



Multimerization domains for antibody phage display and antibody production

H. Thie, S. Binius¹, T. Schirrmann, M. Hust and S. Dübel

Technische Universität Braunschweig, Institut für Biochemie und Biotechnologie, Abteilung Biotechnologie, Spielmannstr. 7, 38106 Braunschweig, Germany

High-throughput generation of antibodies for proteome research has become feasible by using antibody gene libraries and *in vitro* selection methods like phage display. Typically monovalent antibody fragments like scFv, Fab or scFab are obtained by this technology. To mimic the IgG molecule and gain avidity, resulting in stronger binding, multimerization domains can be fused to antibody fragments. Here we systematically analyzed different multimerization domains in respect to three key parameters, crucial for the high-throughput generation of binders. (i) The compatibility to be displayed on phage (assessed for at least three different antibody formats, scFv, Fab and scFab) in combination with five different multimerization domains; (ii) production yields and (iii) oligomerization properties were analyzed for three different scFv fragments. We found that the use of a biotin acceptor domain in combination with an *in vivo* biotinylation system performed best concerning the key parameters and thus would be a useful tool to generate multimeric antibody complexes on demand from phage display selected antibody fragments with the least effort.

Introduction

Recombinant antibody fragments are powerful tools for research, diagnostics and therapy. Specific antibody fragments can be isolated by widely used *in vitro* selection systems like phage display [1,2] and are easy to produce by prokaryotic expression systems (for review see [3]).

These antibody fragments (e.g. selected from naïve antibody gene libraries [4,5]) differ from IgG by their monovalency. In the majority of binding assays used for research projects, the dimeric binding of an IgG causes a significant increase in apparent affinity by the avidity effect [6]. The strength of this effect depends on valency and affinity of the binding events involved in the interaction. For a high-throughput generation of antibody fragments, especially for proteome research projects, it is favorable to have antibody phage display compatible multimerization domains directly included into the antibody gene library. This avoids

further subcloning of selected antibody genes to special expression vectors and will reduce effort and cost.

To date, various strategies have been developed to oligomerize antibody fragments to gain avidity. Based on the scFv format the formation of diabodies [7], triabodies [8], tetrabodies [9] and tandem antibodies [10] is possible because of modifications in the linker region between V_H and V_L. Another strategy is the fusion of antibody fragments to different constant regions of immunoglobulins. Well-known examples are Fc-fusions [11,5], or Minibodies [12].

It is also possible to fuse antibody fragments with heterologous peptides or proteins to achieve multimeric antibody fragments. The use of amphipathic helices or leucine zippers fused to antibody fragments is one strategy for multimerization [13,14] as well as the use of biotin acceptor peptides [15] in combination with streptavidin or fusions directly to streptavidin [16] to build multivalent Streptabodies [17].

In this study we systematically compared different multimerization strategies by generating scFv variants with different multimerization domains (amphipathic helices, leucine zippers and

Corresponding author: Thie, H. (h.thie@tu-bs.de)

¹ Present address: Epigenetic Regulation Group, Helmholtz Centre for Infection Research, Braunschweig, Germany.

TABLE 1

Production properties of soluble antibody fragment production in *E. coli*

Antibody	Production yield [$\mu\text{g/L}$]					
	No domain	p53	dHLX	ZIP	TetraZIP	BAD
D1.3 scFv	290 (100%)	3 (1%)	84 (29%)	102 (35.2%)	nd	112 (38.6%)
α -MUC1 A scFv	458 (100%)	1 (0.2%)	145 (31.7%)	120 (26.2%)	nd	104 (22.7%)
α -MUC1 B scFv	1295 (100%)	10 (0.8%)	61 (4.7%)	38 (2.9%)	nd	834 (64.4%)

Three different scFv fragments were produced in shake flask scale and purified by IMAC from periplasmic fractions. The production yields (absolute and relative) after purification are shown. nd = not detectable.

biotinylation acceptor peptides) C-terminally fused to three different scFvs.

We analyzed three key parameters relevant for their practical use in research: (i) compatibility to phage display, (ii) production yields and (iii) oligomerization properties. The influence of oligomerization on the apparent affinity of these molecules was evaluated by the titration of ELISA. As it is well known that both the size of the antibody fragment and the primary structure influence the production, we used soluble single chain fragments with three different sequences. Further, for phage display experiments, three different formats (scFv, Fab and scFab [18]) of the murine antilysozyme antibody D1.3 were analyzed.

Materials and methods*Bacterial strains and genotypes*

Bacterial strains and phage used in this work:

The antibody fragment genes of D1.3 scFv, anti-MUC1 A scFv and anti-MUC1 B scFv were cloned into the corresponding pOPE101-based expression vectors by using NcoI and NotI [22]. All areas affected by cloning were subsequently sequenced.

Production of phage presenting antibody fragments with multimerization domains

Antibody phage production was achieved by inoculating 50 mL $2\times$ YT medium containing 100 $\mu\text{g/mL}$ ampicillin and 100 mM glucose with an *E. coli* XL1 Blue MRF' clone harboring the corresponding phagemid. The bacteria were grown to an optical density at 600 nm (OD_{600}) of 0.4–0.5 at 37°C and 250 rpm. 2 mL bacterial culture ($\sim 1 \times 10^9$ bacteria) was infected with 2×10^{10} M13K07 phage, incubated at 37°C for 30 min without shaking followed by 30 min at 250 rpm. The infected cells were harvested by centrifugation for 10 min at $3220 \times g$. The pellet was resuspended in

	Genotype	Supplier
Bacterial strain		
<i>E. coli</i> XL1-Blue MRF'	K12: $\Delta(\text{mcrA})183 \Delta(\text{mcrCB-hsdSMR-mrr})173 \text{ endA1 supE44 thi-1 recA1 gyrA96 relA1 lac [F' proAB lacPZ } \Delta\text{M15 Tn10 (Tet}^r\text{)]}$	Stratagene, Amsterdam, Netherlands
<i>E. coli</i> TG1	K12: $\text{supE thi-1(lac-proAB) hsdD5[F' traD36 + proAB + lac}^f \text{ lacZ(M15)]}$	Amersham Biosciences
Helper phage		
M13K07 [19]		GE-Healthcare, Munich, Germany

Cloning of multimerization domain genes

The amino acid sequences (incl. spacer) for the multimerization domains were taken from Plückthun and Pack [13] and Cloutier et al. [17].

250 mL $2\times$ YT containing 100 $\mu\text{g/mL}$ ampicillin and 50 $\mu\text{g/mL}$ kanamycin ($2\times$ YT-AK). The phages were produced at 30°C and 250 rpm over night. On the following day cells were centrifuged for 20 min at $3220 \times g$. Phage particles in the supernatant were

Multimerization domain	Amino acid sequence	Reference
p53	TPLGDTTHTSGKPLDEYFTLQIRGRERFEMFRELNEALELKDAQAGKEPGGGGAPHHHHHH	[13]
dHLX	SPSTPPTSPSTPPASGGLNDIFEAQKIEWHEGSSKYKYHHHHHH	[13]
TetraZIP	PKPSTPPGSSRLKQIEDKLEILSKLYHIENELARIKLLGERGGSGGAPHHHHHH	[13]
ZIP	PKPSTPPGSSRMKQLEDKVEELLSKNYHLENEVARLKKLVGERGGSGGAPHHHHHH	[13]
BAD (AviTag)	SPSTPPTSPSTPPASGGLNDIFEAQKIEWHEGSSKYKYHHHHHH	[17]

The corresponding DNA sequences were codon optimized for *E. coli* K12 by using the JCat-module [20] and cloned into the phagemid pHAL1-D1.3-scFv [21], pHAL1-D1.3-Fab and pHAL1-D1.3-scFabdC, between the antibody fragment and the gIII gene by standard techniques [22]. The multimerization domains were also cloned into the expression vector pOPE101 [23].

precipitated with 1/5 volume of 20% (w/v) polyethylene glycol (PEG)/2.5 M NaCl solution for one hour on ice with gentle shaking and pelleted by centrifugation for one hour at $3220 \times g$ at 4°C. The precipitated phages were resuspended in 500 μL PBS (phosphate buffered saline, pH 7.4) [22]. Residual bacteria and cell debris were removed by additional centrifugation for 5 min at $16,000 \times g$ at 20°C. The supernatants containing the antibody presenting phage

were stored at 4°C. Phage titration was done according to Hust *et al.* [18].

Production of soluble antibody fragments with multimerization domains

Soluble antibody fragment production was performed by inoculating 50 mL 2× YT medium containing 100 µg/mL ampicillin and 100 mM glucose (2× YT-GA) with an *E. coli* TG1 clone harboring the corresponding expression vector. The bacteria were grown in shake flask over night at 37°C and 250 rpm. 10 mL bacterial culture was used to inoculate 600 mL 2× YT-GA to an OD₆₀₀ of 0.1. Cells were grown until OD₆₀₀ reaches 0.9, then the antibody fragment production was induced by adjusting to 50 µM IPTG. The production was performed at 25°C and 250 rpm over night. Cells were harvested by centrifugation at 4225 × *g* for 5 min. The cell pellets were used for a subsequent preparation of periplasmic fractions, which was done by resuspending pelleted cells in 10% of culture volume periplasmic preparation buffer (20% sucrose, 50 mM Tris, 1 mM EDTA, pH 8.0) and incubated for 20 min on ice. The suspension was centrifuged at 30,000 × *g* for 30 min at 4°C. The supernatant (periplasmic fraction) was dialyzed against 5 L PBS over night.

In vivo biotinylated antibody fragments were produced as mentioned above, but the corresponding *E. coli* clones were cotransformed with the helper plasmid pBirAcm (Avidity LLC) that carried an *E. coli* biotin ligase gene. The 2× YT-based media for the production of biotinylated scFvs were supplemented with 34 µg/mL chloramphenicol (2× YT-GAC, preculture) and with 50 µM biotin (2× YT-GACB, production culture).

IMAC purification of soluble antibody fragments

The dialyzed periplasmic extracts were adjusted to 0.5 M NaCl and 10 mM imidazole by adding corresponding volumes of 5 M stock solutions. 0.5–0.7 mL of chelating sepharose FF (GE Healthcare) loaded with Ni²⁺ ions was incubated with the periplasmic extracts for one hour at 4°C in an overhead shaker (12 rpm). The sepharose matrix was separated by centrifugation (500 × *g*, 5 min) after each incubation step. The matrix was washed three times with 4 mL wash buffer (0.5 mM NaCl, 50 mM Tris, pH 8.0), with increasing imidazole concentrations for each washing step (10, 20 and 40 mM). The antibody fragments were eluted by adding 3 mL elution buffer (PBS containing 100 mM EDTA). The elution fractions were aliquoted and stored at –80°C.

ELISA

ELISAs were performed in 96-well MaxisorpTM microtiter plates (Nunc, Langensfeld, Germany). Each well was coated with 1 µg antigen in 100 µL in 50 mM NaHCO₃ buffer, pH 9.6 over night at 4°C. Afterwards the wells were blocked with 2% (w/v) skimmed milk powder (Roth, Karlsruhe, Germany) in PBST (PBS [22] + 0.1% Tween 20) (2% MPBST) for one hour at RT, followed by three washing steps with PBST (PBS + 0.05% Tween 20) using an ELISA washer (Tecan Columbus, Tecan, Crailsheim, Germany). Serial dilutions of the scFv presenting phage or soluble antibody fragments were incubated with the antigen for one hour, followed by three washing steps with PBST. Bound antibody phages were detected by using the murine anti-M13 HRP

conjugate (GE Healthcare, Munich, Germany, 1:5000) that recognizes the pVIII of the phage. Bound soluble antibody fragments were detected by using the murine anti-His5 antibody (Qiagen, Hilden, Germany, 1:5000), which recognizes the C-terminal His tag of the antibody fragments. After three washing steps with PBST, staining was performed with a goat antimouse serum (Fab specific) conjugated to HRP (Sigma, 1:10,000). The visualization in both cases was performed with TMB (3,3',5,5'-tetramethylbenzidine) as substrate. The staining reaction was stopped by adding 100 µL 1 M sulfuric acid. Absorbance at 450 nm (using A₆₂₀ as reference) was measured using a SUNRISETM microtiter plate reader (Tecan).

SDS-PAGE and immunoblot

Phage particles were analyzed by SDS-PAGE under reducing conditions and blotted onto a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 2% (w/v) skimmed milk powder (Roth, Karlsruhe, Germany) in PBST (PBS + 0.1% Tween 20) for one hour at RT. The minor coat protein pIII and its fusion with antibody fragments were detected with mouse anti-pIII monoclonal antibody (mAb) (Mobitec, Göttingen, Germany) (1:2000) for 1.5 hours at RT, followed by 2× washing with PBS supplemented with 0.1% Tween-20 (PBST). Goat anti-mouse IgG (Fc specific) antibody conjugated with alkaline phosphatase (AP) (Sigma, Taufkirchen, Germany) (1:10,000) was used for detection and visualized by nitro-blue tetrazolium chloride/bromo-4-chloro-3-indolylphosphate toluidine (NBT/BCIP).

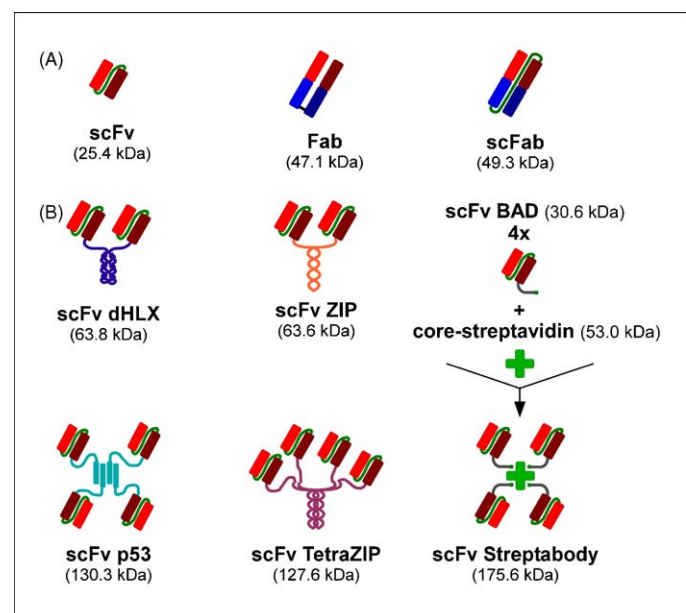


FIGURE 1

(A) Schematic drawings of different antibody formats. V_H and V_L of the scFv fragments were shown red, C_{H1} and C_L in blue, the peptide linker in green. Numbers indicate the calculated molecular mass of the polypeptide chain(s) of the respective construct, given for variants of D1.3. (B) Antibodies with different multimerization domains. The multimerization domains were fused C-terminally to antibody fragments and led to 'auto-multimerized' antibody fragments, except for the BAD domain. *In vivo* biotinylated scFv (BAD) were incubated with core-streptavidin in 6:1 ratio to build Streptabodies. BAD = biotin acceptor domain (AviTag).

Size exclusion chromatography

Size exclusion chromatography was performed using Superdex200 10/300 GL column (GE Healthcare) on an ÄKTA-purifier system (GE Healthcare). 100 μ L of each sample was injected and separated by using PBS [22] as running buffer with a flow rate of 0.5 mL/min. A_{280} was plotted against the retention volume to identify the different antibody fragments, in comparison to a set of marker proteins analyzed in separate runs.

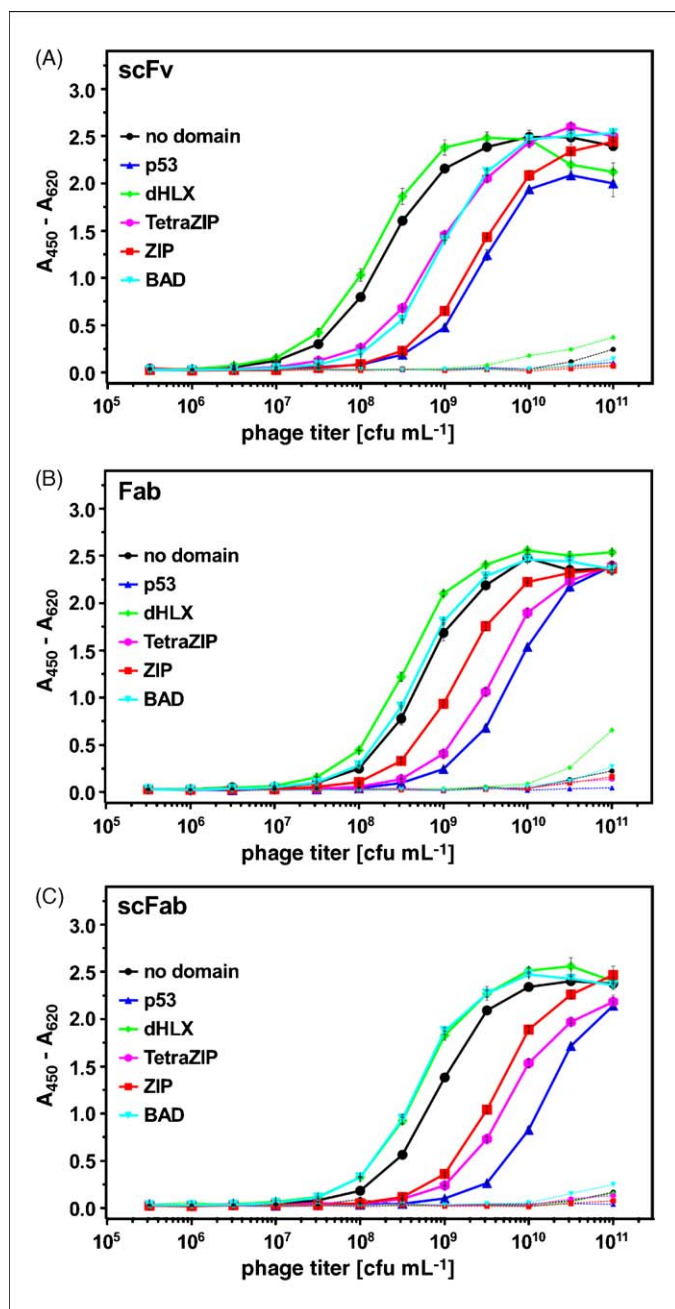


FIGURE 2

Antibody binding of tagged variants of different antibody formats of D1.3 (scFv, Fab and scFab) presented on phage. PEG-purified antibody phages were diluted to defined cfu values and analyzed by phage ELISA. Five different multimerization domains were investigated for their specific influence on the antibody fragment display on M13K07 phage. The mean value of three experiments is given by each data point; error bars represent the standard deviations. The dotted lines show incubation of similar cfu values incubated on BSA (negative control).

Results

Compatibility of antibody fragment multimerization domains with phage display

To determine whether multimerization domains are compatible with antibody phage display these domains were analyzed for their effect on the presentation of the murine lysozyme-specific antibody fragments (D1.3 scFv, D1.3 Fab and D1.3 scFab) on M13 phage. Three different formats of D1.3 were chosen to analyze the effect of antibody size and polypeptide chain composition on multimerization (Fig. 1A).

The genes of ZIP, TetraZIP, dHLX, p53 and BAD (Fig. 1B) were cloned into a pHAL1-based phage display vector [21] at the 3'-terminus of the antibody fragment gene (fd fragment for D1.3 Fab). Antibody phage was produced and purified by PEG-based precipitation. An ELISA with a defined number of cfu of antibody phage was performed to analyze their specific binding to lysozyme.

The ELISA signals are saturated when using 10^{11} phage particles per milliliter (Fig. 2). The investigated multimerization domains did not prevent antibody phage display *per se*. However a decreased

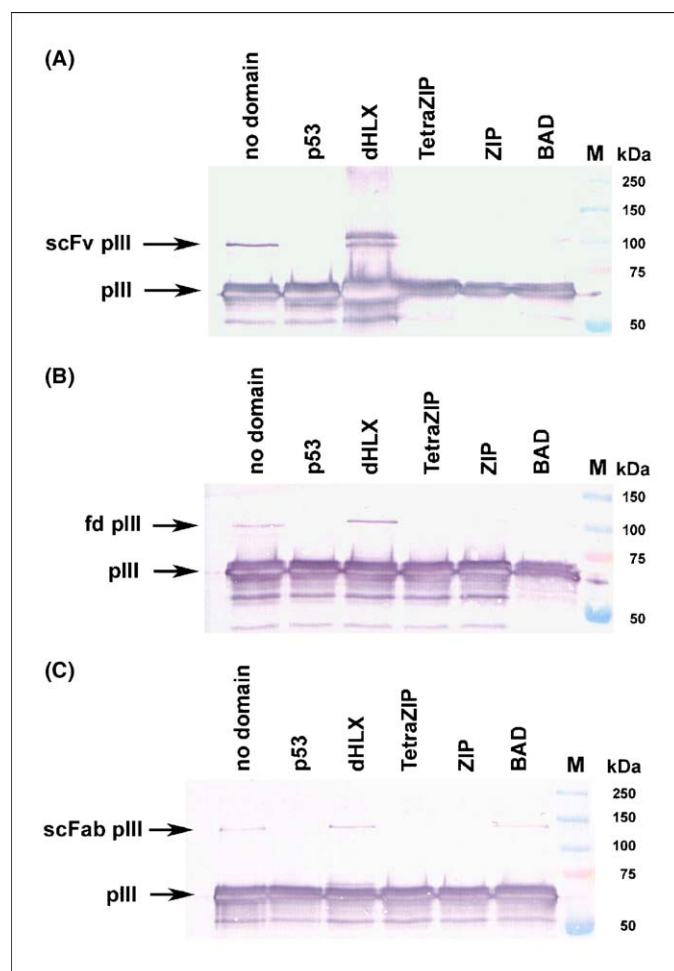


FIGURE 3

Antibody presentation on phage. Three different antibody formats of the lysozyme specific antibody D1.3 (scFv, Fab and scFab) in combination with five different multimerization domains were investigated for the presentation of an antibody pIII fusion protein. 3×10^{10} antibody phage particles were separated on a 10% SDS-PAGE. After electroblotting, pIII and pIII-fusion proteins were detected by immunostaining using a mouse anti-pIII antibody.

display of antibody fragments on phage of more than an order of magnitude was observed mainly for ZIP, TetraZIP and p53 domains in combination with all three antibody formats.

An immunoblot analysis of 3×10^{10} cfu of each antibody phage preparation was performed to determine whether the differences in binding during the phage ELISA depend on the number of antibody fragments presented on phage or on the correct folding of the presented antibody fragments (Fig. 3). For all three analyzed antibody fragments, the variants without any multimerization domain and with dHLX dimerization domain showed the most prominent pIII antibody fusion protein band. A very faint band is detectable for the antibody phage with BAD domain. No pIII fusion protein is detectable for p53, TetraZIP and ZIP domain. These results confirm that reduced phage binding determined by ELISA (Fig. 2) is a result of a reduced presentation efficiency introduced by these domains.

Characterization of soluble multimerized antibody fragments

After proof of principle that antibody phage display is possible with simultaneous use of multimerization domains, multimerized soluble antibody fragments were produced in an *E. coli* expression system. Here, solely the scFv format was used, because expression

rates were usually higher for scFv compared to Fab or scFab fragments.

To analyze the influence of multimerization domains on the production yields of antibody fragments, three different scFvs were analyzed in combination with all multimerization domains. A significant reduction (min. 100-fold) of the production yields was observed when fusing antibody fragment to the tetramerization domains p53 and TetraZIP. The use of other domains also led to reduced production yields, but less pronounced. All three scFv fragments without any multimerization domain showed the highest levels (see Table 1).

To validate whether the scFv fragment without multimerization- or with BAD domain tend to form dimer (diabodies), which would influence the binding, size exclusion chromatography (SEC) was performed. SEC was also used to check the formation of Streptabodies after incubation of *in vivo* biotinylated scFv with core-streptavidin (Fig. 4) D1.3 scFv showed no tendency to form diabodies, anti-MUC1 A showed a high and anti-MUC1 B a low tendency to form dimers. These properties were directly influenced by the use of the BAD domain. An increase of the dimer fraction was observed for D1.3 BAD and anti-MUC1 B-BAD. By contrast anti-MUC1 A-BAD had less dimers.

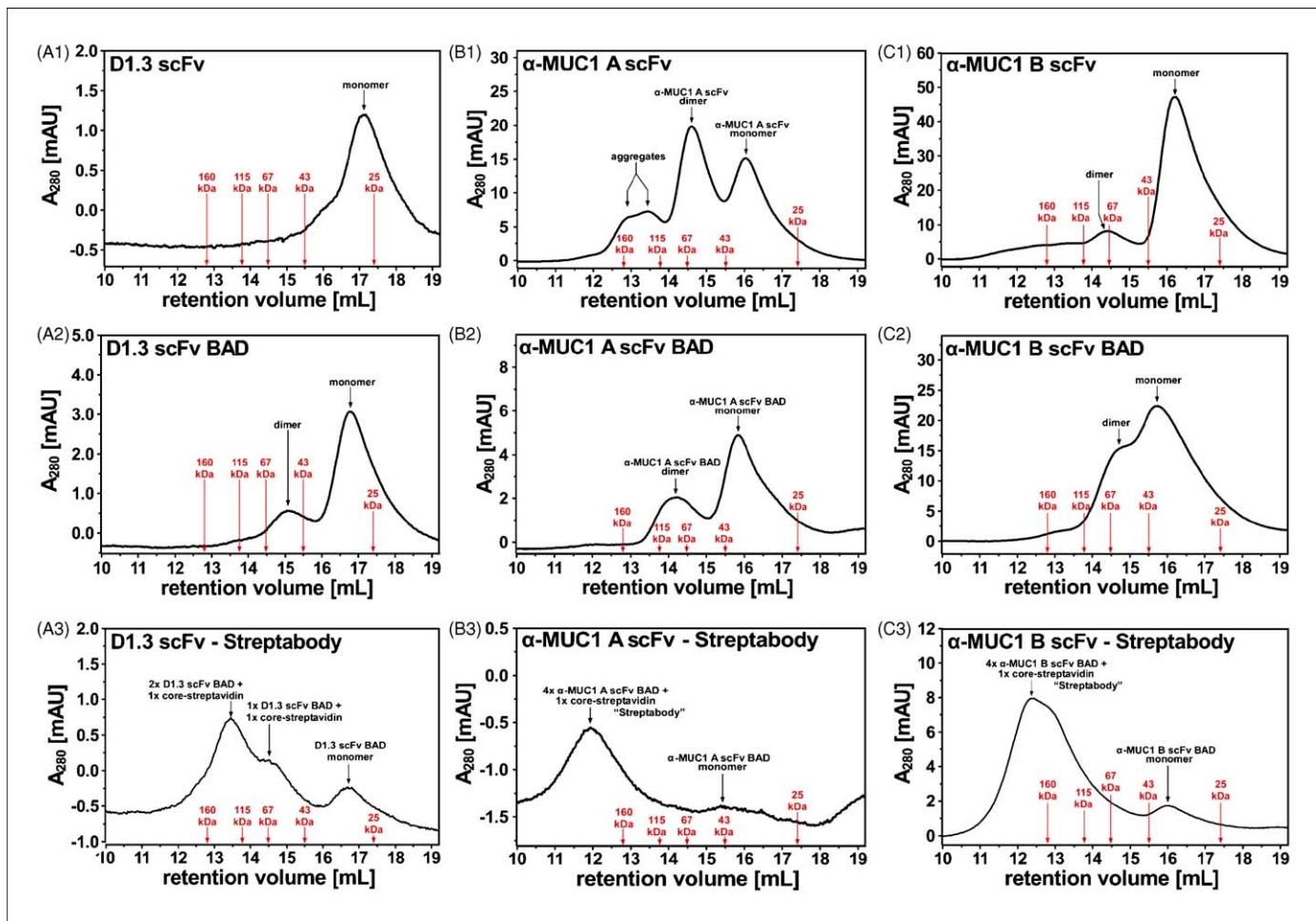
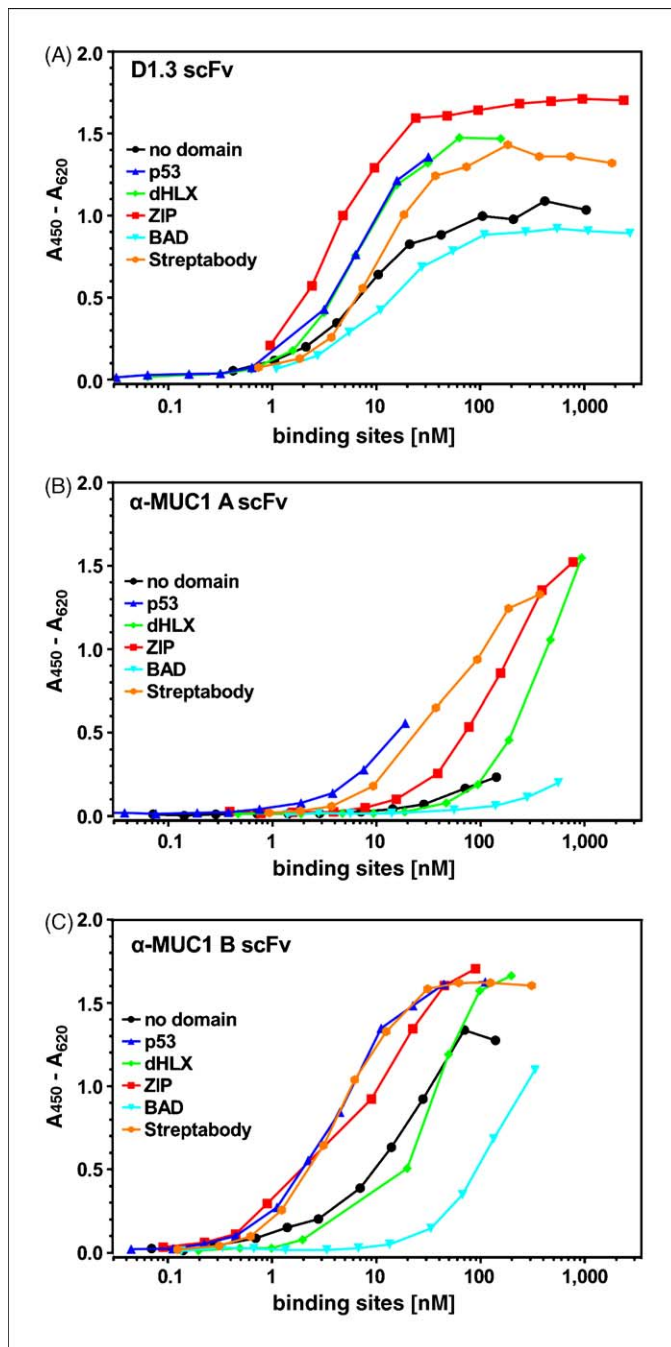


FIGURE 4

Streptobody formation. Size exclusion chromatography analysis of three different antibody fragments (scFv) without any multimerization domain, with BAD domain for *in vivo* biotinylation and after incubation of scFv BAD with core-streptavidin in a 6:1 ratio. Aliquots of purified scFv fragments were analyzed on a Superdex200 10/300 column. The UV-absorption (A_{280}) was drawn against the retention volume. The calibration of the column is shown by red arrows (160 kDa IgG; 115 kDa CRP; 67 kDa Albumin; 43 kDa Ovalbumin; 25 kDa Chymotrypsinogen).

**FIGURE 5**

Antigen binding of three different soluble scFv fragments combined with five different multimerization domains. Soluble scFvs were produced in shake flasks and purified by IMAC. The ELISA signal is normalized to the number of binding sites of each molecule to estimate the avidity effect. The ELISA signal of a BSA negative control was subtracted from the specific ELISA signals.

The formation of Streptabodies was determined by SEC after incubation of *in vivo* biotinylated scFv and streptavidin in 6:1 ratio. Peaks around retention volumes of 16–17 mL in chromatograms (Fig. 4: A3, B3 and C3) were caused by nonstreptavidin-associated scFv. It was shown that Streptabodies could be detected and formed with high efficiency.

To demonstrate the avidity effect of multimeric antibody fragments, antigen ELISA with three antibodies in combination with all multimerization domains was performed. In respect to the

number of antigen binding sites of each antibody molecule the concentration was normalized to the number of antigen binding sites (e.g. D1.3 scFv p53 concentration was multiplied with a factor of four) (Fig. 5). For both anti-MUC1 (A and B) antibody fragments lacking any multimerization domain, monomeric fractions obtained from size exclusion chromatography were used for ELISA analysis.

For the tetravalent antibody variants ELISA signals were detected at significant lower binding site concentrations compared to monovalent antibody fragments. The differences in binding between tetravalent Streptabodies and their corresponding monovalent BAD-only antibody fragments were up to two orders of magnitude (Fig. 5B,C).

By normalizing the antibody concentration to the number of binding sites the avidity effect could be estimated by comparing the corresponding ELISA binding curves.

Discussion

Modern proteomics research projects require large numbers of different antibodies against a multitude of different (often not well characterized) antigens. Antibody gene libraries in combination with *in vitro* selection by phage display offer the possibility to deliver binders with high throughput [24,25]. These antibody fragments usually provide monovalent binding thus lacking the avidity effect provided by the two binding arms of an IgG. To utilize avidity effects for increased binding, multimerization of antibody fragments is a common strategy. We systematically analyzed different multimerization domains for their performance in respect to key parameters that were crucial in the high-throughput generation of recombinant antibodies.

The first key parameter analyzed in this study was the compatibility of multimerization domains with antibody phage display. As a model we used the well-characterized murine anti-lysozyme antibody D1.3 in three different antibody formats (scFv, Fab and scFab [18]). It was shown before that antibody phage display is possible with either multimerization domains or the antibody formats used in this study (Fig. 1), but most of their combinations were not yet tested. Antibody fragment display differed significantly, depending on the choice of multimerization domain. The p53 fusion resulted in a one order of magnitude drop in functional presentation of antibody fragments. A similar drop was caused by the ZIP and TetraZIP domains. In the corresponding immunoblot analysis, pIII fusion protein expression was below the detection limits. The use of 'auto-multimerization' domains like dHLX, ZIP, TetraZIP and p53 might lead to a multivalent presentation of antibody fragments on phage [26]. Even when using an amber stop codon between the multimerization domain gene and the gIII and a suitable *E. coli* suppressor strain for phage production (as done in this study), there should be a multimeric presentation of antibody fragments on phage according to Lee *et al.* [26]. This would result in avidity effects and would also explain that antigen ELISA signals are detectable despite the very low expression. However, for dHLX, ZIP and TetraZIP, the avidity gain cannot compensate the very poor expression. The three different antibody formats did not influence the effect of the multimerization domains, because the results of the phage ELISA on antigen were similar for all three formats. Presentation levels decreased for the two larger and more complex antibody designs (Fab and scFab) by

a factor of around five, except for the BAD constructs, which showed no difference. In conclusion, the compatibility of multimerization domains with antibody phage display was demonstrated.

The BAD is maybe compatible when used with alternative *in vitro* display methods like ribosomal or mRNA display. It will not form multimeric structures after the *in vitro* translation, which might disturb the RNA–ribosome–protein complex, allowing the selection of high affinity binders that afterwards can be biotinylated directly.

Because the results obtained with the three different antibody formats did not show any specific incompatibility with any of the multimerization domains, analysis of soluble multimeric antibody fragments was done for the most widely used scFv format. However, three different scFv fragments, covering a range of production yields were compared to allow a better conclusion on the general applicability of the domains. The expression of these different scFv fragments in all combinations with all multimerization domains again revealed significant differences in production yield, which did not come unexpected.

For the production of scFv dHLX, a decrease around 20–50% in production yield was reported [13]. For the p53 oligomerization domain a maximum reduction by a factor of eight was reported [27]. By contrast our results showed a decrease in production yields of >95% for dHLX fusions and >99% for p53 fusions. When the TetraZIP domain was fused, a production and purification of functional antibodies was not possible. This supports published data where a suitable production yield was shown only for one sequence optimized single chain antibody fragment [28]. We found differences between three different scFv clones used in this study, with the largest variation for TetraZIP. Possible explanations are that the multimerization domains hinder effective translocation through the inner membrane or have an unfavorable influence on protein folding, because high amounts of insoluble antibody were found intracellularly in *E. coli* (data not shown).

SEC analysis was performed to analyze the oligomerization properties of the scFv, that is whether they tend to form dimers spontaneously or not [29], and to analyze the influence of BAD domain on these properties. Interestingly, the BAD domain influenced the dimerization of all three analyzed scFv fragments. In case of D1.3 scFv and α -MUC1 B scFv it increased dimer formation but for α -MUC1 A, it significantly reduced the dimerization tendency. A possible explanation for this observation can be found in the additional amino acids provided by the BAD domain, which might influence the interaction with the folding apparatus for each single antibody fragment in a different way.

The *in vivo* biotinylation is adjustable by the addition of biotin to the culture medium. In our study no significant basal *in vivo* biotinylation has been found even in the presence of the helper plasmid encoding *birA* if culture media without biotin supplementation had been used (data not shown).

Unlike the successful formation of tetravalent Streptabodies for anti-MUC1 A and anti-MUC1 B, the retention of the D1.3

Streptabody in SEC indicates that less than four biotinylated scFv fragments are bound per core-streptavidin, indicating the formation of a di- to trivalent molecule. These Streptabodies are not saturated with biotinylated scFv, which is possibly because of an incomplete biotinylation of scFv antibody fragments. A higher ratio of scFv BAD to core-streptavidin can prevent the formation of di- or trivalent Streptabodies. The use of nonpurified ‘unsaturated’ Streptabodies, that is in multiplexed immunoassays, bears the risk of forming hybrid Streptabodies with multiple specificities, which can be circumvented by purifying the Streptabodies to remove nonstreptavidin-bound scFv BAD.

As expected, multivalent variants yielded stronger antigen ELISA signals compared to monovalent scFv fragments. A comparison between scFv BAD and Streptabody fragments, which differ just by the addition of core-streptavidin, showed a gain in binding up to factor of ~ 400 solely based on avidity, despite less than four binding sites were to be expected per complex. This avidity effect matches the affinity increase observed by direct fusion to core-streptavidin [16]. For the anti-MUC1 antibody fragments this effect is more pronounced because a MUC1 peptide antigen with two epitopes per peptide is used in this experiment, leading to a much higher antigen density compared to lysozyme. We suppose that will result in stronger avidity effects.

In summary, BAD domain in combination with an *in vivo* biotinylation system emerges to be the method of choice for antibody fragment multimerization. First, this system allows monovalent antibody phage display allowing to select for binders with high affinity because of the absence of an avidity effect during panning on antigen. Second, the large variations in yield observed for the individual antibody sequences were least pronounced for BAD fusions. This is maybe because of its smaller size compared to the other multimerization domains. Third, it allows a biotinylation on demand, either *in vitro* or *in vivo*. Fourth, the biotinylated scFv can easily be multimerized by just adding streptavidin or avidin in suitable concentrations. Fifth, biotinylated antibody fragments can be directly detected by a large number of commercially available streptavidin conjugates, depending on the assay of choice.

These advantages recommend the BAD domain to be used in antibody gene libraries optimized for high-throughput delivery of antibodies, when subcloning of the scFv gene to an expression vector has to be avoided to save cost and increase throughput.

Acknowledgements

We thank for the support by the ‘SMP Antibody Factory’ of the German National Genome Research Network (NGFN) funded by the BMBF, the support by EU within the 6th framework program and the ‘Proteome Binders’. We gratefully acknowledge Arne Skerra for providing the vector pASK99-D1.3 encoding the antilysozyme antibody fragment. We also thank Torsten Rülker for crucially reading the manuscript.

References

- McCafferty, J. *et al.* (1990) Phage antibodies: filamentous phage displaying antibody variable domains. *Nature* 348, 552–554
- Breitling, F. *et al.* (1991) A surface expression vector for antibody screening. *Gene* 104, 147–153

- 3 Schirrmann, T. *et al.* (2008) Production systems for recombinant antibodies. *Front. Biosci.* 13, 4576–4594
- 4 Marks, J.D. *et al.* (1991) By-passing immunization. Human antibodies from V-gene libraries displayed on phage. *J. Mol. Biol.* 222, 581–597
- 5 Kirsch, M.I. *et al.* (2008) Development of human antibody fragments using antibody phage display for the detection and diagnosis of Venezuelan equine encephalitis virus (VEEV). *BMC Biotechnol.* 8, 66
- 6 Karush, F. (1970) Affinity and the immune response. *Ann. N. Y. Acad. Sci.* 169, 56–64
- 7 Holliger, P. *et al.* (1993) “Diabodies”: small bivalent and bispecific antibody fragments. *Proc. Natl. Acad. Sci. U. S. A.* 90, 6444–6448
- 8 Iliades, P. *et al.* (1997) Triabodies: single chain Fv fragments without a linker form trivalent trimers. *FEBS Lett.* 409, 437–441
- 9 Le Gall, F. *et al.* (1999) Di-, tri- and tetrameric single chain Fv antibody fragments against human CD19: effect of valency on cell binding. *FEBS Lett.* 453, 164–168
- 10 Gruber, M. *et al.* (1994) Efficient tumor cell lysis mediated by a bispecific single chain antibody expressed in *Escherichia coli*. *J. Immunol.* 152, 5368–5374
- 11 Shu, L. *et al.* (1993) Secretion of a single-gene-encoded immunoglobulin from myeloma cells. *Proc. Natl. Acad. Sci. U. S. A.* 90, 7995–7999
- 12 Hu, S. *et al.* (1996) Minibody: a novel engineered anti-carcinoembryonic antigen antibody fragment (single-chain Fv-CH3) which exhibits rapid, high-level targeting of xenografts. *Cancer Res.* 56, 3055–3061
- 13 Plückthun, A. and Pack, P. (1997) New protein engineering approaches to multivalent and bispecific antibody fragments. *Immunotechnology* 3, 83–105
- 14 Rheinhecker, M. *et al.* (1996) Multivalent antibody fragments with high functional affinity for a tumor-associated carbohydrate antigen. *J. Immunol.* 157, 2989–2997
- 15 Schatz, P.J. (1993) Use of peptide libraries to map the substrate specificity of a peptide-modifying enzyme: a 13 residue consensus peptide specifies biotinylation in *Escherichia coli*. *Biotechnology (N Y)* 11, 1138–1143
- 16 Kipriyanov, S.M. *et al.* (1995) Single-chain antibody streptavidin fusions: tetrameric bifunctional scFv-complexes with biotin binding activity and enhanced affinity to antigen. *Hum. Antibodies Hybridomas* 6, 93–101
- 17 Cloutier, S.M. *et al.* (2000) Streptabody, a high avidity molecule made by tetramerization of in vivo biotinylated, phage display-selected scFv fragments on streptavidin. *Mol. Immunol.* 37, 1067–1077
- 18 Hust, M. *et al.* (2007) Single chain Fab (scFab) fragment. *BMC Biotechnol.* 7, 14
- 19 Vieira, J. and Messing, J. (1987) Production of single-stranded plasmid DNA. *Methods Enzymol.* 153, 3–11
- 20 Grote, A. *et al.* (2005) JCat: a novel tool to adapt codon usage of a target gene to its potential expression host. *Nucleic Acids Res.* 33 (Web Server issue), W526–W531
- 21 Kirsch, M. *et al.* (2005) Parameters affecting the display of antibodies on phage. *J. Immunol. Methods* 301, 173–185
- 22 Sambrook, J. and Russell, D.W. (2001) *Molecular Cloning: A Laboratory Manual* (3rd edn), Cold Spring Harbor Laboratory Press
- 23 Schmiedl, A. *et al.* (2000) Effects of unpaired cysteines on yield, solubility and activity of different recombinant antibody constructs expressed in *E. coli*. *J. Immunol. Methods* 242, 101–114
- 24 Hust, M. and Dübel, S. (2004) Mating antibody phage display with proteomics. *Trends Biotechnol.* 22, 8–14
- 25 Taussig, M.J. *et al.* (2007) Proteome binders: planning a European resource of affinity reagents for analysis of the human proteome. *Nat. Methods* 4, 13–17
- 26 Lee, C.V. *et al.* (2004) Bivalent antibody phage display mimics natural immunoglobulin. *J. Immunol. Methods* 284, 119–132
- 27 Willuda, J. *et al.* (2001) Tumor targeting of mono-, di-, and tetravalent anti-p185(HER-2) miniantibodies multimerized by self-associating peptides. *J. Biol. Chem.* 276, 14385–14392
- 28 Pack, P. *et al.* (1995) Tetravalent miniantibodies with high avidity assembling in *Escherichia coli*. *J. Mol. Biol.* 246, 28–34
- 29 Arndt, K.M. *et al.* (1998) Factors influencing the dimer to monomer transition of an antibody single-chain Fv fragment. *Biochemistry* 37, 12918–12926