

Ribosome Display Selection of a Murine IgG₁ Fab Binding Affibody Molecule Allowing Species Selective Recovery Of Monoclonal Antibodies

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Abstract Affinity reagents recognizing constant parts of antibody molecules are invaluable tools in immunotechnology applications, including purification, immobilization, and detection of immunoglobulins. In this article, murine IgG₁, the primary isotype of monoclonal antibodies (mAbs) was used as target for selection of novel binders from a combinatorial ribosome display (RD) library of 10¹¹ affibody molecules. Four rounds of selection using three different mouse IgG₁ mAbs as alternating targets resulted in the identification of binders with broad mIgG₁ recognition and dissociation constants (K_D) in the low nanomolar to low micromolar range. For one of the binders, denoted Z_{mab25}, competition in binding to full length mIgG₁ by a streptococcal protein G (SPG) fragment and selective affinity capture of mouse IgG₁ Fab fragments after papain cleavage of a full mAb suggest that an epitope functionally overlapping with the SPG-binding site in the CH₁ domain of mouse IgG₁ had been addressed. Interestingly, biosensor-based binding experiments showed that neither human IgG₁ nor bovine Ig, the latter present in fetal bovine serum (FBS) was recognized by Z_{mab25}. This selective binding profile towards murine IgG₁ was successfully exploited in species selective recovery of two different mouse mAbs from complex samples containing FBS, resembling a hybridoma culture supernatant.

Keywords Mouse IgG₁ · Affibody · Selection · Fab fragment · Combinatorial protein engineering · Ribosome display

Introduction

Development of different technologies for handling large collections of genotype–phenotype pairs has provided the field of protein engineering with means for identification of particular entities with desired properties [1]. In the field of affinity biotechnology, this has contributed to the existence of a plethora of different classes of non-immune system-derived binding molecules, based on protein frameworks of different origin, size, structural organization, and biophysical stability [2–4]. One such class of binding proteins is denoted as affibody molecules, which are based on a small 58 aa three-helix bundle protein framework derived from the immunoglobulin binding receptor staphylococcal protein A (SPA). From libraries constructed by combinatorial protein engineering principles, affibody molecules have been isolated based on their capability of selective binding to desired target proteins for later use in a variety of different affinity technology applications, including, for example, bioseparation, protein microarrays, in vivo imaging, and therapy [5–8]. The technologies used for linking genotype and phenotype during these selections have been phage display, cell display, and protein fragment complementation assay (PCA), or combinations thereof, all methods relying on the transformation of bacterial cells with plasmid DNA as one step in the experimental procedure, in practice limiting the sizes of libraries obtained [5, 9–11].

Even if these new classes of reagents are being increasingly used in various experimental modalities, traditional monoclonal antibodies (mAbs) are still the most widely used reagents in numerous in vitro assay formats, including, for example, ELISA, western blotting, immunofluorescence, and immunohistochemistry [12]. Today, several species of laboratory animals including rats and

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rabbits can be immunized and serve as donors of B-cells for fusion to immortal cell lines for a sustained production of mAbs. However, the once pioneering work with murine cells is still the dominant route to how mAbs are generated, with mouse IgG₁, IgG_{2a}, or IgG_{2b} as the most common isotypes of produced reagents. Proteins capable of binding to conserved parts of such mAbs have proven to be of immense importance in immunobiotechnology, for example, in purification, detection, and capture of mAbs and their fragments in different bioseparation and assay formats [13, 14]. Two examples of such reagents are SPA and streptococcal protein G (SPG), each containing several homologous domains, which bind with various strengths to different fragments of immunoglobulins in a highly species, sequence and condition-dependent manner. For example, while SPA binds with high affinity to human IgG₁ Fc under physiological conditions, murine IgG₁ Fc is not or only very weakly bound under the same conditions, requiring the use of a high salt concentration (3 M NaCl) and/or an elevated pH to increase the affinity for practical use [15, 16]. SPG in contrast binds well to murine IgG₁ and is therefore frequently used for purification of mouse mAbs from, e.g., hybridoma supernatants. However, as bovine serum is commonly used as hybridoma culture medium supplement, the inherent strong affinity of SPG for bovine Ig can result in mixed immunoglobulin preparations which may complicate the use of the reagent in immunoassay applications [17, 18].

In this study, we have addressed the possibility of using an affibody molecule library based on a SPA domain scaffold to isolate novel reagents with mouse IgG₁ binding properties. For the first time, a large naïve 10¹¹ library was constructed to use ribosome display (RD) in vitro selection technology for identification of affibody binders, involving an *Escherichia coli* secretion monitor protein-derived sequence (SecM) for stabilization of affibody–ribosome–mRNA (ARM) ternary complexes during selection rounds. The results demonstrate both a biotechnological utility and interesting binding site preferences for identified in vitro evolved binders, which are compared with the binding epitopes for the naturally evolved SPA and SPG.

Materials and Methods

General

All the PCR and extension products were prepared using proofreading Phusion DNA polymerase (Finnzymes) and gel-purified oligonucleotide primers (MWG Biotech). DNA restriction and modifying enzymes were from New England Biolabs. PCR and extension products were purified using the QIAquick DNA purification kit (Qiagen).

Cleaved vectors were gel-extracted using the JETQUICK gel extraction kit (Genomed). DNA sequences of all constructs were verified using an ABI Prism 3700 analyzer (Applied Biosystems). RNA was prepared and purified as described previously [19]. *E. coli* strain RR1ΔM15 was used as host for cloning and *Rosetta* (DE3) for protein expression.

Preparation of DNA Constructs

A plasmid pRD-Z_{WT}dSall was constructed in three steps. First, the gene fragment coding for amino acids 221–308 of M13-phage protein 3 (p3) was amplified from double stranded M13K07 DNA using the primers p3-for (5'-GACA CACTCGAGCTCATATCAAGGCCAATCGTCTGACC-3'; underlined: *Xho*I recognition site) and p3-rev (5'-CTTAT CCAGACGGGCGTGCTGAAAGAATCAAGTTTGCCTT TAGCG-3'; underlined: sequence overlapping with SecM) and a gene fragment encoding amino acids 175–195 of the *E. coli* SecM was amplified from *E. coli* genomic DNA using the primers SecM-for (5'-CGCTAAAGGCCAACTTGATT CTTTCAGCACGCCCGTCTGGATAAG-3'; underlined: sequence overlapping with p3) and SecM-rev (5'-GTAGTA GTCGACGCTCAGCCATTGATGATGTTGACCACTTT GC-3'; underlined: *Bsp*I recognition site). In a second step, using the overlapping sites, the SecM DNA fragment was assembled with the p3 DNA fragment to yield p3-SecM. Third, the p3-SecM fragment was inserted between the *Xho*I and *Bsp*I sites of pET-23d-(Z_{WT})₂ [20]. In order to introduce a *Sall* recognition site between FLAG-tag and Z_{WT}, a fragment of pRD-Z_{WT}dSall was amplified using the primers FLAG-for (5'-TATACCATGGACTACAAAGATGACGA TGATAAAACGTCGACCGTAGAC-3'; underlined: *Nco*I recognition site; italics: *Sall* recognition site) and SecM-rev. The amplified fragment was inserted between the *Nco*I and *Xho*I sites of pRD-Z_{WT}dSall to yield pRD-Z_{WT}. The vector pRD-bla-H3 served as platform for library construction and was prepared by amplifying a dummy DNA fragment (fragment of *bla* gene) from pBR322 using primers Bla-for (5'-GTAGTAGTTCGACCCACCCAGAAACGCTGGTG-3'; underlined: *Sall* recognition site) and Bla-rev (5'-GTAGTA GCTAGCCAATGCTTAATCAGTGAGGC-3'; underlined: *Nhe*I recognition site) and inserting it between the *Sall* and *Nhe*I sites of pRD-Z_{WT}. To prepare a SecM gene fragment with reduced G/C content [21], a fragment of pRD-Z_{WT} was amplified using the primers p3-for and Secmut-rev (5'-GTC GACGCTCAGCTTAAGTTAAACGTTGAGGACCAGCA CGAATACCTTGTGCTTGGCTTATCCAGACGGGCGT G-3'; underlined: *Bsp*I recognition site; bold: mutated positions) and inserted between the *Xho*I and *Bsp*I sites of pRD-bla-H3, yielding pRD2-bla-H3. For construction of pRD2-(Z_{WT})₂ and pRD2-Z_{Taq}, the gene fragment coding for a Z_{WT} dimer, denoted (Z_{WT})₂, was amplified from

pET23-(Z_{WT})₂ using the primers Flag-for and (Z_{Taq})₂-rev (5'-GTAGTAGAGCTCTTCGGCGCCTGAGCATCATTTAG-3'; underlined: *SacI* recognition site), and the gene fragment coding for Z_{Taq} was amplified from pT7-(Z_{Taq})₂ using the primers Z_{Taq}-for (5'-GTAGTAGTCGACCGTAGACAACAAATTCACAAAGAAGCTGGGTTGG-3'; underlined: *SalI* recognition site) and (Z_{Taq})₂-rev. Both fragments were inserted between the *SalI* and *SacI* sites of pRD2-bla-H3 to yield pRD2-(Z_{WT})₂ and pRD2-Z_{Taq}, respectively. Finally, pRD2-Z_{WT}-ABD was constructed by amplifying the gene fragment coding for the albumin-binding domain (ABD) from pAffi1 [22] using the primers Abd-for (5'-CAAGTTCTCGAGTTTAGCTGAAGCTAAAGTCTTAGCTAACAGAG-3'; underlined: *XhoI* recognition site) and Abd-rev (5'-CTAGAAGAGCTCACGTAAGGTAATGCAGCTAAAATTCATCTATC-3'; underlined: *SacI* recognition site) and inserting it between the *XhoI* and *SacI* sites of pRD2-(Z_{WT})₂.

Library Construction

The degenerate library primers Zlib-for (5'-CCTGTAGTA**GTTCGACCGTAGACAA** CAAATTCACAAAGAANNKNNKNNKGGCGNNKNNKGAGATC**NNKNNKCTGCCG AACCTGAAC**-3'; underlined: the overlap with Zlib-rev; bold: the *SalI* recognition site; N stands for A, C, G, or T; K stands for G or T) and Zlib-rev (5'-CTTGTAGTAGCTAGCAAGTTAGCGCTTTGGCTTGGGTCATCMNNTAAAC**TMNNGATGAAGCMNMMNNTTGMNMMNNGTTCA GGTTCGGCAG**-3'; underlined: the overlap with Zlib-for; bold: the *NheI* recognition site; M stands for C or A) were subjected to an extension reaction. In brief, 152 pmol of each primer was extended using 4 μl of DNA polymerase and 60 nmol dNTPs in a volume of 2 × 100 μl and during a heat ramp from 98 to 72°C, 0.1°/s, which was repeated nine times. After cleavage with *SalI* and *NheI*, 3.7 μg of extension product encoding randomized helices one and two of the affibody molecule scaffold and 9.1 μg of pRD-bla-H3 were subjected to ligation using 150 Weiss units of T4 DNA ligase in a 2 ml reaction volume that was distributed in 96 microtiter plate wells. The ligation products were used to generate a pooled library PCR product. In brief, 96 PCR reactions were performed using RD-for (5'-CCGCGAAATTAATACGAC TCACTATAGGGAGACCACAACGG-3') and RD-rev14 (5'-TCTAGTTAAACGTTGAGGACCAGCACGAATACCTTGTGCTTGGCTTATCAGACGGGCGTG-3') as primers, 30 cycles and 5 μl ligation product as template, respectively. The use of the RD-rev14 primer reduced the G/C content of the wild-type SecM gene as described above for the construction of pRD2-bla-H3. PCR products were pooled, and approximately 1.5 μg was used for in vitro transcription to yield library RNA.

Real-Time PCR Analyses

Real-time PCR runs were performed in an iCycler system (Bio-Rad). Per reaction, 12.5 μl Sybr Green mix (Bio-Rad) and 10 pmol of each primer were mixed with template, and the volume was adjusted to 20 μl. A program of 45 cycles with an annealing temperature of 60 °C was run, and Ct-values were determined. For determining the library size, RD-for and RD-rev3 (5'-GTGCTGGCCTTGAGGGCCAGCACGGATGCCTTGCG-3') served as primers; 1 μl ligation, 0.1 μl ligation, and a concentration series of 1000, 100, 10, 1, and 0.1 pg pRD-Z_{WT} were used as templates, respectively. From the concentration series of pRD-Z_{WT}, a standard curve was calculated using linear regression. This standard curve was used to obtain an estimate of the library size, via the concentration of successfully ligated plasmids in two dilutions of a library sample aliquot, using quadruplicate determinations.

DNA Sequencing

Library composition and selected clones after four rounds of biopanning were analyzed by DNA sequencing on an ABI Prism 3700 analyzer (Applied Biosystems) using specific sequencing primers and Big Dye terminators (Amersham Biosciences). Template was prepared from randomly picked *E. coli* colonies by PCR amplification or rolling-circle plasmid amplification using Phi29 DNA polymerase (GE Healthcare).

Target Protein Preparation

Polyclonal human IgG (Pharmacia) was used as target protein during enrichment studies, and the mouse IgG₁ monoclonals ab 18447 (anti-para-nitrophenol, mAb1, Abcam), biotinylated HDL 44 (anti-human Apolipoprotein A1, mAb2, Mabtech), and 1-D1K (anti-human Interferon-γ, mAb3, Mabtech) were used as target proteins for library selections. mAb1 and mAb3 were biotinylated using EZ-LinkTM Sulfo-NHS-LC-biotin (Thermo Fisher Scientific). A 75-fold molar excess of biotin was added to the different immunoglobulins in PBS. The mixture was incubated for 3 h on ice, and excess biotin was removed by extensive dialysis. Stocks of target protein immobilized on Streptavidin or NeutrAvidin-coated paramagnetic Dynabeads[®] M280 (Invitrogen) were prepared. The amount of target immobilized was approximately 4 μg/mg of beads as determined by SDS-PAGE. NeutrAvidin (Thermo Fisher Scientific) was covalently immobilized on tosylactivated Dynabeads[®] M-280 according to manufacturer's recommendations.

Ribosome Display

Ribosome display was performed essentially as described previously [19] with some modifications. Per reaction, 0.5 mg target and control beads (no target) were washed four times with TBST (50 mM Tris–HCl, 150 mM NaCl, 0.05% Tween-20, pH 7.4) and blocked for 20 min with WBT (50 mM Tris–acetate, 150 mM NaCl, 50 mM MgAc, 0.05% Tween-20) containing 0.5% BSA. In vitro translation (IVT) was performed using the PURESYSTEM® classic II (Cosmo Bio). Per reaction, 5 µg of mRNA template, 25 µl solution A, 10 µl solution B, and 80 u RNasin® RNase inhibitor (Promega) were mixed, the volume was adjusted to 50 µl, and the mixture was incubated for 20 min at 37°C. IVT was stopped by transferring the reaction to 220 µl ice-cold WBT containing 0.5% BSA and 2.5 mg/ml Heparin.

For enrichment studies, mRNA coding for Z_{WT} -ABD or Z_{Taq} fused to a p3-SecM spacer was mixed in a ratio of 1:1000 (total amount of 5 µg) and used as template for IVT. The stopped and stabilized IVT was incubated for 1 h with 0.5 mg IgG-coated, blocked Dynabeads® M280 Streptavidin. Beads were washed five times by gentle pipetting before eluting the RNA (see below).

For library selections, 5 µg of library RNA was used as input in IVT. During selection cycle 1, the IVT was incubated with 0.5 mg mAb1-coated Dynabeads® M280 NeutrAvidin for 1 h. During library selection cycle 2, the IVT was first incubated with 0.5 mg Dynabeads® M280 NeutrAvidin for 1 h (pre-selection), immediately followed by incubation with 0.5 mg mAb2-coated Dynabeads® M280 NeutrAvidin for 1 h (selection). During library selection cycles 3 and 4, the IVT was first incubated with 0.5 mg Dynabeads® M280 Streptavidin for 1 h (pre-selection), immediately followed by an incubation with 40 nM (cycle 3) or 4 nM (cycle 4) biotinylated mAb3 in solution for 1 h. Biotinylated mAb3 was then captured with 0.5 mg Dynabeads® M280 Streptavidin for 15 min. The IVT was kept at 4°C at all times. Beads were washed 2, 4, 6, and 10 times during library selection cycles 1, 2, 3, and 4, respectively (for an overview of selection parameters,

see Table 1). After washing, RNA was eluted from beads with 50 mM EDTA in the presence of 50 µg/ml yeast RNA, as described previously [19], followed by purification using the High Pure RNA Isolation Kit (Roche). To avoid DNA contamination, columns were incubated for 10 min with DNaseI after having loaded the eluate. Half of the eluted and purified RNA was immediately reverse transcribed at a temperature of 55°C for 1 h using primer RD-rev11 (5'-TCTAGTTAAACGTTGAGGAC-3') and AffinityScript™ reverse transcriptase (Stratagene). The other half was saved as backup. In order to enter the next selection cycle, the majority of the reverse transcription product was PCR amplified using primers RD-for5 and RD-rev9, and PCR product could directly be used as template for in vitro transcription.

Protein Production and Purification

In order to sequence and produce selected affibody molecules, reverse transcription products obtained after four cycles of selection were PCR amplified using primers Zsub-for2 (5'-AGTAGGGAATTCATATGGTAGACAA CAAATTCACAAAG-3'; underlined: *NdeI* recognition site) and Zsub-rev (5'-TGATATGAGCTCGATTATTTCCGCGCCTGAGCATCATTTAG-3'; underlined: *SacI* recognition site) and the resulting product inserted between the *NdeI* and *SacI* sites of the expression vector pAff8c [23]. After sequencing, the most interesting clones in terms of abundance and amino acid composition were expressed as His₆-tagged fusion proteins in *E. coli* strain Rosetta (DE3). Cells were grown over night at 37°C in 10 ml of TSB medium (30 g/l TSB) containing 50 mg/l kanamycin. Fresh 250 ml TSB medium supplemented with 5 g/l yeast extract (TSBY) and 50 mg/l kanamycin were inoculated with 2.5 ml of over night culture, grown at 37°C to an OD₆₀₀ of 1.5, when gene expression was induced by addition of isopropyl β-D-thiogalactoside (IPTG; Apollo Scientific Ltd) to a final concentration of 1 mM. After 4 h of induction, cultures were harvested by centrifugation (2200 g, 10 min, 4°C) and frozen at –20°C. The next day, cell pellets were suspended in 5 ml denaturing buffer (6 M

Table 1 Selection parameters

Cycle	Beads	Target	Target concentration (nM)	Pre-selection	No. of washes	Phase
1	NA	mAb1	80	No	2	Solid
2	NA	mAb2	80	Yes	4	Solid
3	SA	mAb3	40	Yes	6	Solution
4	SA	mAb3	4	Yes	10	Solution

Abbreviations: NA NeutrAvidin; SA Streptavidin; mAb1 anti-para-nitrophenol mIgG₁ (Kappa); mAb2 anti-Apolipoprotein A1 mIgG₁ (Kappa); mAb3 anti-IFN-γ mIgG₁ (Kappa)

guanidinium hydrochloride, 47 mM Na₂HPO₄, 2.65 mM NaH₂PO₄, 10 mM Tris–HCl, 104 mM NaCl, pH 8.0) and disrupted by sonication. After centrifugation (35000g, 20 min, 4°C), clear cell lysates were collected, and proteins were purified in parallel using Talon Metal Affinity Resin (BD Biosciences) in combination with an ASPEC XL4 liquid handling robot (Gilson). Proteins were released with elution buffer (8 M urea, 0.1 M NaCl, 29.6 mM HAc, 70.4 mM NaAc, 50 mM NaH₂PO₄, pH 5.0). The buffer was changed to HBS, proteins were renatured by gel filtration, and purified proteins were separated by SDS-PAGE using a NuPAGE[®] Novex[®] 4–12% Bis–Tris gel (Invitrogen). The gel was stained using GelCode[®] Blue Stain (Thermo Fisher Scientific) according to manufacturer's recommendations. LMW-SDS Marker (GE Healthcare) was used as molecular weight marker. Protein G C2–C3 fragment was produced in the periplasm of *E. coli* strain O17 transformed with plasmid pK4C2C3 (kind gift from P. Nilsson). 100 ml TSBY-medium was inoculated with a single colony and grown over night at 37°C. Cells were harvested, the periplasmic content separated by osmotic shock as described previously [24], and recombinant protein was purified using an IgG-Sepharose column and 0.2 M acetic acid for elution. The eluate fraction was frozen, lyophilized, and suspended in HBS.

Biosensor Binding Studies

Binding properties of selected affibody molecules were analyzed using a Biacore[®] 2000 or 3000 instrument (GE Healthcare). All measurements were performed in HBS-ET (5 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.005% Tween 20, pH 7.4) at a flowrate of 20 µl/min and 25°C. After each analyte injection, sensor chip surfaces were regenerated by injection of 10 µl 0.1 M glycine–HCl, 0.5 M NaCl, pH 2.5, followed by 10 min stabilization time unless otherwise stated. To correct for systematic deviations and bulk buffer effects, both reference blank surface signal and the signal obtained from the injection of a buffer sample were subtracted from all sensorgrams [25]. For candidate binder screening, the target mAb1, 2, and 3 (20 µg/ml in 10 mM NaAc pH 4.5) were separately immobilized on three flow cells of a CM5 sensor chip (GE Healthcare) to final densities of 3838 RU (mAb1), 3145 RU (mAb2), and 4085 RU (mAb3) using amine coupling chemistry. Another flow cell was activated and deactivated to use as reference. Analyte concentrations were adjusted to 1 µM in HBS-ET, and 80 µl samples were injected. For affinity determination, concentration series of selected affibody molecules from 1.95 nM to 8 µM were prepared, and duplicates of 160 µl were flowed in stepwise order at a flowrate of 40 µl/min over all four flow cells. Assuming a Langmuir 1:1 binding model or 1:1 binding model with

mass transport limitation ($Z_{\text{mab22_25}}$ binding to mAb3), the dissociation constant (K_D), the association rate constant (k_a) and the dissociation rate constant (k_d) were calculated using BIAevaluation 3.2 software (GE Healthcare). For binding analyses to a panel of different antibodies and competition studies, a second CM5 sensor chip was prepared onto which 1871 RU of Z_{mab25} and 1266 RU of $Z_{\text{mab22_25}}$ protein were immobilized on separate flow cells using amine coupling chemistry. The panel of injected antibodies over Z_{mab25} and $Z_{\text{mab22_25}}$ surfaces (100 nM) included four mouse IgG₁s: mAb2, mAb3, mouse IgG₁: HDL110 (anti-human apolipoprotein A1, Mabtech) and mouse IgG₁: 8F11 (anti-special AT-rich sequence-binding protein 2 [26]), one mouse IgG_{2b}: ab 18421 (anti-trinitrophenol, Abcam), one mouse–human chimera: Infliximab (anti-TNF α , Schering-Plough), one human myeloma IgG₁: GTX76676 (GeneTex) and one mouse IgG₁(λ) S1-68.1 (BD Biosciences). For competition studies, a 10 nM solution of mAb3 was incubated with 100 nM, 1, 10 µM or without a recombinant two-domain protein G construct (C2–C3) for 30 min at room temperature, and 100 µl was subsequently injected over the Z_{mab25} surface. C2–C3 (1 µM) was also injected over the target mIgG₁ chip surfaces to confirm binding to mIgG₁. For analysis of Z_{mab25} or protein G C2–C3 domain interaction with fetal bovine serum (FBS), a third CM5 sensor chip was prepared onto which 488 RU of protein G C2–C3 and 1817 RU of Z_{mab25} were immobilized on separate surfaces, and 100 µl of 10% FBS (Gibco) or 10% FBS and 133 nM mAb3 were flowed over both surfaces. Sensor chip surfaces were regenerated with 10 µl 0.1 M glycine–HCl pH 2.0. For species selective immunoglobulin binding analysis of eluate fractions from affinity purification studies, a fourth CM5 sensor chip was prepared onto which 5330 RU of an anti-cow IgG antibody (ab6934, Abcam) or 5354 RU of an anti-mouse Ig antibody (P0447, Dako) was immobilized on separate surfaces. 100 µl of neutralized affinity purification eluate fractions was flowed over both surfaces.

Isotyping of Antibodies

An IsoStrip Mouse Monoclonal Antibody Isotyping Kit (Roche) was used for typing of antibody heavy and light chains when not specified in their respective data sheets provided by the supplier.

Papain Cleavage of Antibodies

To obtain Fab and Fc fragments of mAb3, 100 µg of the antibody was cleaved with papain immobilized on agarose-beads (Thermo Fisher Scientific) according to manufacturer's recommendations.

Affinity Purification Studies

A HiTrapTM Protein G HP column with 1 ml bed volume coupled with more than 2 mg recombinant protein G lacking the albumin-binding region was bought from GE Healthcare. The NHS-activated agarose matrix of a second HiTrapTM HP Column with 1 ml bed volume (GE Healthcare) was coupled with approximately 5 mg of Z_{mab25} according to manufacturer's recommendations. All purification studies were performed on an Äkta Explorer (GE Healthcare) at flow rates of 0.1 or 0.5 ml/min (sample loading) or 1 ml/min (washing/elution) using TST (25 mM tris(hydroxymethyl)aminomethane, 1 mM EDTA, 200 mM sodium chloride, and 0.05% v/v Tween20, pH 8.0) as running buffer. The following samples were loaded on the protein G or Z_{mab25} columns or both (see Results section): (1) 50 µg of mAb3 after papain cleavage in 1 ml TST; (2) 5 ml TST supplemented with 10% FBS (Gibco); (3) 100 µg of mAb3 in 5 ml TST supplemented with 10% FBS; (4) 100 µg of mAb 8F11 in 5 ml TST supplemented with 10% FBS. The chromatography system was equilibrated with TST, samples were injected and after the flow-through had passed, the column was washed with 3 ml 5 mM ammonium acetate pH 6, and bound protein was eluted with 0.5 M acetic acid, pH 2.2. For sample (1), eluate fractions of 0.5 ml were collected, pooled, immediately frozen to -80°C , lyophilized, and taken up in reducing loading buffer for SDS-PAGE analysis. For samples (2–4), four eluate fractions of 0.5 ml were pooled, one half was neutralized by adding 760 µl 1 M tris(hydroxymethyl)aminomethane pH 9.0 and dialyzed extensively against HBS-ET for subsequent biosensor analysis. The other half was lyophilized and suspended in 100 µl reducing loading buffer for SDS-PAGE analysis.

SDS-PAGE and Western Blot Analysis of Affinity Purification Studies

Sample (1)-derived fractions were separated together with the SeeBlue[®] Plus2 Pre-Stained Standard (Invitrogen) by SDS-PAGE under reducing conditions using two NuPAGE[®] Novex 4–12% Bis-Tris gels (Invitrogen). 200 V was applied for 45 min in MES running buffer at 4°C . Separated proteins were transferred on two PVDF membranes with 0.45 µm pore size (Invitrogen) for 1 h at 30 V and 4°C . Membranes were blocked for 1 h with 5% milk powder in de-ionized water supplemented with 0.5% v/v Tween 20 and incubated with either HRP-conjugated goat polyclonal antibody to mouse IgG Fc (F(ab)₂ fragment, ab 5879, Abcam) or goat polyclonal to mouse IgG F(ab)₂ (F(ab)₂ fragment, ab 5887, Abcam) diluted 1:5000 in blocking solution. The membranes were subsequently washed with TBS supplemented with 0.5% v/v Tween 20

and developed using the TMBM-500 substrate (MOSS). Sample (2), (3), or (4)-derived fractions (4 µl applied sample, 4 µl flow-through fraction, 10% of the eluate fraction, respectively) were separated by SDS-PAGE using a Mini-PROTEAN[®] TGXTM Any kD gel (Bio-Rad) under reducing conditions at 100 V for 75 min at 4°C . The gel was fixed in 40% v/v ethanol and 10% v/v acetic acid and stained with GelCode[®] Blue Stain (Thermo Fisher Scientific) according to manufacturer's recommendations.

Results

Ribosome Display System Design and Evaluation

A plasmid construct denoted pRD was constructed to use as assembly vector and PCR amplification template of linear expression cassettes for RD-mediated selection of binding proteins from libraries of affibody molecules (Fig. 1a). Following sequences corresponding to a standard T7 RNA polymerase promoter (PT7), a Shine Dalgarno (SD) ribosomal-binding site and a FLAG peptide encoding sequence, restriction sites allowing for introduction of gene fragments encoding different model proteins or affibody molecule library members were incorporated for in-frame fusion to a downstream element encoding a bipartite spacer and ribosome tunnel spanning sequence. The first part of this sequence corresponds to a stretch of 88 residues from the bacteriophage M13 protein 3 (p3 spacer), previously utilized as spacer element in RD selections [19]. This sequence was linked to a second element corresponding to a 21-residue stretch (aa 175 to 195) of the *E. coli* SecM, earlier reported to interact with the ribosome tunnel and capable of providing stable peptide-ribosome-mRNA ternary complexes even when followed by a downstream in-frame termination codon [27–30]. Here, the natural stop codon in the SecM gene was, however, replaced by an AGA triplet, corresponding to a rare arginine codon in *E. coli* to possibly further avoid disruption of ternary complexes during selection.

For system evaluation, test enrichment experiments were performed involving an mRNA mixture, prepared from mRNA obtained after in vitro transcription using PCR product expression cassettes encoding the model proteins Z_{WT}-ABD (IgG and HSA binding fusion protein; Fig. 1b) or Z_{Taq} (affibody molecule binding to *Taq* DNA polymerase [31]; Fig. 1c) fused to the p3-SecM spacer. Two rounds of RD selection were performed using human IgG-coated beads for enrichment of the rare IgG-binding Z_{WT}-ABD encoding constructs present in an initial 1000-fold molar excess of *Taq* DNA polymerase binder encoding control constructs, and the results monitored via analysis of PCR products obtained after post-selection RT-PCR. An

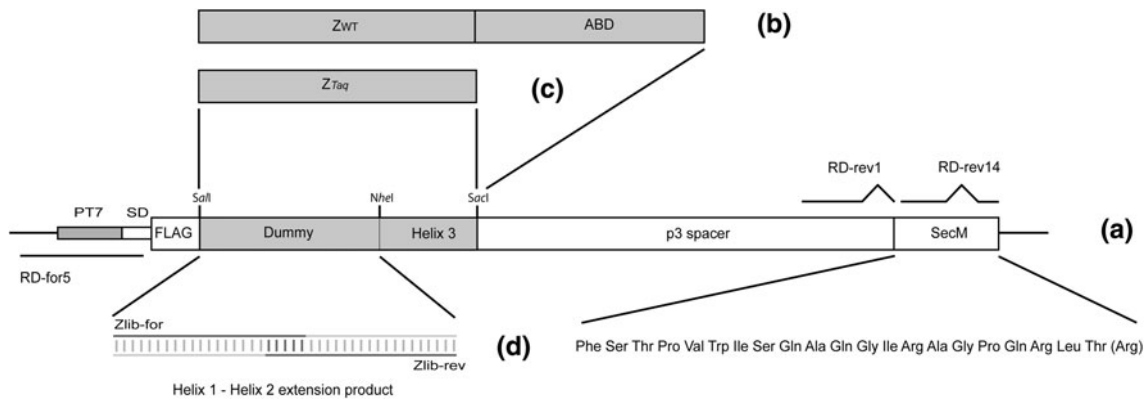


Fig. 1 Affibody molecule ribosome display system design. Schematic overview of the constructed ribosome display system. **a** An expression cassette was assembled in a vector denoted pRD, containing a T7 promoter, a Shine Dalgarno sequence and a FLAG epitope encoding sequence, followed by a cloning window and a sequence encoding a bipartite spacer element containing a 88-residue phage M13 protein 3 sequence (p3 spacer) followed by a 21-residue element derived from the *E. coli* SecM. The Arg residue (*in brackets*) indicates the extension of the SecM motif by this codon during PCR amplification prior to transcription; **b**, **c** for initial enrichment studies, gene fragments encoding either Z_{WT} -ABD or Z_{Taq} proteins were

inserted in the expression cassette as indicated; **d** for affibody molecule library construction, two oligonucleotides (Zlib-for and Zlib-rev) encoding randomized helices one and two of the three-helix bundle scaffold were extended together utilizing mutually overlapping sequences for annealing, followed by cloning into the expression cassette where the extension products replaced a dummy fragment. A gene fragment encoding helix three was here already present in the pRD vector. Restriction sites and annealing sites for some of the oligonucleotide primers used during cloning, reverse transcription and PCR work are indicated (see text for details)

efficient enrichment for Z_{WT} -ABD encoding constructs was repeatedly seen (Fig. 2), indicating that the system was functional.

An affibody molecule library was constructed based on a previously described randomization strategy of the Z domain, involving an unbiased combinatorial NNK codon-based variegation of 13 surface-located positions in helices one and two of the three-helix bundle scaffold [5]. Through

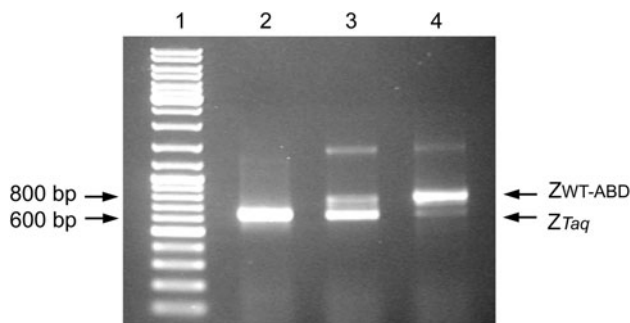


Fig. 2 Results from initial enrichment experiments. Agarose gel electrophoresis analysis of PCR products obtained using DNA templates produced after reverse transcription of mRNA recovered after different numbers of rounds of IgG affinity selection of ternary complexes containing Z_{WT} -ABD proteins. *Lane 1*: marker DNA; *lane 2*: PCR product obtained after reverse transcription and amplification of an initial 1:1000 mixture of Z_{WT} -ABD mRNA in a background of Z_{Taq} mRNA; *lane 3*: PCR product obtained after a first round of enrichment; *lane 4*: PCR product obtained after a second round of enrichment. *Arrows* indicate marker DNA bands and the expected sizes for amplicons corresponding to amplification of Z_{WT} -ABD and Z_{Taq} constructs, respectively

replacement of a previously inserted dummy DNA fragment in the pRD vector for a primer extension product encoding the library of randomized helices one and two, a plasmid format library with the size of 10^{11} was obtained (Fig. 1d), as determined via quantification of ligation product DNA by real-time PCR (Fig. 3). Transformation of bacteria with an aliquot of the library allowed for a clone-based analysis of the library quality, which showed that 62 out of 87 sequenced clones (71%) were correctly assembled and that remaining 25 clones (29%) contained nucleotide insertions and/or deletions at different positions. The plasmid format of the library served as template pool for the generation of linear expression cassettes by PCR (Fig. 1 and [Material and Methods](#) section).

Selections

In order to direct a selection of binders towards general murine IgG₁-specific epitopes, three different mIgG₁ antibody proteins (mAbs 1–3) were used as targets during selections. In a first cycle, an anti-para-nitrophenol monoclonal antibody (mAb1) was used, followed by an anti-human Apolipoprotein A1 mAb (mAb2) in cycle 2, and an anti-human Interferon- γ mAb (mAb3) in cycles 3 and 4. The selection stringency was gradually increased by introducing a pre-selection step using non-target containing beads in cycle 2, and gradually decreasing target protein concentration and increasing the number of washing steps (Table 1). Analysis of 206 selected clones obtained

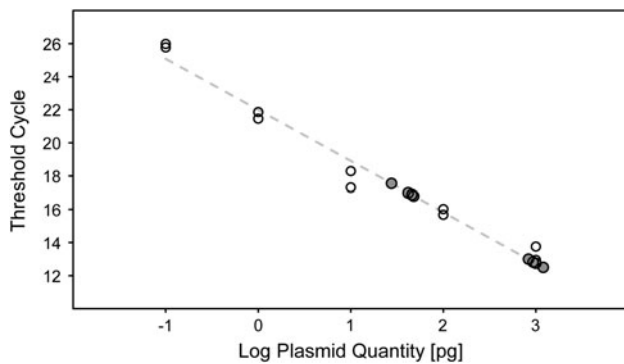


Fig. 3 Real-time PCR analysis of the library size. The real-time PCR threshold cycle (Ct) values obtained from five different pRD- Z_{WT} template amounts (0.1, 1, 10, 100, and 1000 pg) were plotted in a logarithmic scale. The data were fitted using linear regression (*gray dashed line*) and from the regression line, the amount of successfully ligated plasmids in two different dilutions of the library ligation reaction was determined (quadruplicates, *filled circles*). A low estimate of the library size, calculated using the mean value from the quadruplicate referring to the more diluted sample, was approximately 1×10^{11}

exclusively from selection cycle 4 by DNA sequencing showed that three library members (Z_{mab22} , Z_{mab25} , and Z_{mab26}) had been more enriched than other members and appeared 173 (Z_{mab22}), 13 (Z_{mab25}), and 2 times (Z_{mab26}), respectively. Furthermore, a hybrid clone denoted Z_{mab22_25} , probably created by recombination of Z_{mab22} and Z_{mab25} during post-selection cloning work, was found three times (Fig. 4). Clone Z_{mab22} contains two cysteines that give rise to oligomerization (dimers and trimers) under non-reducing conditions as confirmed by non-reducing SDS-PAGE analysis (data not shown). In addition, clones corresponding to the wild-type Z domain sequence appeared, presumably corresponding to a contamination during the selection.

Binding Studies

Binding to mAb1, mAb2, and mAb3 was analyzed using surface plasmon resonance where the three variants Z_{mab22} ,

Z_{mab25} , and Z_{mab22_25} all showed binding to all three mAb, whereas the Z_{mab26} variant did not bind any of the three investigated antibodies under these conditions. For determination of binding affinities, serial dilutions of the Z_{mab22} , Z_{mab25} , and Z_{mab22_25} variants ranging from 1.95 nM to 8 μ M were injected over sensor chip surfaces coated with mAb1, mAb2, and mAb3, respectively. The results showed that the three affibody variants bind the three different mIgG₁ antibodies mAb1–3 with dissociation constants (K_D) of 1.3 μ M, 487, 8.1 nM (Z_{mab22}); 371, 190, 19.2 nM (Z_{mab25}), and 191, 125, 1.8 nM (Z_{mab22_25}) (Fig. 5).

To further investigate the selectivity in binding, a reversed format was used in which the Z_{mab22} , Z_{mab25} , and Z_{mab22_25} variants were covalently immobilized onto sensor chip surfaces and analyzed for binding to various injected mouse and human IgG antibodies. The Z_{mab25} variant bound the antibody targets used during selection as well as two other mIgG₁/kappa light chain antibodies not used as targets during selection, indicating a broad mIgG₁ binding capacity. The binding responses obtained from the two non-target mIgG₁/kappa mAbs included here were lower than those obtained from the antibodies used as targets during selection, indicating a lower affinity of Z_{mab25} for these particular antibodies (see **Discussion**). Also, as judged from the shape of the traces, the interaction with the non-target mIgG₁ 8F11 mAb shows significantly slower off-rate kinetics than seen for the other non-target mIgG₁ mAb included in the analysis (HDL110). Interestingly, a mouse IgG_{2b}/kappa isotype control, a purified myeloma-derived human IgG₁ antibody and a chimeric mouse–human IgG₁ (murine Fv part) were not recognized by the Z_{mab25} variant, indicating a narrow binding preference for murine IgG₁. Notably, a mouse IgG₁ isotype control mAb containing a lambda light chain was not recognized under these conditions, implying a possible influence also from the light chain type on binding (Fig. 6). The Z_{mab22} and Z_{mab22_25} variants, however, did either not show any or only a limited binding ability in this format, suggesting that the covalent immobilization via primary amines affected the molecular properties.

		Helix 1		Helix 2		Helix 3	
Z_{WT}	VDNKFNK	EQQNAFYEILH	LPNLNE	EQRNAFTQSLKD	DPSQ	SANLLAEAKKLNDA	QAPK
Z_{mab22}	-----	-AWR-HM--MC	-----W	S-TT---F--C-	----	-----	----
Z_{mab25}	-----	-LWV-HM--IR	-----A	F-AY---A--I-	----	-----	----
Z_{mab26}	-----	-SPT-IE--LV	-----L	W-ML---H--F-	----	-----	----
Z_{mab22_25}	-----	-AWR-HM--IR	-----A	F-AY---A--I-	----	-----	----
							173
							13
							2
							3

Fig. 4 Sequences of selected affibody molecules. Deduced amino acid sequences of in total 206 sequenced clones appearing two or more times. The wild-type Z domain sequence is shown, with *dots* over the positions randomized to construct the library and the three

alpha helices of the scaffold are *boxed*. The numbers to the right indicate the number of times with which a particular library member appeared among the sequenced clones

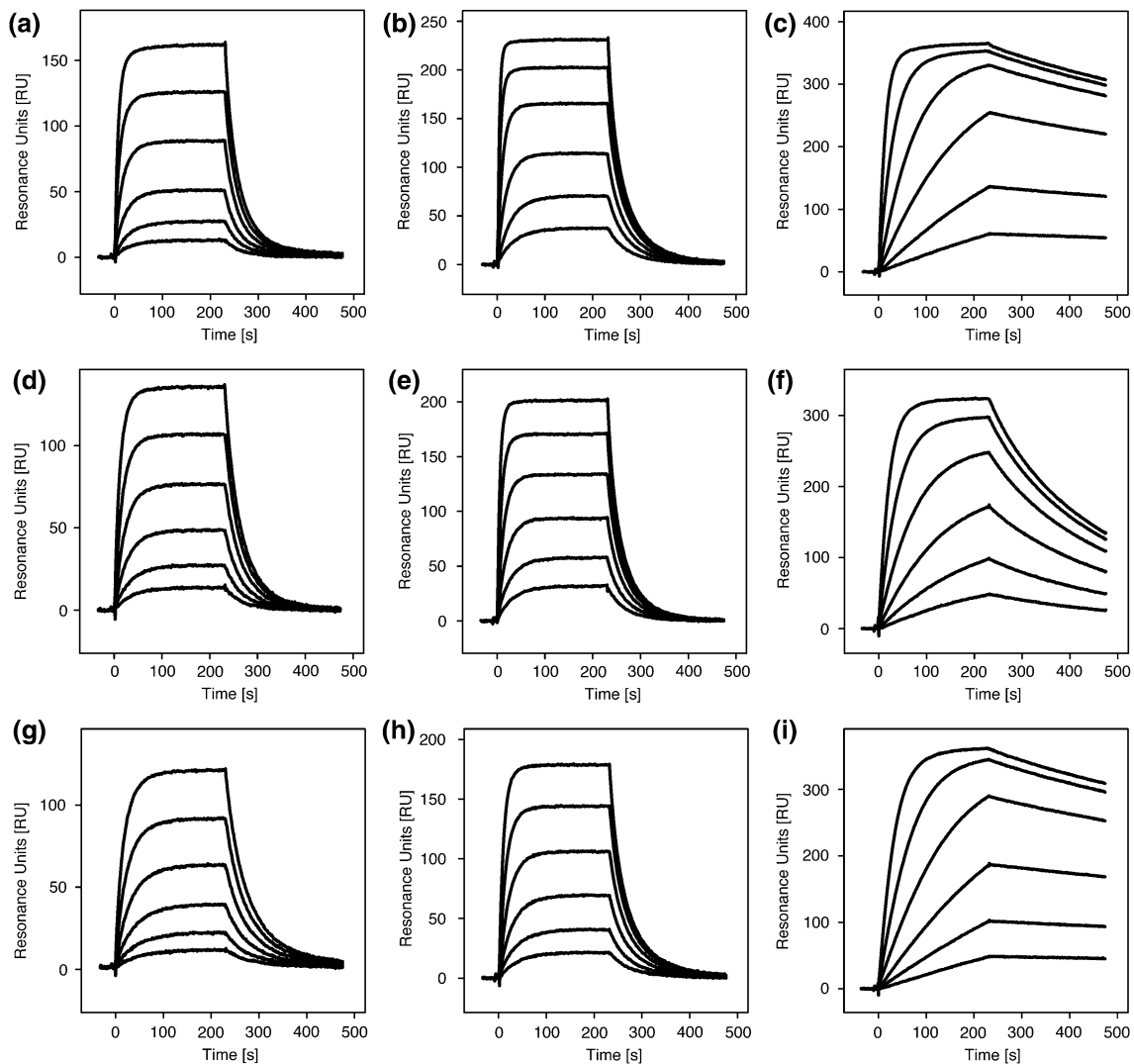


Fig. 5 Biosensor analysis of affibody molecule variants Z_{mab22} , Z_{mab25} , and $Z_{\text{mab22_25}}$ binding to mAb1, mAb2, and mAb3. Panels show recorded concentration dependent responses after injection of affibody molecules Z_{mab22} (a–c), Z_{mab25} (d–f), and $Z_{\text{mab22_25}}$ (g–i) over sensor chip immobilized antibodies mAb1 (a, d, g), mAb2 (b, e,

h), or mAb3 (c, f, i). Biosensor sensorgram traces were obtained during injection of a series of concentrations ranging between 4 μM and 125 nM (a, b), 1 μM and 31 nM (c, d, e), 250 and 7.8 nM (f), 500 and 16 nM (g, h) as well as 125 and 4 nM (i) of monomeric affibody variants produced as hexahistidyl (His_6) fusion proteins

Mapping of Binding Site

A biosensor-based binding competition assay was performed as an attempt to roughly map the binding site for the Z_{mab25} variant on mIgG₁. Here, a recombinantly produced fragment of the Ig binding protein SPG containing two Ig binding domains (C2–C3) was used. SPG is known to bind IgG in a species-dependent manner both to CH₁ domains in Fab regions [32] and to the CH₂–CH₃ domain interface in Fc [33]. In the competition assay, the binding between sensor chip immobilized Z_{mab25} protein and mAb3, used either as free monoclonal antibody or after pre-incubation with different molar excesses of SPG fragment were analyzed. The

results showed that a mixing of the mAb3 with a 100-fold molar excess of SPG fragment significantly reduced the mAb3 binding to Z_{mab25} , and that a mixing with a 1000-fold molar excess of SPG fragment almost completely blocked the binding (Fig. 7). This indicated that the binding site for Z_{mab25} on mouse IgG₁ is located at a site sterically interfering with SPG at one of the two binding sites of SPG. To prepare a sample that could be used to exclude one of the two sites, mAb3 was enzymatically digested with papain, yielding a mixture of Fc and Fab fragments. After applying this sample onto an affinity column containing Z_{mab25} ligands, the Fab fragments were selectively captured and eluted from the column (Fig. 8), indicating that the selected

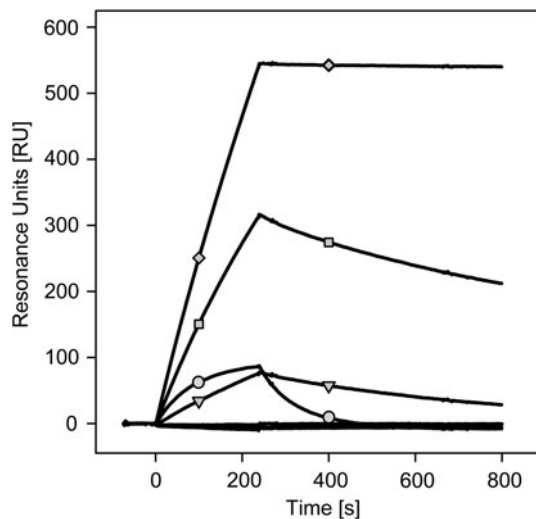


Fig. 6 Binding studies of $Z_{\text{mab}25}$ with a panel of eight antibody samples. $Z_{\text{mab}25}$ was recombinantly produced as a hexahistidyl (His)₆ fusion protein, IMAC purified and immobilized onto a CM5 sensor chip surface and various immunoglobulin samples of a common 100 nM concentration injected. Response curves obtained on samples flowed over the $Z_{\text{mab}25}$ surface are labeled as follows: anti-human interferon- γ mIgG₁ mAb3 (*gray diamonds*), anti-human apolipoprotein mIgG₁ mAb2 (*gray squares*), anti-human apolipoprotein mIgG₁ HDL110 (*gray circles*), and anti-special AT-rich sequence-binding protein 2 mIgG₁ 8F11 (*gray triangles*). Responses obtained for anti-trinitrophenol mIgG_{2b} ab18421, anti-TNF α mouse–human chimera Infliximab, human myeloma IgG₁ GTX76676, and mIgG₁(λ) S1-68.1 can be seen as flat traces at the bottom of the graph. In a parallel experiment, also the mAb1 reagent was found to be recognized by the $Z_{\text{mab}25}$ variant in this format (data not shown)

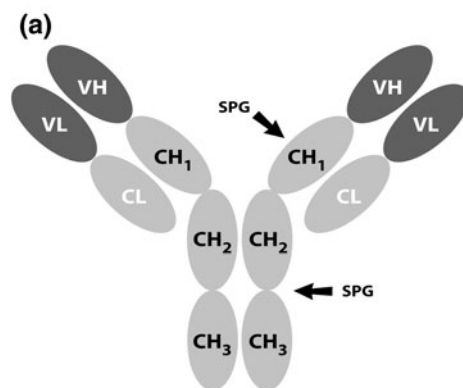
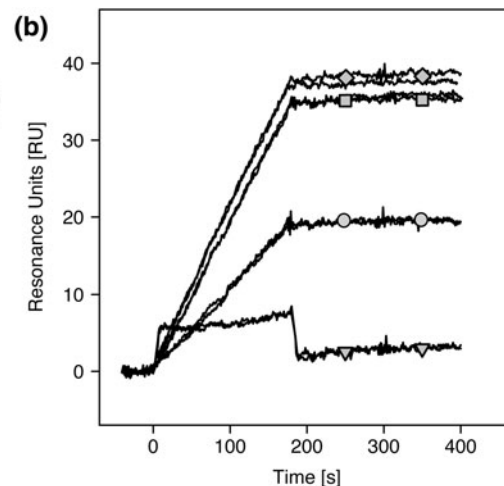


Fig. 7 Mapping of $Z_{\text{mab}25}$ binding site by competitive binding analyses. Possible binding sites on mouse IgG₁ for the $Z_{\text{mab}25}$ variant were mapped using a competitive binding analysis employing an immunoglobulin binding protein with known binding sites. **a** Schematic figure showing an IgG antibody with its different regions and the binding sites for SPG in Fc and Fab, respectively,

$Z_{\text{mab}25}$ variant binds to a site functionally overlapping with SPG at its binding site on the CH₁ domain of mouse IgG₁ Fab.

Affinity Chromatography

As indicated by the successful column-based recovery of the Fab fragment, one possible biotechnological use of the $Z_{\text{mab}25}$ affibody protein could be as affinity ligand in the recovery of mouse IgG₁ mAbs from hybridoma culture supernatants. However, such feed stocks frequently contain, in addition to the secreted mAb, also bovine immunoglobulins originating from the hybridoma growth medium supplement FBS. Thus, bovine Ig can become co-purified with mAbs using group specific affinity ligands with broad Ig binding spectra, such as wild-type protein A or G. Such contamination has been shown to frequently contribute to false-positive readouts in sandwich immunoassays involving human sera samples containing human anti-bovine IgG antibodies (HABIAs) [18]. To investigate if the $Z_{\text{mab}25}$ affibody protein showed any binding to bovine Ig, a sample of 10% FBS was injected over both a $Z_{\text{mab}25}$ and a protein G C2–C3 domain reference sensor chip surface. Interestingly, whereas the reference surface showed a significant response, no binding response was observed for the $Z_{\text{mab}25}$ surface, indicating that bovine Ig, or any other FBS component, was not recognized by the variant selected using mouse IgG₁ as target (Fig. 9a). Only after spiking of the 10% FBS sample with mouse IgG₁



indicated. **b** Biosensor sensorgrams resulting from duplicate injections of 10 nM mAb3 alone (*gray diamonds*); mAb3 in a 100 nM (*gray squares*), 1 μM (*gray circles*), or 10 μM (*gray triangles*) solution of the recombinant two-domain protein G construct (C2–C3) over a sensor chip surface containing the $Z_{\text{mab}25}$ variant

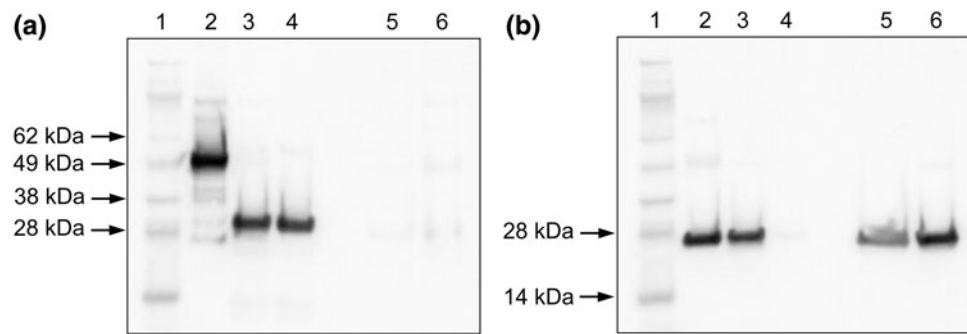


Fig. 8 Western Blot analysis of mAb3 Fab fragment capture after papain cleavage. The mouse IgG₁ mAb3 was papain-digested and the sample applied on a HiTrapTM HP column with Z_{mab25} immobilized as ligand for capture of the targets for the ligand. Panel **a** was stained with a polyclonal goat anti-mouse IgG Fc HRP conjugate. *Lane 1*: SeeBlue[®] Plus2 Pre-Stained standard; *lane 2*: mAb3 sample before cleavage; *lane 3*: mAb3 sample after cleavage; *lane 4*: column flow-

through fraction; *lanes 5, 6*: eluate fractions. Panel **b** was stained with a polyclonal goat anti-mouse IgG F(ab)₂ HRP conjugate. *Lane 1*: SeeBlue[®] Plus2 Pre-Stained standard; *lane 2*: mAb3 sample before cleavage; *lane 3*: mAb3 sample after cleavage; *lane 4*: column flow-through fraction; *lanes 5, 6*: eluate fractions. The *black arrows* to the left indicate molecular weights in kilo Dalton (kDa)

(mAb3, 133 nM) a detectable binding response was obtained, suggesting that the Z_{mab25} variant could be used for selective affinity recovery of mouse IgG₁ from samples also containing bovine Ig and high concentrations of bovine serum albumin originating from added FBS (Fig. 9a). To investigate this possibility, affinity columns containing either SPG or Z_{mab25} ligands were in separate experiments loaded with 5 ml samples of 10% FBS alone or 10% FBS spiked with 100 µg of mAb3, followed by washing and elution. Analysis of eluates from both experiments by SDS-PAGE under reduced conditions showed that the SPG column had retained proteins corresponding to characteristic sizes of heavy and light chains of immunoglobulins, approximately 25 kDa for light chains and 50–60 kDa for heavy chains, irrespective of the sample used (Fig. 9b). In contrast, the Z_{mab25} column eluates only contained such bands when the mAb3-spiked sample was applied, indicating a species selective capture of mouse IgG₁. To investigate if also a murine IgG₁ monoclonal antibody not used as target during selection and with lower apparent affinity for Z_{mab25} could be recovered, the experiment was repeated with a sample of 10% FBS spiked with 100 µg of mAb 8F11. Again, proteins corresponding to characteristic sizes of heavy and light chains were selectively retained (Fig. 9b). To confirm these findings, the compositions of pH neutralized eluates obtained from the mAb3 or mAb 8F11-spiked samples were further analyzed by biosensor technology, utilizing antibodies of anti-cow IgG or anti-mouse Ig specificities immobilized on separate sensor chip surfaces. In this analysis, significant binding responses to both surfaces were obtained with the SPG column eluate, showing that this eluate contained both bovine and mouse Ig. In contrast, the eluates from the Z_{mab25} column both only gave a response to the anti-mouse Ig surface (Fig. 9c). Taken together, this demonstrated the

applicability of the Z_{mab25} affibody molecule as ligand for selective capture of two different mouse IgG₁ mAbs from a sample resembling a supernatant from hybridoma cultured in medium containing FBS.

Discussion

This study describes the selection of novel proteinaceous affinity ligands to mouse IgG₁ by RD technology. A binding experiment performed with the most thoroughly investigated variant Z_{mab25} showed that the resulting binding profile for this ligand, among the tested immunoglobulin variants, was restricted to mouse IgG₁ only. This corresponds to very narrow binding spectra involving only the immunoglobulin class used for selection. This is in contrast to the widely used natural immunoglobulin binding proteins SPG and SPA which both show much broader binding spectra in terms of Ig origin and subclass identity, and which have been evolved under the pressure of complex biological and immunological aspects in several different hosts. It is interesting to note that although the scaffold upon which the affibody binding molecule class is based on is derived from SPA, showing a species-dependent and well-characterized binding to the CH2–CH3 region in Fc and to some VH domain families, no preference for the corresponding region in the mouse antibody targets was observed in this study. In fact, an attempt to map the binding site for the Z_{mab25} binder indicated that an epitope on Fab was recognized, overlapping with the SPG-binding site. The structure of the co-complex between a single SPG domain and human IgG₁ Fab has been determined by X-ray crystallography and shows that the SPG domain with its mixed beta-sheet/alpha-helix secondary structure elements content predominantly interacts with the

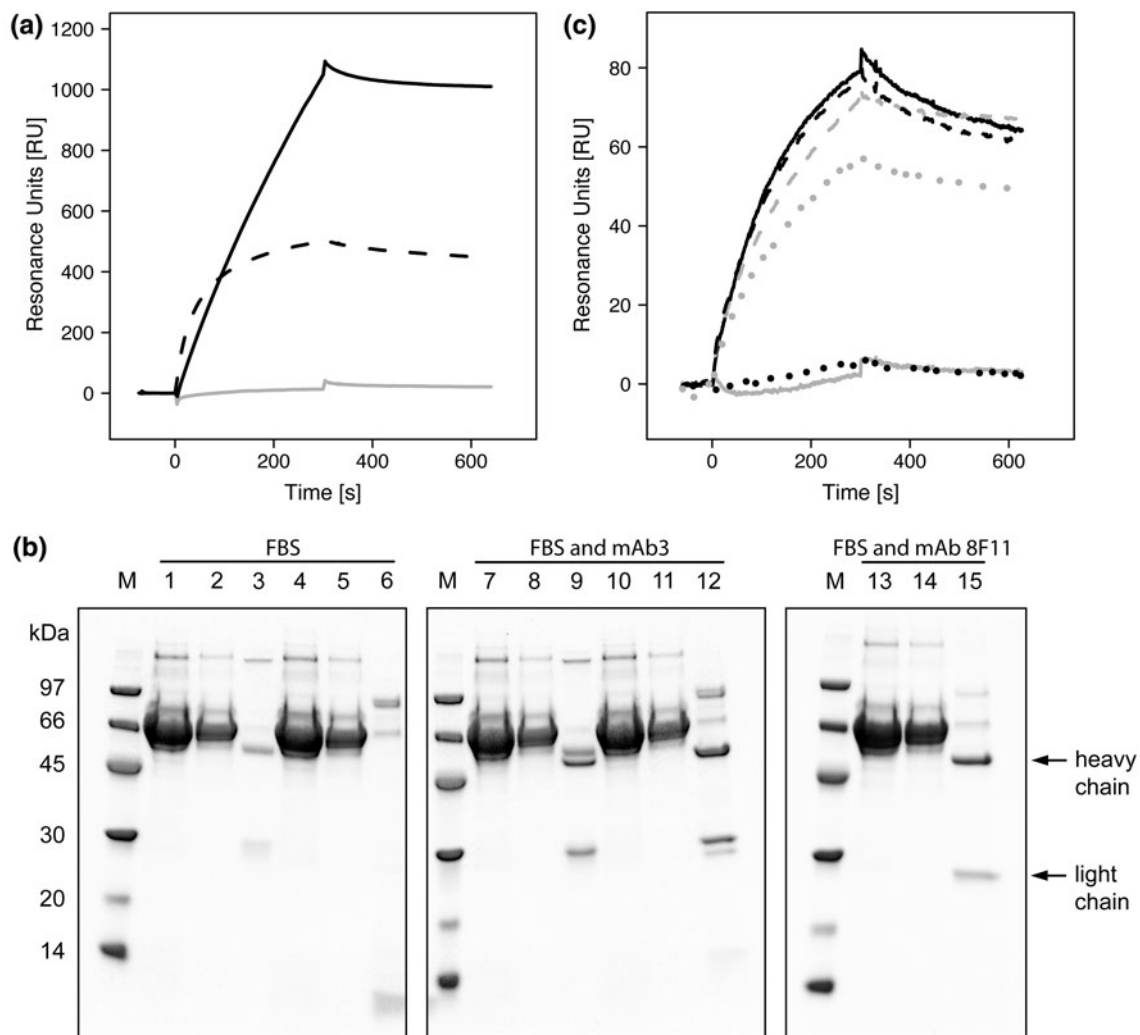


Fig. 9 Binding studies of Z_{mab25} or protein G with fetal bovine serum and species selective affinity recovery of two monoclonal antibodies. **a** 10% FBS was flowed over a Z_{mab25} (gray solid trace) or protein G C2–C3 fragment (SPG) (black dashed trace) BIAcore biosensor surface. For comparison, a sample containing 10% FBS spiked with mouse IgG₁ mAb3 was also flowed over the Z_{mab25} surface (black solid trace). **b**: SDS-PAGE analysis (reducing conditions) of samples, flow-through (FT), and eluate (E) fractions from affinity chromatography experiments using either a protein G (SPG) column or a Z_{mab25} column, and samples of 10% FBS only (lanes 1–6) or 10% FBS with in-spiked mouse IgG₁ mAb3 (lanes 7–12) or 10% FBS with in-spiked mouse IgG₁ 8F11 (lanes 13–15). *M* LMW-SDS molecular weight marker; lane 1: 10% FBS sample; lane 2: SPG column, FT; lane 3: SPG column, E; lane 4: 10% FBS sample; lane 5: Z_{mab25} column, FT; lane 6: Z_{mab25} column, E; lane 7: 10% FBS + mAb3 sample; lane 8: SPG column, FT; lane 9: SPG column, E; lane 10: 10% FBS + mAb3 sample; lane 11: Z_{mab25} column, FT; lane 12: Z_{mab25}

column, E; lane 13: 10% FBS + mAb 8F11 sample; lane 14: Z_{mab25} column, FT; lane 15: Z_{mab25} column, E. The black arrows to the right indicate nominal molecular weights of antibody heavy and light chains, respectively. The numbers to the left indicate molecular weights in kilo Dalton (kDa). **c** Results from a biosensor analysis of pH neutralized eluates from the SPG and Z_{mab25} columns, originating from the use of the 10% FBS sample containing in-spiked mAb3 (1–4) or mAb 8F11 (5, 6). On separate flow cell surfaces, anti-cow IgG and anti-mouse Ig antibodies were immobilized, and samples were injected. (1) black solid trace SPG column eluate, anti-cow IgG surface; (2) black dashed trace SPG column eluate, anti-mouse Ig surface; (3) gray solid trace Z_{mab25} column eluate, anti-cow IgG surface; (4) gray dashed trace Z_{mab25} column eluate, anti-mouse Ig surface; (5) black dotted trace Z_{mab25} column eluate, anti-cow IgG surface; (6) gray dotted trace Z_{mab25} column eluate, anti-mouse Ig surface

CH₁ domain. This interaction is preferentially mediated through main chain atom-mediated beta-zipper interactions, which in turn has been proposed to contribute to the observed promiscuity in terms of binding to Fab fragments derived from antibodies of different origin and subclass identities [32, 34]. The indication from the competition

experiment that the binding site for the Z_{mab25} variant resides in the Fab CH₁ domain together with the binding preference for mouse IgG₁ over mouse IgG_{2b} is notable, considering the high sequence homology between murine Fab CH₁ domains. However, in a study by Sheriff et al. [35], the three-dimensional structures of CH₁ domains

from different Fab fragments, including four murine IgG subclasses (IgG₁, IgG_{2a}, IgG_{2b}, and IgG₃) were compared, and although similar in sequence, local structural differences were observed, which may explain the observed binding characteristics of Z_{mab25}.

Furthermore, a difference in binding strengths to different individual mouse IgG₁ clones were also observed, including the three mAbs used during the selection. This could possibly be explained by differences in post-translational modification or allotypic variance. However, the comparison of relative affinities to target and non-target antibodies based on the analysis where the mAb analytes were injected over a surface containing the Z_{mab25} affinity ligand should be done with some caution. The comparison may have been influenced by difficulties in estimating the true analyte (mouse mAb) concentration in the injected sample. As shown in this study, preparations of mouse monoclonals purified by conventional methods may contain an unknown level of FBS-derived bovine Ig, whose presence may contribute to an overestimation of the mAb protein concentration as determined by spectroscopic measurements or other methods. Actually, contamination of mAbs with bovine immunoglobulin originating from the culture medium supplement FBS is a general concern in monoclonal antibody production. To date, this has been addressed by the screening for FBS containing low natural levels of bovine immunoglobulin or via protein G-mediated Ig depletion [17]. The observed discrimination between murine IgG₁ and bovine serum immunoglobulins by the Z_{mab25} affibody ligand developed in this study, in combination with its ease of recombinant production, envision the use of Z_{mab25} as an alternative solution for selective murine IgG₁ recovery from different hybridoma culture media.

The dominant affibody variant found after four selection cycles was Z_{mab22} containing two cysteines. It could be speculated that this could result in the formation of an intrachain disulfide, contributing to increased stability of the protein, in turn leading to dominance during selection. However, assuming a native-like fold of this affibody variant, the distance between these cysteines would be approximately 15 Å, by far exceeding the ca. 2 Å regarded as a productive distance for disulfide formation [36]. Rather, the observation that the Z_{mab22} variant can form both dimeric and trimeric assemblies suggest that the two cysteines are accessible for inter- rather than intra-chain linkages. Hypothetically, the enrichment of Z_{mab22} may in fact have been driven by avidity effects due to oligomerization during the panning procedure. During the IVT procedure in RD, several ribosomes may line up on the very same mRNA molecule (polysomes) [37]. Thus, it is possible that two or more individual Z_{mab22} polypeptides, emerging from separate but closely positioned ribosomes,

may have become linked during the procedure and favoured during selections due to avidity effects in target binding.

The dominance of Z_{mab22} may have influenced negatively on the selection of other good binders devoid of cysteines, potentially present in the library. Such clones could be expected to increase in number during early cycles while the target amount still is non-limiting, while later become gradually outcompeted by a dominant clone. In fact, some clones which appeared only once after selection cycle 4, and thus potentially could correspond to such clones, were produced and tested for mIgG₁ binding. However, they were all found to be either non-binders or very weak binders (data not shown). This may indicate that potentially good binders were not missed due to dominance by the Z_{mab22} clone.

The described study represents the first study where affibody molecule libraries have been explored in conjunction with RD technology. One of the benefits with this display technology is that transformation of cells is not needed for either the library construction or during the selection process, which facilitates the generation of large libraries. The library size in this study was determined to 10¹¹ using Q-PCR, corresponding to a complexity 30- to 2000-fold larger than previously described naïve affibody molecule libraries [5, 9]. The design of the expression cassette in the present RD system allowed for an optional extension of a standard spacer element by a ribosome tunnel interacting SecM sequence in the translated gene product. In the development phase of the system, this element was shown to result in higher yields of recovered RT-PCR products after selection, interpreted as a capability to contribute to the stabilization of formed ternary complexes under the experimental conditions used. This may allow for future development of alternative selection protocols involving higher temperatures or different chemical compositions of selection buffers, without causing complex disintegration.

In conclusion, this study shows that RD in vitro selection technology is a viable option for constructing and working with large affibody molecule libraries. Further, a novel mouse IgG₁ Fab binding reagent is described which should be a valuable tool in biotechnology applications.

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