A dimeric bispecific miniantibody combines two specificities with avidity

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Received 15 May 1998; revised version received 1 July 1998

Abstract Bispecific antibodies extend the capabilities of nature and might be applied in immunotherapy and biotechnology. By fusing the gene of a single-chain Fv (scFv) fragment to a helical dimerization domain, followed by a second scFv fragment of different specificity, we were able to express a functional protein in *E. coli*, which is bispecific and has two valencies for each specificity. The dimeric bispecific (DiBi) miniantibody preserves the natural avidity of antibodies in a very small-sized molecule of only 120 kDa. The generality of the principle was shown with a scFv fragment binding the EGF-receptor (named scFv 425) in three combinations with scFv fragments either directed against CD2 (ACID2.M1), phosphorylcholine (McPC603) or fluorescein (FITC-E2). Binding was analyzed by sandwich surface plasmon resonance biosensor (BIAcore) measurements.

Key words: Protein design; Dimerization domain; Bispecific antibody; scFv fragment

1. Introduction

In natural antibodies the Fc part is utilized as the second effector site and also serves to dimerize the antigen binding sites. A potentially much wider scope of applications becomes accessible by engineered antibody constructs with two different specificities. So far, most of the bispecific antibodies have been produced as hybrid hybridoma IgG [1] or chemically crosslinked [2] molecules, which assemble as heterodimeric molecules with one binding site for each specificity. However, this approach results in the loss of avidity, also named functional affinity, which arises from the simultaneous binding to two antigens. Depending on the spatial arrangement of the antigen binding sites as well as the antigen surface distribution, the avidity can be significantly higher than the intrinsic affinity [3,4]. In addition, divalency is required for several biological functions such as receptor crosslinking on cell surfaces. For some antibody action divalency is therefore essential [5]. Furthermore, for therapeutic approaches the presence of an Fc domain might not always be desired. We therefore constructed a miniantibody, consisting of four single-chain (scFv) fragments and a dimerization domain, which is at the same time bispecific and bivalent for each specificity.

Bispecific antibodies have been considered as useful agents for example in the treatment of cancer. They can bind with one side to a tumor cell and with the second specificity to immune effector cells, which are thereby redirected against the tumor, or further molecules of pharmacological interest. Several markers on tumor cells (e.g. EGF-R, HER-2/neu, Ep-CAM/EGP-2, CEA, CD19, CD30) and on immune cells (e.g. CD2, CD3, CD16, CD64) have been investigated for this purpose [6]. The immune therapy approach with bispecific antibodies has come of age and is already evaluated in clinical trials [7].

For targeting of tumors overexpressing the epidermal growth factor receptor (EGF-receptor), such as the head and neck tumor, the well characterized murine IgG2a antibody 425 is a promising molecule. It binds to the EGF-receptor while preventing growth stimulation [8] and has already been used in clinical trials [9,10]. We utilize a scFv fragment [11–13] derived from the monoclonal antibody 425 [14] for designing bispecific molecules. For the potential redirection of cytotoxic T-cells and natural killer cells, a scFv fragment has been used which binds CD2 and has been derived from a monoclonal antibody named ACID2.M1 [15–17]. To test the generality of this concept of combining various specificities two additional model scFv fragments have been studied in a fusion protein together with the 425 scFv fragment. One is derived from the monoclonal antibody McPC603 [18,19], which binds the hapten phosphorylcholine, and has been engineered for improved in vivo expression [20]. The other scFv fragment is named FITC-E2, binds FITC, and has been derived from a phage display library [21]. As a dimerization domain we used a helix-loop-helix motif, based on designed peptides [22], which has been shown previously to work well for the functional dimerization of miniantibodies [23,24].

Here we demonstrate that a protein resulting from a gene fusion of a scFv fragment to a helix-loop-helix motif and further to a second scFv fragment can be functionally expressed in *E. coli*. We show that the molecule is both bispecific and shows avidity resulting from divalency for each specificity. As expression and purification worked with all three combinations of specificities tested so far, the fusion strategy presented might be applicable to a wide range of scFv fragments.

2. Materials and methods

2.1. Plasmid construction

The expression vector was based on pASK30 [25], in which the *Xba*I/*Hin*dIII cassette has been replaced by an insert as shown in Fig. 1B and C. The new vector was assembled in the first step from the plasmids pACK9-9p53his and pACKdhlxAscI (P. Pack, unpublished) to give the intermediate plasmid pKM309-9dhlxhis2. In this plasmid, a scFv fragment binding CD2, named M1, with a preceding *pelB* leader sequence was cloned from the plasmid pEG3M1FH (W. Strittmatter, unpublished) via *Xba*I/*Eco*RI to give pKM30M1dhlxhis2. As the second, C-terminal specificity a scFv fragment binding EGF-receptor, named 425 [14], was amplified from the plasmid pFEG1T (W. Strittmatter, unpublished) by PCR with add-on tails on both sides containing *Asc*I sites using the primers ak425_1:

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Abbreviations: 425, anti-EGF-receptor scFv; dhlx, helix-turn-helix dimerization domain; DiBi, dimeric bispecific; EGF-R, epidermal growth factor receptor; FITC-E2, anti-fluorescein scFv; IMAC, immobilized metal ion affinity chromatography; PC, phosphorylcholine; RU, resonance units; M1, anti-CD2 scFv; scFv, single-chain Fv fragment of an antibody

PII: S0014-5793(98)00829-1

AGGCGCGCCG GAAGTGCAAC TGCAGCAGTC and ak425_1-rev: AGGCGCGCCC CGTTTGATCT CGAGTTCTG. The PCR fragment was cloned via the *Asc*I sites to give plasmid pKM30M1dhlx425h. To test additional N-terminal specificities the scFv fragment M1 was replaced via *Xba*I/*Eco*RI by two further scFv fragments. First, a scFv fragment binding phosphorylcholine, named McPC603_H11_L, which is preceded by an *ompA* signal sequence, contains three stabilizing mutations (H11) [20], a long glycine-rich linker of 25 amino acids (L) and was taken from the plasmid pKA30H11-25 to give pKM30PCLdhlx425. Second, in an improved vector (see below), scFv M1 was replaced by a scFv fragment binding fluorescein, named FITC-E2 [21], which was first subcloned in pAK500 via *Sfi*I [26], to give pKM310Fitcdhlx425h.

The improved vector was constructed containing a minimal *hok*/*sok* system [27] for killing of plasmid-free cells, and the *skp* gene [28] for higher product yield. First the *hok*/*sok* was amplified by PCR using the primers hoksok: TCGGAAGATC TCCCGGGACA AACTCCGGGA GGCAGC, and hoksok_rev: TCGGAAGATC TCAACATCAG CAAGGAGAAA GG. Both primers carried a *Bgl*II site and the first primer an additional *Sma*I site as add-on tail. The *Bgl*II *hok*/*sok* fragment was cloned into a *Bam*HI site behind the *lpp* terminator of the scFv fragment in the plasmid pAK100 [26] to give pKM100hs. The *skp* gene was cloned as *Spe*I/*Spe*I insert filled with Klenow fragment from plasmid pHB112 [28] in the newly established *Sma*I site to give pKM100hsskp. The *hok*/*sok*-*skp* insert was cloned via *Hin*dIII/*Bsa*I in the pKM30 series to give pKM310, thus resulting in pKM310M1dhlx425h. All PCR steps were verified by sequencing and all cloning steps by analytical restriction digests.

2.2. Protein expression

Plasmids were transformed either into *E. coli* JM83 (F^- *ara (lac-proAB) rpsL* (str^r) [*Φ80dlac Δ(lacZ)M15] thi*) or BF18-61 (R. Wenderoth, unpublished), a RV308 (*lac74-gal*ISII::OP308-*strA*) derivative. Protein expression was carried out in LB broth in shake flasks, containing 0.1 g/l ampicillin. Overnight cultures were grown at 26°C. The main culture was inoculated to an OD_{550} of 0.15 (typical dilution of 1:25–1:30) and grown at 24°C. Cells were induced at an OD_{550} of 0.5 to 0.6 and harvested by centrifugation at $5000 \times g$ after 4 h. Cell pellets were immediately frozen at −80°C and stored until use.

2.3. Protein purification

Cell pellets were thawed in a seven-fold volume of PBST (50 mM Na-phosphate, 150 mM NaCl, 0.005% Tween-20) containing 10 mM imidazole at pH 7.4 in case of the miniantibodies M1dhlx425 and FITCE2dhlx425, and in BBST buffer (200 mM H_3BO_3, 160 mM NaCl, 0.005% Tween-20, adjusted to pH 8.0 with NaOH) in case of the miniantibody PCLdhlx425. Cells were disrupted by sonification three times with continuous temperature monitoring in an ice-ethanol bath between 3 and 10°C. The DiBi miniantibodies containing scFv M1 and scFv FITC-E2 miniantibodies were purified by immobilized metal ion affinity chromatography (IMAC) using Ni-NTA superflow material (Qiagen, Germany). After washing steps with 12 mM and 30 mM imidazole the protein was eluted at 250 mM imidazole in PBST buffer, pH 7.4. After IMAC the miniantibody FITCE2dhlx425 was dialyzed against PBST with 1 mM EDTA and a second time against PBST and used for analysis. The miniantibody M1dhlx425 was further purified using thiophilic adsorption chromatography. In this case, 3 M $(NH_4)_2SO_4$ was added to the IMAC-eluted sample to a final concentration of 1 M. The protein was loaded on a thioether column (Fractogel EMD TA, Merck Darmstadt, Germany), washed with 500 mM $(NH_4)_2SO_4$ in PBS and eluted with 150 mM $(NH_4)_2SO_4$ in PBS buffer. The sample was dialyzed against PBST buffer. The miniantibody PCLdhlx425 was purified by phosphorylcholine affinity chromatography as described [29,30] in borate buffered saline (BBS) with a washing step with buffer containing 1 M NaCl and elution with 20 mM PC. In addition, miniantibodies have also been purified by anti-425 anti-idiotypic immunoaffinity chromatography [17].

2.4. SDS-PAGE and size exclusion chromatography

Protein samples were analyzed by SDS-PAGE (12% mini-gels with 5% stacking gels) and stained with Coomassie Brilliant Blue according to standard methods. The size exclusion chromatography column (Superose-12 PC3.2/30, Pharmacia, Sweden) was run with a SMART system (Pharmacia, Sweden) and calibrated with a molecular weight standard (cytochrome c, 12.4 kDa; carbonic anhydrase, 29 kDa; alcohol dehydrogenase, 150 kDa; β-amylase, 200 kDa) (Sigma, USA). Samples of 20 μl were injected, the running buffer was PBST, and elution was monitored at 280 nm and 254 nm.

2.5. Analysis of binding

Binding capabilities of the miniantibodies were analyzed with a BIAcore instrument (Biacore, Sweden) [31]. FITC (Fluorescein isothiocyanate) (Fluka, Switzerland) was coupled to BSA (Imject, Pierce, USA) at a molar ration of 3 to 1 to give BSA-FITC using a standard procedure [32] and dialyzed against PBS buffer. Phosphorylcholine, coupled to the tyrosines of BSA and prepared according to [23], was taken from the lab stock. EGF-receptor (EGF-R) extracellular domain was kindly provided by Dr. W. Strittmatter (Merck KGaA). BSA-FITC and EGF-R were diluted in 10 mM Na-acetate, pH 4.5 to a final concentration of approximately 0.2 mg/ml and 70 μl were coupled to a CM5 sensor chip (Biacore, Sweden) using standard EDC (1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide-HCl) NHS (*N*-hydroxysuccinimide) coupling chemistry with subsequent blocking by ethanolamine. Running buffer for the BIAcore was PBST in the case of the miniantibodies M1dhlx425 and FITCE2dhlx425, and BBS buffer with 0.005% Tween for the miniantibody PCLdhlx425. Surfaces were regenerated with a glycine/HCl buffer (100 mM glycine, 500 mM NaCl, adjusted to pH 2.8 with HCl).

3. Results

3.1. Plasmid construction, expression and purification

The concept of homodimerizing scFv fragments via a fused homodimerization domain, named miniantibodies, was extended by fusing a second scFv fragment behind the homo-

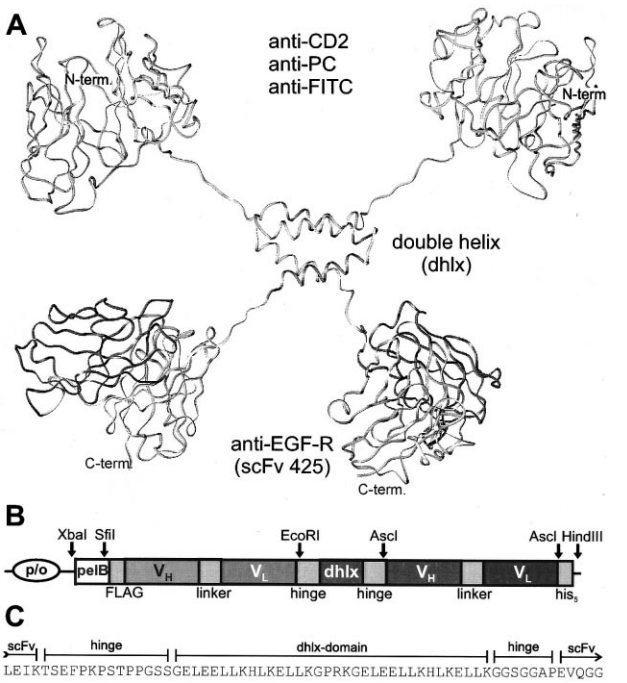

Fig. 1. Model of a dimeric bispecific (DiBi) miniantibody (A), scheme of the gene arrangement (B), and sequence of the dhlx dimerization domain [23] with its adjacent hinge regions (C). A: The protein model was composed from a minimized structure of a variable domain (PDB file 4FAB) to which a linker was attached, a hinge region modeled in extended conformation, a preliminary structure of a designed helix-loop-helix motif with sequence similar to the actually used domain (kindly provided by W.F. DeGrado), a linker (the second hinge) modeled in extended conformation, and finally the variable domains again. B: The gene depicts the modular arrangement of the dimeric bispecific miniantibody including singular restriction sites, which was cloned into various vectors. The scFv fragments used all have V_H-linker-V_L orientation. C: A part of the amino acid sequence of a DiBi miniantibody is shown.

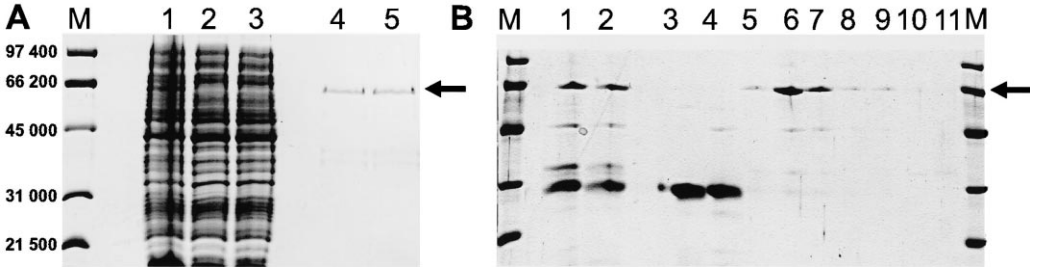

Fig. 2. SDS-PAGE of the purification of (A) the DiBi miniantibodies PCLdhlx425 by PC affinity chromatography and (B) the DiBi miniantibody M1dhlx425 by IMAC and thiophilic adsorption chromatography (TAC). A: Lane M, marker; lane 1, crude lysate; lane 2, centrifugation supernatant; lane 3, flow through; lanes 4–5, PC elution fractions. B: Lane M, marker; lane 1, sample eluted from IMAC; lane 2, sample supplemented with 1 M $(NH_4)_2SO_4$; lanes 3–4, wash fractions at 500 mM $(NH_4)_2SO_4$; lanes 5–8, fractions of the TAC elution peak at 150 mM $(NH_4)_2SO_4$; lanes 9–10, residual protein eluting in pure PBS buffer; lane 11, column wash with H_2O. Arrows indicate the size of the DiBi miniantibody band.

dimerization domain (Fig. 1). Unique restriction sites flanking each domain facilitate easy combination of various scFv fragments of different specificity, giving convenient access to several dimeric bispecific (DiBi) miniantibodies. This concept has been tested with three different N-terminal scFv fragments: a better folding variant of the scFv fragment McPC603 [19] with a 25-mer linker, named PCL, which binds phosphorylcholine, the scFv FITC-E2 [21], which binds fluorescein, and the scFv ACID2.M1 [16], named M1, which binds CD2. These are followed by a murine IgG3 upper hinge and a helix-loop-helix (dhlx) dimerization domain [23]. As the C-terminal specificity we used the scFv fragment 425 [14], which binds to the EGF-receptor.

All three miniantibodies were directed to the periplasm with a PelB or OmpA signal sequence. Functional protein could be purified from crude cell lysates by affinity chromatography (Fig. 2A) or by a combination of IMAC and thiophilic adsorption chromatography (Fig. 2B). The thiophilic adsorption chromatography worked well with two out of three miniantibodies tested, including miniantibody M1dhlx425. Typical yields of purified protein were 100 μg l^{-1} OD^{-1} from *E. coli* shake flask culture.

3.2. Protein analysis

The dimeric nature of the bispecific scFv constructs was determined by size exclusion chromatography. The DiBi miniantibody M1dhlx425 had an apparent molecular weight of 144 kDa and the DiBi miniantibody PCLdhlx425 one of 140 kDa, which is consistent with the calculated weight of about 120 kDa (Fig. 3). For comparison, the monospecific miniantibodies 425dhlx and M1dhlx, dimerized via the dhlx domain, but not carrying the C-terminal scFv, were also investigated and corresponded to the expected mass of 67 and 66 kDa, respectively [17].

To analyze the bispecificity of the miniantibodies a sandwich assay was performed by immobilizing either the antigen of the first or the second antibody on a BIAcore biosensor chip. Upon binding of the miniantibody, the signal (measured in resonance units (RU)) increased as expected, and when the second antigen was added, a further increase in the signal was seen (Fig. 4A, B). As an example, the antigen of the N-terminal scFv fragment, BSA-FITC, was immobilized. When EGF-receptor was injected alone onto this surface as a control, only a background signal (20 RU) was obtained (Fig. 4A, phase I). Injection of a DiBi miniantibody comprising an anti-FITC and an anti-EGF-R scFv fragment resulted in a significant signal (1400 RU, Fig. 4A, phase II). When the bulk effect (due to change in the buffer composition) had passed, a stable binding of the scFv FITC-E2 was seen, which is a result of its high affinity [21], further amplified by the high coating density of the FITC-BSA, and the bivalent binding (phase III). Injection of EGF-receptor extracellular domain onto the bound DiBi miniantibody (phase IV) gave then rise to another increase in signal, due to association of this antigen to the DiBi miniantibody, which stopped at the end of injection (additional 480 RU), and was followed by a dissociation phase of the EGF-receptor (phase V).

Three points from these results are to be noted. First, the DiBi miniantibody is indeed functionally bispecific. Second, the anti-EGF-R scFv 425 is C-terminal in the construct, and thus both variable domains of this scFv are blocked by linkers at their respective N-termini, but it is still able to bind a large (approx. 100 kDa) molecule. Third, in this situation the EGF-R is bound only monovalently, which results in a higher off-rate (phase V) than when it is bivalently bound (see below). A control experiment, in which an anti-425 idiotypic monoclonal antibody was injected instead of the EGF-R antigen confirmed bispecificity as well (data not shown).

Immobilization of the antigen of the C-terminal scFv is exemplified in Fig. 4B with the anti-PC-anti-EGF-R DiBi miniantibody. EGF-receptor was immobilized and the DiBi

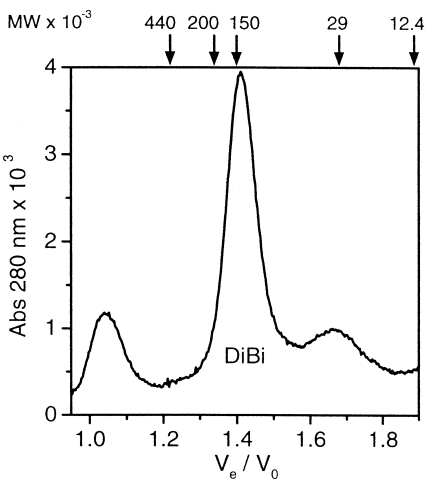

Fig. 3. Determination of protein size by size exclusion chromatography. Samples of 20 μl were injected onto a Superose-12 column, and the molecular weight was determined according to a calibration curve of markers shown on top.

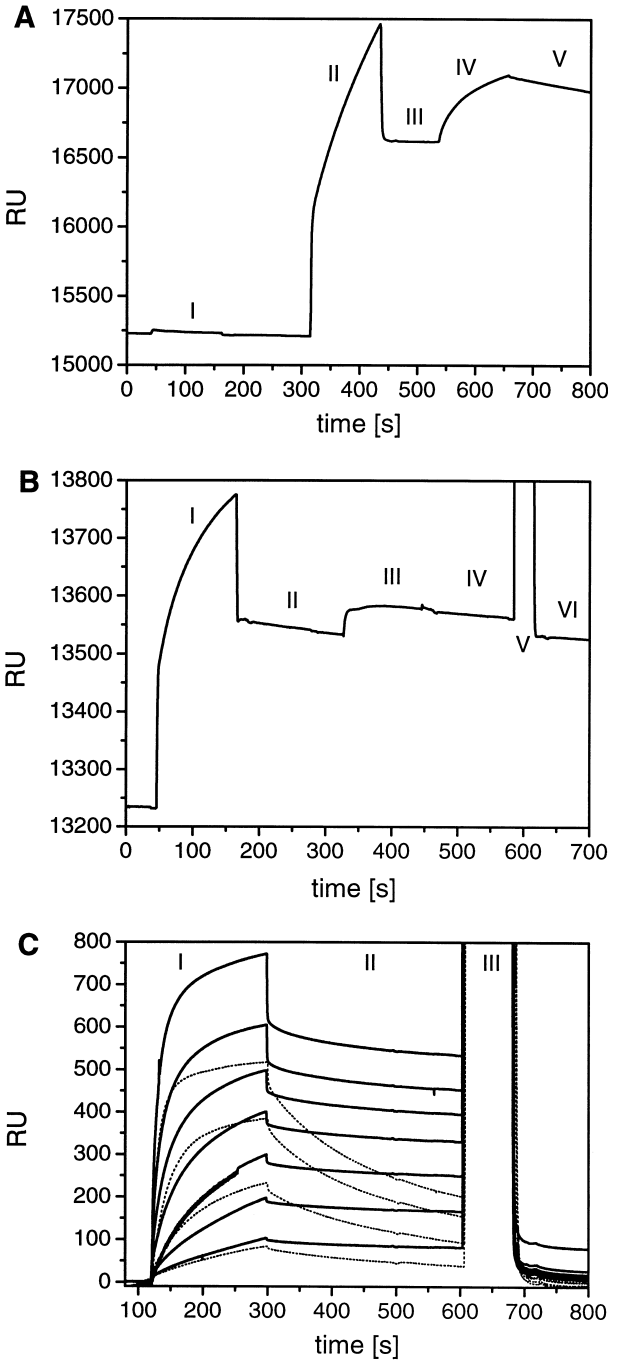

Fig. 4. BIAcore sensorgrams demonstrating the bispecificity (A, B) and avidity (C) of the DiBi miniantibodies. A: BSA-FITC was coupled to the surface and the anti-FITC-anti-EGF-R miniantibody was tested. Phase I, injection of EGF-receptor (control); II, injection of DiBi miniantibody FITCE2dhlx425; III, dissociation of DiBi miniantibody; IV, injection of EGF-receptor; V, dissociation of EGF-receptor. B: EGF-receptor was coupled to the surface and the anti-PC-anti-EGF-R miniantibody was tested; phase I, injection of DiBi miniantibody PCLdhlx425; II, dissociation of DiBi miniantibody; III, injection of BSA-PC; IV, dissociation of BSA-PC and DiBi miniantibody; V, injection of 20 mM PC (large bulk effect) causing BSA-PC elution; VI, signal after PC has been washed out. C: Anti-CD2-anti-EGF-R DiBi miniantibody M1dhlx425 was injected at increasing concentrations (25, 50, 100, 200, 400, 800, 1600 nM) onto a surface with EGF-receptor coupled to 1600 RU. Phase I, injection of M1dhlx425; II, dissociation; III, regeneration with Gly/HCl. For comparison, the data of a monovalent, C_HC_L-heterodimerized miniantibody containing scFv 425 and M1, measured under identical conditions (25, 100, 400, 1600 nM) on the same chip [17], are shown as dotted lines.
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miniantibody bound as expected. Dissociation was slow (Fig. 4B, phase II), presumably since the binding was bivalent and the intrinsic binding to EGF-R is tight. Injecting BSA-PC on this DiBi miniantibody resulted in a further signal increase (phase III). The comparatively small increase can be explained by each BSA molecule carrying on average seven PC molecules (data not shown), which results in a high valency of a single BSA-PC molecule, reducing the total number of bound BSA-PC molecules. The high avidity of bound BSA-PC is also evidenced by the slow dissociation (phase IV), despite the intrinsically very fast off-rate of 10 to 38 s^{-1} of PC from the antibody McPC603 [33]. That this binding is nevertheless very specific is demonstrated by the fact that soluble PC can displace bound BSA-PC immediately (phase V and VI), since the stable binding of the latter is caused by multivalency, and PC can interfere with the highly dynamic binding (see also [4,34]).

Finally, the binding of the miniantibody M1dhlx425 to the EGF-R was analyzed to visualize the bivalent binding behavior using a range of miniantibody concentrations (25 nM to 1600 nM) and a surface coated with 1600 RU EGF-R (Fig. 4C). Measurements under identical conditions were performed with a monovalent, C_HC_L-heterodimerized miniantibody containing scFv M1 and 425 (some curves are shown for comparison in Fig. 4C), the homodimeric miniantibody 425dhlx, and the monoclonal antibody 425, which all have been published previously [17]. The comparison of these series reveals that the C-terminal scFv in the DiBi miniantibody binds comparably to the parental monoclonal antibody, but dissociates much more slowly than the monovalent constructs, suggesting a significant contribution of bivalent binding.

4. Discussion

The results presented here demonstrate that bivalent and bispecific scFv-based molecules can be obtained in functional form *E. coli* by encoding two scFv fragments in a single peptide. Furthermore, a helix-loop-helix motif encoded in the same peptide leads to homodimerization, resulting in tetravalent species with two specificities (Fig. 1). Up to now, expression in *E. coli* of two scFv fragments in a single peptide chain was mainly achieved by cytoplasmic inclusion bodies [35], and it was even noted, depending on the antibody, that functional expression can be impossible, while some of the molecules can still be produced in mammalian expression systems [36]. However, the three DiBi miniantibodies described here demonstrate that the functional expression approach is feasible.

We think that three factors may account for this success. First, the scFv fragments on their own need to be well expressed, and scFv fragments with a high tendency to form aggregates in the periplasm are unfavorable candidates for more complex fusion proteins. Second, we speculate that the additional dimerization domain might serve as structured spacer between the scFv fragments, thereby reducing intramolecular aggregation and incorrect domain swapping. Third,

efficient purification schemes with high specificity and low product loss are essential. Of course, antigen or anti-idiotypic immunoaffinity purification are most advantageous, but depending on the scFv fragments involved, thiophilic adsorption chromatography has also been found useful. A combined IMAC/anti-His-tag immunoaffinity purification may provide a general strategy [37].

The idea of bispecific antibodies is associated with high expectations for therapeutic applications, and thus basic science has striven for a long time for a facile generation of these molecules [38]. Therefore, various methods are already available such as the hybrid hybridoma technology [1], the chemical crosslinking of Fab fragments [2], diabodies consisting of dimerized scFv fragments [39], scFv fused to leucine zipper domains [40], scFv fused to whole antibodies [41], and C_HC_L-heterodimerized scFv [17]. Nonetheless, our dimeric bispecific miniantibody concept features two advantageous properties. To our knowledge it is currently the smallest available antibody-based molecule to combine bispecificity with the avidity of two binding sites, which are, moreover, connected via flexible linkers. Depending on the application, size is an important factor, for example for tissue penetration of molecules circulating in the bloodstream. Bivalent binding can be important in several aspects. Bivalent binding increases avidity, which can compensate for weak binding, but might also be used to increase selectivity for targets with multiple antigen display, such as tumor cells overexpressing certain markers. Furthermore, bivalent binding is often required for receptor crosslinking and subsequent signal transduction. Thus, we believe that the dimeric bispecific miniantibody is a valuable tool for research and a format suitable for future clinical testing.

Acknowledgements: We would like to thank Dr. W. Strittmatter (Merck KGaA, Darmstadt) for providing EGF-receptor and clones of the scFv fragments 425 and M1, Dr. P. Pack (Morphosys, München) for providing clones with *Asc*I restriction site, and Dr. W.F. DeGrado for providing the structure of a helix-loop-helix motif before publication, Prof. D. Riesenberg and Dr. U. Horn for helpful discussions, and the Deutsches Bundesministerium für Bildung und Forschung (BMBF) for financial support.

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