshly prepared solutions.

Subcloning, protein production and purification

DNA encoding for the three affibody molecules was sub-cloned into the E. coli expression vector, pAY430 (Grönwall et al., 2007), by sticky-end PCR and ligation. The affibody molecules were expressed in E. coli strain BL21(DE3) with an N-terminal His-tag fusion and a C-terminal cysteine, yielding fusion proteins of ~8 kDa. Over-night cultures were used to inoculate 100 ml of TSB+Y supplemented with 50 μg ml⁻¹ kanamycin. The cells were cultivated at 37°C until OD₆₀₀ₙₘ = 1, when expression was induced using IPTG followed by cultivation for 4 h. Cells were pelleted by centrifugation and then resuspended in PBS containing 30 mM imidazole. Prior to purification, cells were lysed by sonication, and after centrifugation, the proteins were purified by loading the supernatant onto a 5 ml His-trap column (GE Healthcare Bio-sciences AB, Uppsala, Sweden) on an ÄKTA explorer system (GE Healthcare Bio-sciences AB). The column was washed using PBS containing 30 mM imidazole, and proteins were finally eluted using PBS containing 200 mM imidazole.

Biosensor analysis

The different proteins were immobilized onto carbamidomethylated dextran chips (CM5, BIACORE AB, Uppsala, Sweden) by thiol coupling using a Biacore 2000 instrument (BIACORE AB) according to the manufacturer’s suggestions. Around 40 RU (Biacore resonance units) of Z₉TNF-alpha1, Z₉TNF-alpha2 and Z₉TNF-alpha3 were immobilized to flow cells 2, 3 and 4 respectively and with the blank surface (flow cell 1) treated as the other surfaces on the chip only with no protein injected. Biosensor analysis was carried out using a Biacore 2000 instrument (BIACORE AB) to determine the equilibrium dissociation constants of the three affibody molecules. All experiments were performed using Hepes-buffered saline (10 mM HEPES, 0.15 M NaCl, 3.4 mM EDTA and 0.005% Surfactant P20 (BIACORE AB), pH 7.4) as running buffer and 10 mM HCl for regeneration of the chip surface. A series of different concentrations of TNF-alpha (0.02–300 nM) were injected over the flow cells of the chip with the immobilized affibody molecules. The injections were performed in duplicate, in a random order and at a flow rate of 20 μl min⁻¹, and the responses at equilibrium binding were collected. The entire experiment was carried out in duplicates on different days using freshly prepared solutions.

Circular dichroism spectroscopy

Circular dichroism spectroscopy on a Jasco-810 spectropolarimeter (Jasco Scandinavia AB, Mölndal, Sweden) was used to measure the secondary structure content and to monitor the thermal denaturation of the three affibody molecules. Denaturation experiments were conducted in a temperature range of 20–90°C at a wavelength of 220 nm and in PBS at pH 7.4.

On-cell competition

Staphylococcal cells, displaying the three different affibody molecules respectively, were grown and prepared as described above. Biotinylated TNF-alpha at a concentration of 33 nM was pre-incubated with 330 nM of etanercept (Enbrel; Immunex, Seattle, WA, USA), adalimumab (Humira; Abbott Laboratories, Abbott Park, IL, USA) or infliximab (Remicade; Centocor, Inc., Malvern, PA, USA) for 15 min at room temperature before incubation with the staphylococcal-displayed affibody molecules. Polyclonal human IgG at 330 nM was used as a negative control in the experiments. Labeling with secondary fluorescent reagent and flow-cytometric analysis was performed as described earlier.

Results

Pre-enrichment using phage display

Although modern high-speed flow cytometers can screen up to 100 000 cells per second, sortings from large libraries containing billions of variants are currently not feasible. In order to circumvent this limitation and reduce the complexity of our protein library, we performed one round of phage display-mediated selection prior to the flow-cytometric sorting. As protein scaffold for the combinatorial
randomizations, a three-helical, 58 residue bacterial domain, derived from an IgG-binding domain of SpA was used. Thirteen amino acids in two of the helices are randomized, and specific binders, denoted affibody molecules, have previously been selected for several targets using phage display (Nord et al., 1997; Nilsson and Tolmachev, 2007). A previously described affibody library (3 × 10^6 variants) (Grönwall et al., 2007), displayed on the surface of M13 filamentous phages, was incubated with 33 nM of biotinylated TNF-alpha (soluble part, trimer concentration) and captured using SA-coated magnetic beads for the enrichment of phages displaying antigen-binding affibody molecules. After elution (4 × 10^9 pfu; 25 000-fold amplification), the phages were used to infect E. coli cells, which were then cultivated for the preparation of the plasmid library.

**Construction of a staphylococcal-displayed affibody library**

In order to avoid PCR-induced biases in the library repertoire upon library subcloning, we redesigned the staphylococcal display vector to allow for direct, in-frame digestion and ligation from the phage display vector. We incorporated two new restriction sites into the original staphylococcal surface display vector (pSCXm) (Wernérus and Ståhl, 2002), matching the restriction sites flanking the affibody library in the phage display vector (Grönwall et al., 2007) (Fig. 1A). The DNA encoding the pre-enriched affibody library was transferred from the phage display vector by restriction-enzymatic digestion and subsequent ligation to the novel staphylococcal surface display vector (pSCZ1) digested with the same enzymes. The staphylococcal vector contains an albumin-binding protein (ABP) from streptococcal protein G (Sjölander et al., 1997) introduced to allow surface expression-level normalization of the target-binding signal (Löfbloom et al., 2005) and export signals and anchoring sequences required for efficient expression, translocation and display on the bacterial cell surface (Fig. 1A and B) (Mazmanian et al., 1999; Wernérus and Ståhl, 2002). We transformed the library to the staphylococcal host using electroporation as described previously (Löfbloom et al., 2007a) and the library size was estimated to 1.1 × 10^6 individual transformants. Ninety-six clones from the library were sequenced and the results showed that 70% of the clones had an insert of correct length and, as expected, that all inserts were unique (data not shown). In order to verify that the affibody library was functionally displayed on the bacterial surface, a culture containing 10^7 staphylococcal cells from the library pool was grown overnight, incubated with fluorescently labeled HSA, and specific binders, denoted affibody molecules, have previously been selected for several targets using phage display (Nord et al., 1997; Nilsson and Tolmachev, 2007). A previously described affibody library (3 × 10^6 variants) (Grönwall et al., 2007), displayed on the surface of M13 filamentous phages, was incubated with 33 nM of biotinylated TNF-alpha (soluble part, trimer concentration) and captured using SA-coated magnetic beads for the enrichment of phages displaying antigen-binding affibody molecules. After elution (4 × 10^9 pfu; 25 000-fold amplification), the phages were used to infect E. coli cells, which were then cultivated for the preparation of the plasmid library.

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and the results showed a stable level of $\sim 20\%$, demonstrating no detectable growth bias (Fig. 1D).

Selection using flow-cytometric sorting

We screened and sorted the staphylococcal-displayed affibody library using flow cytometry to enrich for TNF-alpha binders. Briefly, staphylococcal cells (at least 10 times the library size) were incubated with biotinylated TNF-alpha for 1 h at room temperature. After washing, cells were incubated for 1 h with SA-fluorophore conjugate for the detection of bound TNF-alpha as well as fluorescently labeled HSA to monitor the surface expression level of individual clones and allow for real-time normalization of the antigen-binding signal as described previously (Löfblom et al., 2005). The cells were washed again, and positive TNF-alpha-binding cells were sorted using flow cytometry and spread on agar plates for amplification by growth before the next sorting cycle (Fig. 2A). In the first screening round, a concentration of 33 nM of labeled TNF-alpha (trimer concentration) was used in combination with a non-stringent sorting mode (enrichment mode) to obtain a high yield of positive clones. In subsequent rounds, the antigen concentration was decreased (8 nM), the washing time was prolonged to 4 h and a more stringent sorting mode (normal-C mode) was used to increase the selection pressure. TNF-alpha is a homotrimer at the concentrations used in the screenings (Corti et al., 1992), and in order to minimize the avidity effect, an excess of unlabeled TNF-alpha was added to the washing buffer in rounds 2–3. In the last screening round, positive cells were sorted into individual wells in 96-well plates containing semi-solid medium and grown overnight to form colonies. A portion of the colonies was transferred to new 96-well plates for PCR amplification and sequencing, which yielded 15 unique full-length affibody sequences. Among those 15 clones, 3 sequences appeared more than once (Fig. 2B). Since one clone, $Z_{\text{TNF-alpha1}}$, dominated the

![Fig. 2](https://academic.oup.com/peds/article-abstract/21/4/247/1506832/attachment/1506832?fileId=204)

**Fig. 2** (A) Dot plots showing the results from flow-cytometric sortings of Sc$Z_{\text{TNF-alpha1}}$. FL-4 channel fluorescence intensity corresponding to surface expression level (monitored via HSA binding) on the x-axis and FL-2 channel fluorescence corresponding to TNF-alpha binding on the y-axis. The dot plots show the staphylococcal library before flow-cytometric sorting rounds 1, 2 and 3, respectively, with regions used in gating. (B) Amino acid sequences of the parental $Z_{\text{WT}}$ with randomized positions indicated with filled dots and isolated TNF-alpha binding affibody molecules $Z_{\text{TNF-alpha1}}$, $Z_{\text{TNF-alpha2}}$ and $Z_{\text{TNF-alpha3}}$. Approximate representation (%) among sequenced clones is shown after each sequence.
repertoire of sequences (~95%), a growth-rate experiment was conducted in order to verify that the clone was selected on the basis of high affinity for TNF-alpha and not due to faster growth compared with the rest of the library. Staphylococcal cells displaying Z\textsubscript{TNF-alpha\textsubscript{1}} were mixed with the unsorted library (to ~5% of Sc\textsubscript{Z\textsubscript{TNF-alpha\textsubscript{1}}}), the cells were amplified by growth in two passages (a total amplification of approximately 10\textsuperscript{6}) and the percentage of TNF-alpha-binding clones was analyzed each day by flow cytometry. The analysis showed that the proportion of cells in the TNF-alpha-positive gate was 3.8% the first day, 5.1% the second day and 4.4% the third day (data not shown), demonstrating that cells displaying Z\textsubscript{TNF-alpha\textsubscript{1}} had a growth rate comparable with the unsorted library and hence indicating that the clonal overrepresentation of Z\textsubscript{TNF-alpha\textsubscript{1}}, observed after flow-cytometric sorting, was due to high affinity for TNF-alpha.

**On-cell affinity screening and K\textsubscript{D} determination**

The 15 clones with unique sequences from the flow-cytometric sorting were screened individually for antigen-binding activity in a whole-cell ELISA set-up as described previously (Löfblom et al., 2005). Briefly, over-night cultures of staphylococcal cells expressing each candidate clone respectively were incubated with biotinylated TNF-alpha and then with SA-alkaline phosphatase conjugate. Substrate was added and the difference in absorbance was measured in a 96-well format using a spectrophotometric plate reader. The results showed positive ELISA signals for the three most frequently occurring clones (data not shown). Altogether, >99% of the sorted cells were TNF-alpha-binding clones, demonstrating the high purity obtained in the flow-cytometric sorting. The apparent equilibrium-affinity constants (K\textsubscript{D}) of the three clones were determined on-cell using flow cytometry as described previously (Löfblom et al., 2007b). Staphylococcal cells displaying the three affibody molecules respectively were incubated in 11 different concentrations of labeled TNF-alpha, spanning the expected K\textsubscript{D}. After flow-cytometric analysis, the MFI was plotted against the TNF-alpha concentration and the data were fitted to a monovalent binding equation to determine the apparent K\textsubscript{D} (Fig. 3A). All three affibody binders had affinities <3 nM (trimeric TNF-alpha concentration) and the strongest binder, Z\textsubscript{TNF-alpha\textsubscript{1}}, an apparent K\textsubscript{D} of ~95 pM (Table I).

**Biacore analysis**

To verify the results from the on-cell affinity determinations, the three TNF-alpha-binding affibody molecules were subcloned to an expression vector and expressed in E. coli with an N-terminal His-tag for protein purification and a C-terminal cysteine for site-directed immobilization on a biacore chip surface. The proteins were purified with immobilized metal-ion affinity chromatography yielding pure proteins of correct size without any detectable degradation (data not shown). The three binders were immobilized to one surface each on a carboxylated biacore chip using thiol coupling. Eleven different concentrations of TNF-alpha were injected over the surface and the responses at equilibrium binding were determined. The RU-values were then plotted against the TNF-alpha concentrations (trimer) and the data were fitted to the same monovalent binding equation as used earlier for the flow-cytometric data to determine the apparent K\textsubscript{D} values (Fig. 3B). The results were in accordance with the values obtained from on-cell analysis using flow cytometry. However, a difference (~6-fold) was observed in absolute values determined with the two methods (Table I). As has been shown in a previous study (Löfblom et al., 2007b), this might be explained by the difference in recombinant protein density on the staphylococcal cell surface compared with the biacore chip resulting in different degrees of avidity effect when interactions with multivalent antigens are analyzed.

**Biophysical characterization**

The affibody library is randomized in 13 of 58 amino acid positions and some of these variants may potentially acquire mutations that may alter the structure and stability in a negative manner. Consequently, a brief biophysical characterization of the selected clones was performed in order to ensure that the selected candidate clones were stable proteins with the same three-helical fold as the parental scaffold. CD spectroscopy was used to analyze the secondary structure content and thermal stability of the TNF-alpha-binding affibody molecules. All three affibody molecules showed a CD spectrum comparable with the parental IgG-binding Z-domain (Wahlberg et al., 2003), suggesting intact three-helical structure (Fig. 3C). Moreover, the melting temperature (T\textsubscript{m}) was determined to 54°C for Z\textsubscript{TNF-alpha\textsubscript{1}}, 53°C for Z\textsubscript{TNF-alpha\textsubscript{2}} and 78°C for Z\textsubscript{TNF-alpha\textsubscript{3}} (Fig. 3D, Table I).

**On-cell competition studies**

A competitive assay with three different anti-TNF-alpha proteins, presently in therapeutic use, was performed to further analyze the interaction between the affibody molecules and TNF-alpha. Staphylococcal cells expressing the different affibody binders, respectively, were incubated with soluble biotinylated TNF-alpha, pre-incubated with a 10 times molar excess of either one of two different monoclonal antibodies [infliximab (Targan et al., 1997) or adalimumab (Furst et al., 2003)] or a recombinant TNF-alpha receptor construct [etanercept (Moreland et al., 1997)]. Since both antibodies and the recombinant receptor have extremely high affinity for TNF-alpha (Sakorafas et al., 2007), almost all TNF-alpha is in complex with the antibodies or the receptor when incubated with the staphylococcal cells. An unrelated antibody was used as a negative control in the assay to account for unspecific effects. After incubation and secondary reagent labeling, the cell populations were analyzed using flow cytometry to determine any decrease in TNF-alpha-binding signal. The results demonstrated that pre-incubation with adalimumab almost totally blocked the affibody/TNF-alpha interaction, indicating an overlapping epitope (Fig. 4A). Pre-incubation with the recombinant receptor, etanercept, decreased the TNF-alpha-binding signal to ~20% (Fig. 4B). The partial reduction seen for the receptor is likely to be due to the mechanism by which it interacts with TNF-alpha. The receptor is binding TNF-alpha only in the biological active, trimeric form and when doing so, blocking two TNF-alpha subunits (Scallon et al., 2002). However, the third subunit will thereby always be unblocked and hence available for interaction with the affibody, and a total blocking will not occur even if the two binders share the same epitope. In contrast to the other two therapeutic proteins,
infliximab showed a smaller impact on the TNF-alpha-binding signal, indicating perhaps a partial overlapping epitope or a small allosteric effect (Fig. 4C). The results from the negative control experiment showed no reduction in TNF-alpha binding, indicating that the earlier observed competitions were specific (Fig. 4D).

Interestingly, all three affibody molecules demonstrated similar patterns in the competitive experiments, indicating that they bind to the same region on TNF-alpha. This result is not surprising, since isolated affinity reagents generally recognize only one or a few interaction hot spots on the target protein, which also in many cases overlap with the epitope of the natural ligand.

Table I. Affinities of the three affibody molecules for TNF-alpha and melting temperatures

<table>
<thead>
<tr>
<th>Clone</th>
<th>(K_D) (on-cell analysis, nM, mean ± SD)(^a)</th>
<th>(K_D) (biosensor analysis, nM, mean ± SD)(^b)</th>
<th>(T_m) (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Z_{\text{TNF-alpha}1})</td>
<td>0.095 ± 0.0085</td>
<td>0.77 ± 0.15</td>
<td>54</td>
</tr>
<tr>
<td>(Z_{\text{TNF-alpha}2})</td>
<td>0.3 ± 0.05</td>
<td>1.7 ± 0.08</td>
<td>53</td>
</tr>
<tr>
<td>(Z_{\text{TNF-alpha}3})</td>
<td>2.2 ± 0.8</td>
<td>6.6 ± 0.6</td>
<td>78</td>
</tr>
</tbody>
</table>

\(^a\)Performed in triplicate on different days.

\(^b\)Performed in duplicate on different days with duplicate of each concentration.
Discussion

In this study, we describe the development and application of a novel combinatorial selection method for protein engineering purposes. The method is based on display of a combinatorial affibody library on the surface of the Gram-positive bacteria *S. carnosus*, followed by fluorescence-activated cell sorting (FACS) to generate specific affinity proteins. Three affibody molecules with high affinity for human TNF-alpha were successfully isolated and characterized using this method.

Library diversity is an important parameter in combinatorial protein engineering for the generation of high affinity binders, and although staphylococcal display, as other cell display technologies, has several advantages over phage display, the higher transformation efficiency for *E. coli* results in larger combinatorial libraries using the latter. In order to circumvent the lower transformation efficiency, a new strategy was employed here. Generally for cell display, FACS from libraries with a complexity over $10^8$ variants is not feasible due to the time aspect, and a biopanning step is

Fig. 4 On-cell competition analysis using flow cytometry. FL-2 fluorescence intensity corresponding to the TNF-alpha-binding signal on the x-axis. Filled (right most) peaks in histograms are showing TNF-alpha binding without competition (control). Lined (left most) peaks in histograms show TNF-alpha binding with competition. The percentage of remaining binding activity after blocking is shown in each histogram and calculated as $\frac{MFI_{competition}}{MFI_{control}} \times 100$. (A) Competition with a 10-fold molar excess of adalimumab over TNF-alpha. (B) Competition with a 10-fold molar excess of etanercept over TNF-alpha. (C) Competition with a 10-fold molar excess of infliximab over TNF-alpha. (D) Competition with a 10-fold molar excess of human polyclonal IgG over TNF-alpha.
therefore often included using, for example, magnetic bead enrichment of antigen-binding cells to reduce the library size (Feldhaus et al., 2003; Bessette et al., 2004). In this study, we combined our new staphylococcal method with one cycle of phage display to perform the essential reduction of the library size and then continue with the Gram-positive bacterial display of the pre-enriched library for sorting and on-cell candidate characterization. In this way, the same combinatorial complexity as can be obtained with phage display was screened in a more quantitative manner using our new technology. Moreover, by employing this flexible strategy, phage display can be replaced by a cell-free selection system [i.e. ribosome or microbead display (Nord et al., 2003)] in the pre-enrichment step to increase the library size even further. During subcloning to the staphylococcal host, it would also be feasible to introduce new mutations by error-prone PCR for a more evolutionary approach.

Even though Gram-positive bacteria have several properties that make them excellent candidates for combinatorial cell display purposes (e.g. a single-cell membrane architecture, high viability and C-terminal anchoring to the cell wall of recombinant fusion proteins) so far, large library applications have not been possible due to the low transformation efficiency. However, in a recent study, we optimized the electroporation-mediated transformation frequency of S. carnosus to up to 10^6 cfu per transformation (Löfblom et al., 2007a). Using the improved protocol here, an affibody library of 1.1 × 10^9 variants was transformed to the staphylococcal host and displayed on the cell surface, creating the first reported combinatorial protein library to be displayed on Gram-positive bacteria. In addition, since only a few transformations are required to cover the size of the pre-enriched library, subcloning to the staphylococci is rather straightforward.

As for other cell display methods, the actual selection is performed using FACS, by which sortings are based on a quantitative measure of the individual affinity of each library member in contrast to the capture and elution principle used in, for example, phage display (Feldhaus et al., 2003). Three cycles of FACS yielded three different affibody molecules, with affinities (95 pM–2.2 nM) in the same range as for affinity proteins generated with alternative methods from repertoires of similar size.

Although the staphylococcal system is ideal for correct display and selection of staphylococcal-derived affibody molecules due to the staphylococcal origin of both the host cell and the cell surface-anchoring cassette, individual scFvs have previously been functionally expressed on the staphylococcal surface (Gunneriusson et al., 1996), indicating that selections from protein libraries based on other scaffolds should also be possible. We believe that the novel staphylococcal selection system is a powerful alternative to existing display technologies, and the successful results obtained here might open the field to include other Gram-positive species in the future, expanding and improving the area of combinatorial protein engineering.

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**References**


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