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# Design of multivalent complexes using the barnase•barstar module

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**The ribonuclease barnase (12 kDa) and its inhibitor barstar (10 kDa) form a very tight complex in which all N and C termini are accessible for fusion. Here we exploit this system to create modular targeting molecules based on antibody scFv fragment fusions to barnase, to two barnase molecules in series and to barstar. We describe the construction, production and purification of defined dimeric and trimeric complexes. Immobilized barnase fusions are used to capture barstar fusions from crude extracts to yield homogeneous, heterodimeric fusion proteins. These proteins are stable, soluble and resistant to proteolysis. Using fusions with anti-p185$^{HER2-ECD}$ 4D5 scFv, we show that the anticipated gain in avidity from monomer to dimer to trimer is obtained and that favorable tumor targeting properties are achieved. Many permutations of engineered multispecific fusion proteins become accessible with this technology of quasi-covalent heterodimers.**

Innovative protein therapeutics will in many cases have to consist of targeting and effector moieties. In addition, the need for high efficacy and minimal side effects will require high-affinity and highly selective binding[1]. If the molecular target is located on a cell surface, as in the usual strategy for tumor targeting, this can often be most easily achieved by multivalent and/or multispecific targeting moieties, which simultaneously bind to several of the same or different target epitopes on the cell[1–3]. It follows that we need a method that allows the linking of protein domains not only in a linear form, as in fusion proteins, but also in more complicated assemblies.

Over the last few years several approaches to engineering multivalency have appeared, especially for recombinant antibodies[2,3]. First, linked domains of antibodies can exploit a domain-swapping phenomenon[4], yielding dia-, tria- and tetrabodies[3]. However, this approach is not of uniform efficiency, and the particular molecular constraints are not fully understood. Second, the linear fusion of two or more antibody scFv fragments might seem convenient, but may lead to unintended domain swapping and often requires low-yield eukaryotic production[5]. Third, molecules can be linked by an engineered disulfide bridge or by thioether crosslinks[6,7], but the reproducible large-scale manufacture of such assemblies is difficult. Fourth, fusions to dimerizing or oligomerizing peptides can be engineered[2,8–11]. However, long-term *in vivo* stability requires very high-affinity peptides because dilution of the complexes in the bloodstream promotes their dissociation. This has been countered by engineering intermolecular disulfides[12,13], but at the price of much more difficult production. Fifth, natural dimeric or oligomeric protein domains can be used, but the same considerations for interference of the extra domains with folding of the antibody domains and the additional complications introduced by intermolecular disulfides hold, usually requiring eukaryotic production[14,15]. Another constraint for dimerizing proteins and peptides is that usually they cannot exist as monomers, and thus a dimerization on demand is impossible. Finally, most systems cannot easily be extended from homodimeric to heterodimeric assemblies.

We describe a multimerization system based on the very tight, defined interaction between barnase and barstar. The approach allows inexpensive large-scale production in bacteria, is extendable to a wide range of fusion proteins and avoids eukaryotic expression, chemical modification, intermolecular disulfide bond formation and difficult separations of oligomers. Barnase (110 amino acids) is a secreted ribonuclease from *Bacillus amyloliquefaciens*. Barstar (89 amino acids) is a cytoplasmic barnase inhibitor with which the host protects itself. Both can be efficiently expressed in bacteria in recombinant form[16,17]. They rapidly form a complex with a $K_D$ of ~$10^{-14}$ M (refs. 16–18), comparable only to that of biotin and streptavidin (~$10^{-15}$ M (ref. 19)). Unlike the latter, however, our system does not require covalent modification of any of the proteins, and both partners are monomeric. The three-dimensional structure of the barnase•barstar complex shows that the N and C termini of both proteins are localized away from the dimerization interface[20,21]. Therefore, all four termini are accessible and available for fusions.

Barnase and barstar, when attached to an scFv fragment via a hinge region, can thus serve as building blocks for multivalent miniantibodies that mimic the rotational and segmental flexibility of the natural antibody binding arms. By fusing more than one barnase (or barstar) in series, complexes of higher valency can be created (see Results), as each one will complex with a partner. Unlike other interacting peptides, both barnase and barstar are just as soluble and stable in the uncomplexed form, and thus fusion proteins can be kept separate and

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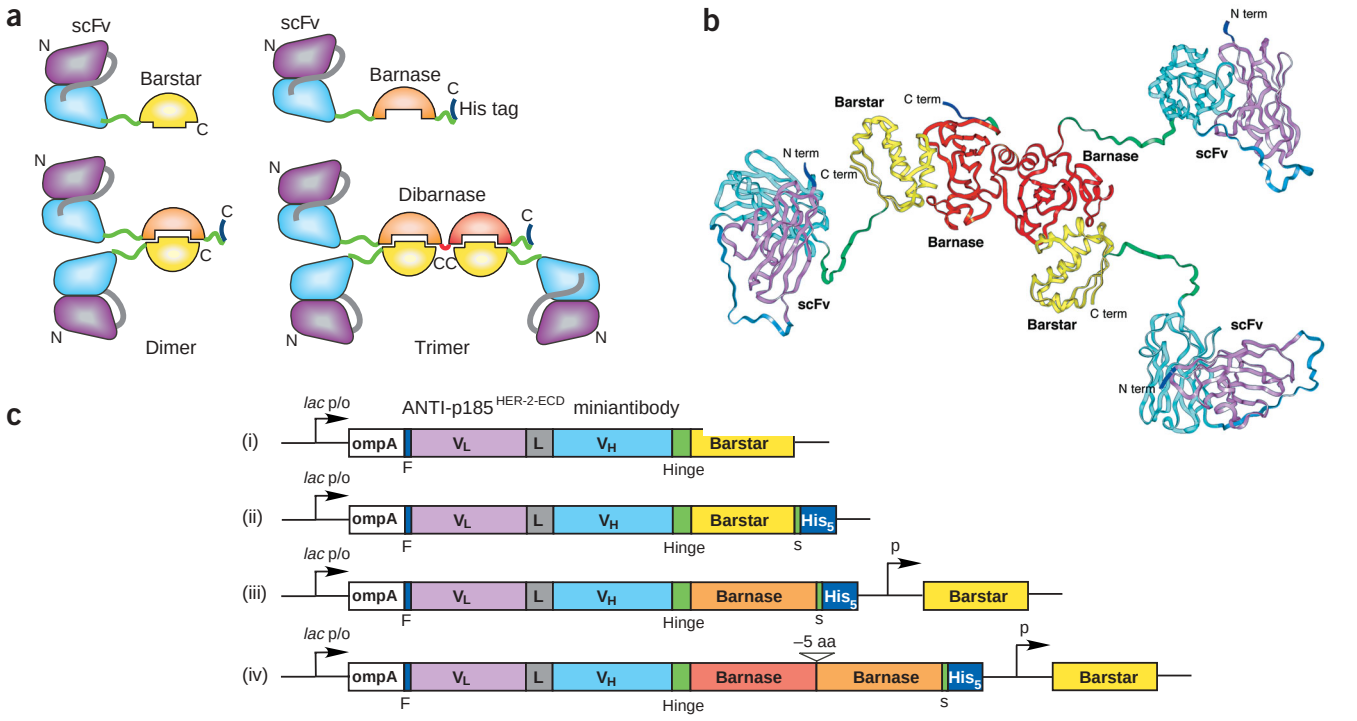


**Figure 1** The concept of creating multimeric miniantibodies using heterodimeric barnase·barstar modules and scFv fragments. (**a**) scFv fusion proteins with barstar and barnase or dibarnase yield dimeric and trimeric complexes owing to specific complex formation. (**b**) Molecular model (ribbon representation) of the ternary complex of 4D5 scFv–dibarnase-$His_5$ (corresponding to the 'trimer' in **a**, and construct (iv) in **c**) with two molecules of 4D5 scFv–barstar (construct (i)). The 4D5 scFv–dibarnase-$His_5$ construct starts with an N-terminal short FLAG tag (F, blue), followed by the 4D5 scFv in VL-linker-VH orientation (VL, magenta; linker, gray; VH, cyan), a 16-amino-acid hinge linker (green) and two barnases (red, orange) connected directly. The construct terminates in a $His_5$-tag (blue) attached through a short spacer (s) with the sequence Gly-Ala-Pro (green) to the C-terminus of the scFv-dibarnase fusion protein. The 4D5 scFv–barstar construct starts with the same FLAG-tagged 4D5 scFv connected by the linker to the barstar moiety (yellow). (**c**) Overview of the gene constructs for expression of the 4D5 scFv–barstar (i), 4D5 scFv–barstar-$His_5$ (ii), 4D5 scFv–barnase-$His_5$ (iii) and 4D5 scFv–dibarnase-$His_5$ (iv) fusion proteins. F, FLAG sequence; s, spacer GAP. The triangle in construct (iv) indicates a deletion of the five N-terminal amino acids of the second barnase. The gene fusions are under control of the *lac* promoter and the ompA signal peptide is used to direct secretion of the recombinant proteins to the periplasmic space of *E. coli*. Barstar coexpression under the control of its own constitutive promoter (p) is required to suppress cytotoxicity of barnase fusions. P/o, promoter/operator.

complexes can be formed only on demand. Fusions of barnase or barstar to scFvs are compatible with transport to the bacterial periplasm. Proteolytic cleavage of the linkers is essentially not observed in the protocol reported here, in contrast to other fusion protein strategies. The extremely specific and strong association of barnase and barstar makes it possible to obtain a precise 1:1 ratio of partners.

Barstar has two cysteines, which do not form a disulfide bond[16,17], whereas barnase has no cysteine[16,17]. Barstar can tolerate replacement of both cysteine residues with alanine and fold correctly[16,17]. The resulting protein still forms a very tight complex with barnase. Therefore, both barnase and the barstar mutant (called 'barstar' hereafter) can be linked to disulfide-containing proteins such as antibody fragments, which need to be secreted, without the danger of forming incorrect disulfides. This modular system therefore allows the convenient linkage of proteins produced in different cellular compartments or in different production hosts.

We demonstrate the utility of this system with fusions of barnase and barstar to the antibody scFv fragment 4D5 (refs. 22,23), which recognizes $p185^{HER2}$, a protein that is overexpressed in many carcinomas and is correlated with poor prognosis[24,25]. This work describes the convenient production and purification of well defined di- and trivalent constructs, their properties and their favorable *in vivo* targeting properties.

## RESULTS

### Design of constructs

We placed scFv fragments at the N terminus of both barnase and barstar to exploit the periplasmic secretion of the antibody fragment[23] and avoid obstruction of its binding site. The incorporation of flexible hinge peptides helps simultaneous binding to several antigens[2]. We used the peptide EFPKPSTPPGSSGGAP, which is derived from the murine IgG3 hinge region and can span 2.5–2.7 nm. To avoid interference with the formation of intradomain disulfides in the scFv fragment during periplasmic expression of the fusion protein, we used the barstar (C40A, C82A) double mutant.

The fusions scFv-hinge-barnase and scFv-hinge-barstar are monomeric and soluble by themselves and form divalent miniantibodies after mixing (**Fig. 1a**). We also designed a scFv-(barnase-barnase) fusion protein (termed 'dibarnase'), in which the two barnase units are directly fused head-to-tail without a linker and the first five flexible residues of the second barnase are deleted. The three-dimensional model of this barnase dimer shows a compact structure with no steric hindrance for attachment of three scFv fragments (**Fig. 1b**). To facilitate subsequent metal ion affinity chromatography as well as $^{99m}$Tc-labeling[26], the fusion proteins carried, separated by the short spacer GAP, a C-terminal penta-histidine tag (**Fig. 1c**).

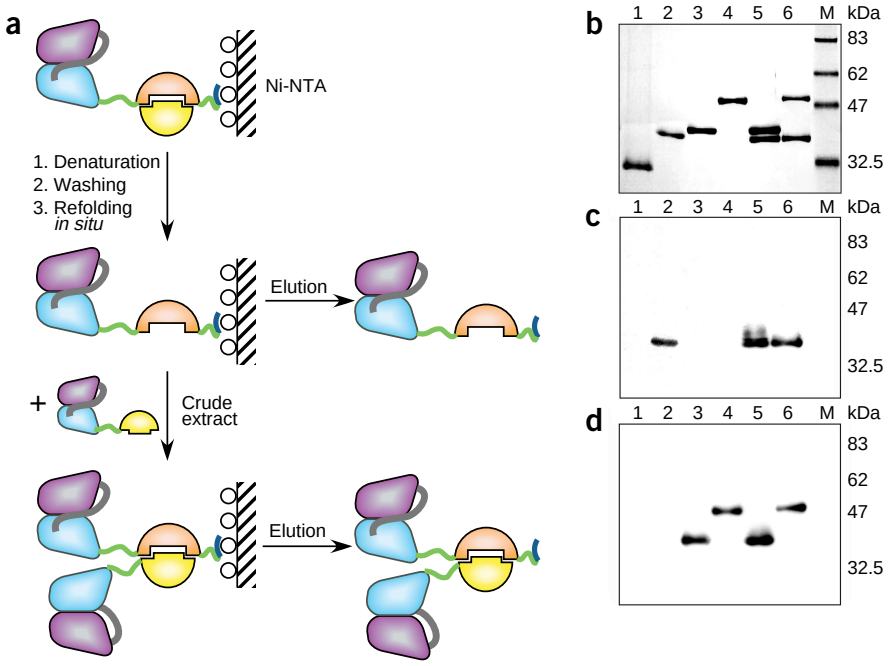


**Figure 2** The preparation of homogeneous mono- and multivalent proteins. (**a**) Schematic representation of the procedure. Expression of scFv–barnase fusion proteins requires coexpression of the barnase inhibitor, barstar; denaturation of the His-tag-immobilized scFv-barnase•barstar complex removes the inhibitor and allows stringent washing. Refolding of the $Ni^{2+}$-NTA adsorbed fusion protein *in situ* results in functional protein preparation. The His-tag-immobilized scFv-barnase fusions can be saturated on the column with scFv-barstar protein (without His-tag) from crude extract resulting in a one-step preparation of oligomeric complexes with an exact stoichiometric ratio of subunits. (**b**–**d**) 12% SDS-PAGE documenting the purification of monovalent fusion proteins and multimeric complexes. Coomassie stained gel (**b**). Western blot carried out with anti-barstar and anti-barnase sera, respectively (**c**,**d**). The same blot was used for both analyses after stripping the first detecting antiserum. Lanes are: (1) 4D5 scFv fragment, (2) 4D5 scFv–barstar-$His_5$, (3) 4D5 scFv–barnase-$His_5$, (4) 4D5 scFv–dibarnase-$His_5$, (5) dimeric complex, (6) trimeric complex, M, molecular weight markers. The same analysis using an 18% SDS gel (not shown) also verified the absence of any proteolytic degradation products or other contaminants of the purified proteins.

### The necessity of barstar coexpression

Expression of active barnase is lethal to the bacterial host but can be suppressed by coexpression of barstar[16,27]. We confirmed this necessity, as it has important implications for the design of the optimal production strategy. When the gene for the scFv-barnase fusion was assembled on the same plasmid vector with the barstar gene under the control of its own constitutive promoter and with an efficient ribosome binding site[28] (**Fig. 1c**), intact clones were obtained. Thus, a reliable system for production of completely active barnase fusions requires inducible expression of the target gene coupled with a sufficient constitutive basal level of barstar.

### Removal of barstar, denaturation and refolding of fusions

We worked out a convenient, scaleable production method that can be used for individual proteins or complexes under the same conditions. It makes use of the fact that barnase refolds completely and extremely rapidly[29] after exposure to denaturants. Antibodies fold efficiently, provided the disulfides are already formed correctly, as is the case in periplasmic expression[30]. The cell extract containing barnase•barstar was adsorbed to a $Ni^{2+}$-NTA matrix, and the fusion protein was purified by denaturation with 6 M guanidine HCl to remove the barstar inhibitor and refolded on the column. This process eliminates the majority of contaminants and results in up to 95% purity of the target protein (**Fig. 2**). Subsequently, Protein-A affinity chromatography was used to remove residual contaminants[31]. Protein A specifically binds human $V_H3$ domains in their native form, such as those present in the antibody 4D5, and thus allows removal of traces of nonnative and denatured forms of 4D5 scFv fusions. The purification yielded approximately 1.5 mg/l from shake flask culture for both the 4D5 scFv–barnase and 4D5 scFv–dibarnase fusions. The His-tagged 4D5 scFv–barstar fusion was purified analogously using $Ni^{2+}$-NTA affinity chromatography under denaturing conditions from insoluble periplasmic protein with subsequent folding by dilution.

We verified the integrity of the monomeric constructs and showed that neither moiety of the fusion proteins (barstar or barnase and the 4D5 scFv fragment) interfered with the functionality of the other.

We investigated the association of radiolabeled 4D5 scFv–barnase and 4D5 scFv–dibarnase fusions with their partner 4D5 scFv–barstar fusion. 4D5 scFv–barnase and 4D5 scFv–dibarnase constructs could be conveniently labeled with $^{99m}Tc(I)$-carbonyl at their His-tag[26]. The efficient complex formation of barnase and barstar in the constructs was demonstrated by analytical gel filtration on a Superdex-75 column (**Fig. 3a**). Characteristic shifts of each complex were observed. The functionality of the 4D5 scFv moiety was demonstrated by gel filtration with antigen (**Figs. 3b–e**), enzyme-linked immunosorbent assay (ELISA) (see **Supplementary Fig. 1** online) and by surface plasmon resonance (BIAcore) measurements on recombinant antigen (**Fig. 4**).

### Homogeneous preparation of oligomeric complexes

We exploited the purification strategy to directly prepare defined oligomeric complexes. We immobilized the 4D5 scFv–barnase-$His_5$ protein or the 4D5 scFv–dibarnase-$His_5$ on a $Ni^{2+}$-NTA column, removed barstar and refolded the fusion proteins *in situ* as above, and subsequently added 4D5 scFv–barstar protein (without His-tag) to bind to the immobilized barnase fusion, followed by extensive washing and final elution (**Fig. 2a**).

This approach allows the use of crude lysates without the need for prior partner purification. We applied to the scFv-barnase and scFv-dibarnase columns 4D5 scFv–barstar in (i) cleared *Escherichia coli* lysate or (ii) solubilized lysate pellet, because the barstar-containing fusion was mostly accumulated as insoluble periplasmic protein. In the latter case, to avoid any dialysis steps, we solubilized the pellet in 6 M guanidine HCl, diluted it 1:200 with PBS buffer and loaded it directly on the barnase fusion affinity columns. In both cases the same homogeneous production of oligomers was achieved. The expression of the 4D5 scFv–barstar fusion (about 2 mg/l) was slightly higher than that of 4D5 scFv–barnase. We used approximately equal volumes of both shake flask cultures to prepare the oligomer, and the overall yield of dimer was ~1 mg/l of each shake flask culture.

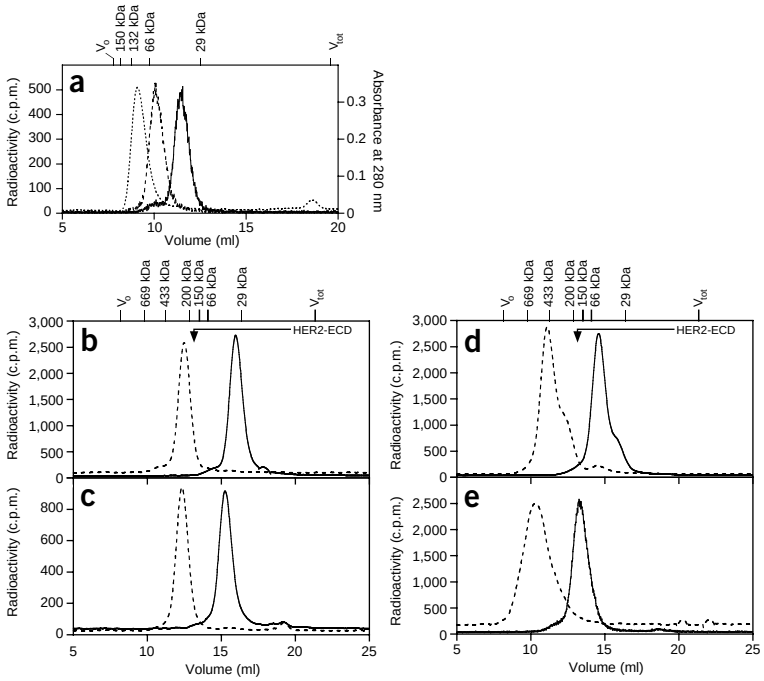


**Figure 3** Assembly of multimeric proteins and their antigen binding. (**a**) Association of 4D5 scFv–barnase-$His_5$ and 4D5 scFv–dibarnase-$His_5$ with their partner 4D5 scFv–barstar fusion. The preparations were separated on a Superdex-75 gel filtration column. In this assay the elution peak of nonlabeled 4D5 scFv–barstar (solid line) was shifted from an apparent molecular weight of 39.9 kDa to 81 kDa when associated with radiolabeled 4D5 scFv–barnase-$His_5$ (dashed line), and to 132 kDa when associated with radiolabeled 4D5 scFv–dibarnase-$His_5$ (dotted line). Calibration of the column was done with radioiodinated bovine serum albumin (66 kDa), its dimer (132 kDa) and IgG (150 kDa). (**b**–**e**) Antigen binding of radiolabeled mono-, di- and trimeric 4D5 scFv–barnase•barstar constructs. The shift in molecular weight was followed on a Superdex-200 gel filtration column when the antibodies were mixed with soluble recombinant $p185^{HER2\text{-}ECD}$ antigen. 4D5 scFv–barnase-$His_5$ (**b**), 4D5 scFV–dibarnase-$His_5$ (**c**), 4D5 scFv–barnase-$His_5$•4D5 scFv–barstar, (dimer) (**d**), 4D5 scFv–dibarnase-$His_5$•$(4D5\ scFv\text{–}barstar)_2$, (trimer) (**e**). Antibody constructs are shown with a solid line, the same constructs after interaction with the antigen with a dashed line. The column was calibrated with radioiodinated carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa), β-amylase (200 kDa), apoferritin (443 kDa) and thyroglobulin (669 kDa). The small shoulder in **d** is most likely a small excess of uncomplexed scFv-barnase (cf., **c**), as no proteolysis is apparent (cf., **Fig. 2b**). The elution volume of $p185^{HER2\text{-}ECD}$ is shown by an arrow. While it runs at higher molecular weight than observed by equilibrium centrifugation[42], this is most likely due to its elongated shape and the limited resolution of this column in this molecular weight range. The observed molecular weight of the complexes are consistent with one molecule $p185^{HER2\text{-}ECD}$ bound in **b** and **c**, two molecules in **d** and three molecules in **e**.



Coomassie staining of SDS-PAGE gels showed no detectable contaminants in any preparation of the affinity-purified proteins (**Fig. 2**). Significant proteolytic activity is normally present in crude *E. coli* lysates. To investigate whether 4D5 scFv–barnase-$His_5$ and 4D5 scFv–dibarnase-$His_5$ fusion proteins are resistant to proteolytic digestion when coupled to a solid support during the purification process, we tested the proteins by SDS-PAGE (**Fig. 2b**) and Western blot analysis, screening with either anti-barstar (**Fig. 2c**) or anti-barnase (**Fig. 2d**) serum. No noticeable degradation was observed even in the absence of protease inhibitors. This result suggested that the immobilized fusion proteins, despite having rather long interdomain linker sequences, were highly stable to proteases and/or that the process removes proteases efficiently. Storing samples over a long period (6 months) did not lead to aggregation or monomer dissociation.

In summary, this affinity procedure resulted in efficient production of milligram quantities of the oligomeric proteins. The barnase•barstar complexes were resistant to concentration-dependent dissociation and aggregation. The essentially irreversible association of barnase and barstar under physiological conditions resulted in homogenous preparations of exact stoichiometric ratios of subunits.

The ability of designed constructs to associate with one, two or three antigen moieties and the correct folding of all scFv moieties in the molecules were directly demonstrated on a Superdex-200 gel filtration column by a gel-shift assay with radiolabeled constructs and purified recombinant $p185^{HER2\text{-}ECD}$ antigen (**Figs. 3b–e**).

## Enhancement of avidity due to multimerization

The most important advantage of multivalent miniantibodies over monovalent ones is the enhancement of specific binding avidity towards antigens. We tested the binding properties of our constructs with purified recombinant $p185^{HER2\text{-}ECD}$ using both ELISA and BIAcore experiments. ELISA plates were coated with $p185^{HER2\text{-}ECD}$ and binding was analyzed in a sandwich ELISA using an anti-His-tag antibody[32] (see **Supplementary Methods** and **Supplementary Fig. 1** online). As every construct or assembly has only a single His tag, the binding signal could be directly compared. All constructs have the same 4D5 scFv moiety and demonstrated specific binding, with the lowest apparent functional affinity for the monomers, intermediate affinity for dimeric complexes and highest functional affinity for the trimers.

The functionality study using BIAcore was designed to evaluate differences in the off-rates of multivalent complexes compared with monovalent 4D5 scFv–barnase and 4D5 scFv–dibarnase fusions. Because we did not want to confirm the monovalent affinity of 4D5 but rather test the effect of multimerization, we coupled $p185^{HER2\text{-}ECD}$ antigen to the sensor chip at the high density of 4,500 resonance units (RU) to allow maximal multivalent contact of the miniantibodies without limitation owing to the distance between $p185^{HER2\text{-}ECD}$ molecules. A high flow rate (30 μl/min) was used to minimize rebinding of whole scFv or miniantibody constructs after dissociation from the antigen. Analysis of all analytes tested was performed under identical conditions and at the same molar concentrations. Because of the intrinsically tight binding of the 4D5 scFv fragment to $p185^{HER2\text{-}ECD}$ (ref. 31), the dissociation was followed for 120 min and revealed a slower dissociation for the dimeric construct (96% of residual binding, **Fig. 4**) and the trimeric construct (98%, **Fig. 4**) compared with the monovalent constructs (84% and 85%), indicating a significant contribution from multivalent binding. These data clearly reflected a gain in avidity for the designed multivalent 4D5 scFv constructs, as anticipated.

## $^{99m}$Tc-radiolabeling and high serum stability of multimers

All constructs were radiolabeled with $^{99m}$Tc-tricarbonyl-trihydrate with the His-tag-specific labeling method[26] to a specific activity of 2 GBq/mg. Immunoreactivity was fully retained (94%–99%) as determined by a gel-shift assay with soluble antigen (**Fig. 3b–e**). Gel-filtration assay of radiolabeled dimer, 4D5 scFv–barnase-$His_5$•4D5

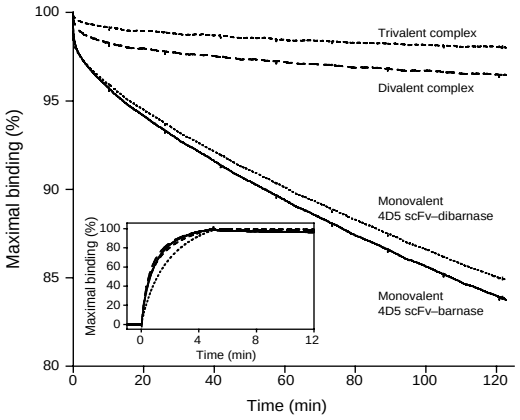


**Figure 4** Comparison of dissociation kinetics of monomeric, dimeric and trimeric 4D5 scFv–constructs measured by surface plasmon resonance (BIAcore). Recombinant $p185^{HER2\text{-}ECD}$ was immobilized on a CM5 sensor chip surface at density of about 4,500 RU to provide the opportunity for multivalent binding, rather than determining the true monovalent dissociation kinetics, and binding was measured at a concentration of 160 nM (mol of protein) for each construct. The time point "0" corresponds to the start of the dissociation phase. The insert shows the initial phase of the association/dissociation sensorgram. Because of the multivalency effect and rebinding at the high coating density needed for this experiment, the dissociation phases are multiphasic and it would clearly not be meaningful to report monophasic apparent $k_{off}$ values. Duplicate injections reproduce the retained signal after 120 min better than within 1% (data not shown), with relative $RU_{120\ min}$ of 84.3% for 4D5 scFv–barnase, 85% for 4D5 scFv–dibarnase, 96.2% for the dimer and 97.8% for the trimer when the RU at time 0 are taken as 100%.

scFv–barstar, and trimer, 4D5 scFv–dibarnase-$His_5$•(4D5 scFv–barstar)$_2$, incubated for 24 h in human sera at 37 °C and in PBS at 4 °C, revealed no sign of aggregation or dissociation, indicating a high thermal and serum stability (data not shown).

### Pharmacokinetics and tumor localization

To test whether our multivalent molecules have improved *in vivo* properties, we carried out biodistribution studies in xenografted mice by intravenous injection of labeled construct. All three constructs displayed bi-exponential time/activity curves with a fast equilibration rate and a subsequent elimination phase. The blood clearance profiles of the constructs showed half-lives of 1 min for the monomeric, 6 min for the dimeric and 49 min for the trimeric construct for the respective fast alpha phase, with slow beta (elimination) phases of 35, 135 and 225 min for the monomeric, dimeric and trimeric constructs, respectively. The organ distribution was investigated in nu/nu mice xenografted with SK-OV-3 and SK-BR-3 cells highly expressing HER2/neu antigen. At the tumor site, the higher the degree of multimerization, the greater the percentage accumulation of injected dose per gram of tissue at 24 h: 2% for monomer, 6% for dimer and 10% for trimer; whereas, in kidney, the higher the degree of multimerization, the lower the percentage accumulation: 140% for monomer, 56% for dimer and 21% for trimer. After 48 h no substantial decrease of tumor accumulation for dimer and trimer occurred, and tumor-to-blood ratios of 27 were reached (**Table 1**).

## DISCUSSION

The strategy presented here is in principle applicable to any protein that can be attached in functional form to the barstar and barnase molecules. It seems particularly well suited to the production of heterooligomeric constructs because the extremely specific barnase-barstar interaction reliably eliminates mispairing problems. The linking of proteins and components of the module via the flexible hinge peptide allows rotational and segmental flexibility, enhancing the simultaneous binding of different receptor molecules.

The proposed approach has several advantages over current techniques. First, it allows effective purification of protein multimers (**Fig. 2a**). In the first step one partner—for instance, the barnase fusion—is bound to the support via a function that does not dissociate during subsequent affinity chromatography. A $Ni^{2+}$-NTA matrix is convenient for immobilizing the His-tagged barnase. The intermittent exposure to denaturing conditions, needed to remove barstar, also

**Table 1 Biodistribution of $^{99m}$Tc-labeled mono-, di- and trimeric 4D5 scFv–barnase•barstar constructs in nu/nu mice[a]**

| | Percentage of injected dose per gram of organ | | | | | |
|---|---|---|---|---|---|---|
| Organ | Monomer | Dimer | Trimer | Monomer | Dimer | Trimer |
| | 24 h | | | 48 h | | |
| Blood | 0.09 ± 0.01 | 0.57 ± 0.17 | 0.88 ± 0.15 | 0.05 ± 0.01 | 0.20 ± 0.04 | 0.30 ± 0.04 |
| Heart | 0.20 ± 0.07 | 1.84 ± 0.34 | 2.47 ± 0.62 | 0.14 ± 0.01 | 1.16 ± 0.25 | 1.39 ± 0.24 |
| Lung | 0.31 ± 0.07 | 1.25 ± 0.28 | 2.54 ± 0.30 | 0.17 ± 0.01 | 0.81 ± 0.03 | 1.19 ± 0.12 |
| Spleen | 0.24 ± 0.21 | 2.90 ± 0.58 | 4.91 ± 1.14 | 0.46 ± 0.07 | 3.64 ± 1.72 | 5.00 ± 1.28 |
| Kidney | 140.63 ± 2.83 | 56.43 ± 7.64 | 21.16 ± 0.72 | 106.00 ± 6.04 | 40.49 ± 6.47 | 15.95 ± 0.98 |
| Stomach | 0.31 ± 0.11 | 0.80 ± 0.17 | 0.91 ± 0.26 | 0.18 ± 0.05 | 0.48 ± 0.15 | 0.33 ± 0.13 |
| Intestine | 0.31 ± 0.01 | 1.15 ± 0.03 | 1.73 ± 0.38 | 0.15 ± 0.01 | 0.97 ± 0.25 | 1.00 ± 0.10 |
| Liver | 1.61 ± 0.21 | 4.56 ± 0.42 | 14.29 ± 0.51 | 1.04 ± 0.06 | 3.44 ± 0.43 | 12.92 ± 2.80 |
| Muscle | 0.18 ± 0.05 | 0.57 ± 0.09 | 0.72 ± 0.15 | 0.08 ± 0.01 | 0.26 ± 0.07 | 0.46 ± 0.11 |
| Bone | 0.16 ± 0.11 | 0.67 ± 0.49 | 1.71 ± 0.59 | 0.12 ± 0.10 | 0.54 ± 0.58 | 1.06 ± 0.18 |
| SK-OV-3 | 0.97 ± 0.32 | 3.43 ± 1.01 | 7.04 ± 2.35 | 0.47 ± 0.16 | 3.34 ± 0.44 | 4.56 ± 1.00 |
| SK-BR-3 | 1.91 ± 0.79 | 5.91 ± 2.30 | 9.80 ± 0.82 | 0.65 ± 0.20 | 5.38 ± 1.39 | 8.11 ± 0.41 |
| T/B SK-OV-3 [b] | 10.80 ± 4.70 | 6.01 ± 3.56 | 8.00 ± 4.03 | 9.41 ± 5.08 | 16.70 ± 5.54 | 15.20 ± 5.32 |
| T/B SK-BR-3 [b] | 21.20 ± 11.05 | 10.37 ± 7.12 | 11.10 ± 2.83 | 13.00 ± 6.60 | 26.9 ± 12.33 | 27.03 ± 4.96 |

[a]Biodistributions of $^{99m}$Tc labeled constructs were studied in xenografted nu/nu mice ($n = 3$) 24 h and 48 h after injection of the radiolabel into the animal. The results express the mean ± s.d. SK-OV-3 and SK-BR-3 are xenografted tumors on the left and right flank of the mice. Monomer, 4D5 scFv–barnase-$His_5$; dimer, 4D5 scFv–barnase-$His_5$•4D5 scFv–barstar; and trimer, 4D5 scFv–dibarnase-$His_5$·(4D5 scFv-barstar)$_2$. [b]T/B, tumor/blood ratio.

removes a large fraction of all impurities. Then, the second partner, a barstar fusion, is added to the column and associates with the first one, forming in an exact stoichiometric ratio oligomers that are then eluted by imidazole. The high affinity of the barnase-barstar pair makes it possible to obtain a homogeneous preparation of multimers directly from very crude cell extracts in one step. Although the method requires an unfolding and refolding step, many proteins can be reversibly unfolded and refolded on a solid support as done here.

Second, the barnase•barstar complex can be reliably used both *in vitro* and *in vivo*. In contrast, multimeric scFvs (diabodies, triabodies and tetrabodies) based on shortened linkers between V-domains often exist as an equilibrium mixture in which subunits can dissociate, shuffle and realign[3]. Furthermore, this strategy is not applicable to non-scFv molecules.

Third, unlike other oligomerizing proteins and peptides, the barnase and barstar constructs can also exist in monovalent form, allowing a controlled association process. The interaction is precisely 1:1, and both partners by themselves are not aggregation prone. The trimeric miniantibody based on an engineered barnase dimer presents an example of a higher order oligomer design.

Finally, the heterodimeric nature of the barnase-barstar approach allows a robust preparation of both homo- and heterooligomers, unlike most other approaches (refs. 1–3 and references contained therein).

The improvement of tumor retention properties when going from monomers to dimers or trimers or tetramers has been reviewed[2] and documented in several studies[23,33–35]. It is difficult to compare the merit of different formats when different antigens are used because of differences in antigen expression on the tumor and in normal tissues. However, when the values are compared with anti-HER2 diabodies, comparable or somewhat better results are obtained with the present strategy for both total tumor accumulation and the tumor-to-blood ratio[34]. About twofold higher tumor accumulations were seen in anti–carcinoembryonic antigen models with bivalent[33] or trivalent[35] molecules.

An increase in the size of the molecules above the kidney filtration threshold will lead to increased accumulation of radiometal-labeled metabolites in the liver, as this becomes the organ of catabolism. This is consistent with similar observation in other systems[23,33,36]. It should be noted that the observed kidney and liver accumulation is also a function of the labeling strategy used and depends on whether the label, after uptake and degradation of the protein molecules by renal or hepatic cells, is retained or excreted again[37]. The $^{99m}$Tc–tricarbonyl strategy does not lead to resecretion of the $^{99m}$Tc label and thus faithfully documents the history of the protein molecules.

Taken together, our data show that the barnase•barstar module constitutes a useful tool for the design of oligomeric proteins for basic research as well as for imaging and therapeutic applications.

## METHODS

**Molecular modeling.** The models are based on the X-ray structures of humanized anti-p185$^{HER2-ECD}$ antibody 4D5 version 8 (PDB entry 1FVC, 2.2 Å resolution)[22] and the structure of the barnase•barstar complex (PDB entry 1BGS, 2.6 Å resolution)[20]. The flexible tags, linkers and hinge residues were modeled as β-strands and shaped by simulated melting and annealing using the Discover module of the InsightII molecular modeling suite (MSI/Biosym).

**Construction of expression plasmids.** The plasmids for the periplasmic expression of scFv-barnase and barstar fusion proteins are based on the vector pIG6-4D5 (refs. 23,38,39) and the barstar and barnase genes were amplified out of the plasmid pMT413 (ref. 40). The details are given in the **Supplementary Methods** online.

**Production of proteins.** Freshly transformed *E. coli* SB536 cells[41] ($F^-$, WG1, Δ*fhu*A (*ton* Δ), Δ*hho*AB (SacII), *shh*) were grown in Super Broth medium containing 0.1 g/l ampicillin and the *lac* promoter was induced with 1 mM IPTG at an $OD_{550}$ of 0.7. Expression was allowed to continue for 16 h at 27 °C. Cells were harvested by centrifugation at 4,000*g* for 15 min at 4 °C. Cell extracts were prepared by French press lysis at 10,000 p.s.i. and cleared by centrifugation at 30,000*g* for 1 h. The supernatants were applied to a $Ni^{2+}$-NTA column (Qiagen) according to the manufacturer's instructions. The immobilized proteins were denatured during ~1 h with 6 M guanidine HCl in PBS, pH 8.0, and then refolded during 7 h *in situ* using a linear gradient from 6 to 0 M guanidine HCl in the same buffer, washed with 25 mM imidazole and eluted with 200 mM imidazole. For final purification of 4D5 scFv–barnase-$His_5$ and 4D5 scFv–dibarnase-$His_5$, elution fractions were directly applied onto a Protein-A Sepharose column (Pharmacia) and eluted with 0.1 M sodium citrate, pH 3.5; the eluate was immediately neutralized with 1 M Tris. For preparation of dimeric and trimeric complexes, 4D5 scFv–barnase-$His_5$ or 4D5 scFv–dibarnase-$His_5$, respectively, were immobilized on the $Ni^{2+}$-NTA column and saturated with cleared cell lysate containing 4D5 scFv–barstar that had been diluted 1:100 with PBS. Alternatively, the insoluble periplasmic 4D5 scFv–barstar was solubilized with 6 M guanidine HCl in PBS, pH 8, centrifuged at 30,000*g* for 30 min and diluted 1:200 with PBS, pH 8, and added to the column. After extensive washing with PBS containing 25 mM imidazole and 1 M NaCl, the resulting complexes were eluted with PBS containing 200 mM imidazole and 300 mM NaCl and finally dialyzed against PBS containing 300 mM NaCl and 10% glycerol. Molecular weights were confirmed by matrix-assisted laser desorption ionization/mass spectrometry: 4D5 scFv–barstar, 38,830 Da (39,109 theor.); 4D5 scFv–barstar-$His_5$ 39,880 Da (39,885 theor.); 4D5 scFv–barnase-$His_5$, 42110 Da (42,120 theor.); and 4D5 scFv–dibarnase-$His_5$, 54,060 Da (54,074 theor.).

**Surface plasmon resonance biosensor.** Measurements were performed with a BIAcore instrument (BIACORE 3000). Recombinant p185$^{HER2-ECD}$ was coupled onto a CM5 chip at a density of 4,500 RU by standard amine coupling chemistry. All proteins were used at the same concentration, 160 nM in PBS, pH 7.4, with 0.005% Tween-20. The sensorgrams were obtained at a flow rate of 30 µl/min at 15 °C and the dissociation phase was followed for 120 min.

**SDS-PAGE and western blot analysis.** SDS-PAGE analyses were performed under reducing conditions according to standard protocols using 12% and 18% polyacrylamide gels. Immunoblots on Immobilon-P transfer membrane (Millipore) were carried out according to the manufacturer's instructions using rabbit anti-barstar serum (kindly provided by R.W. Hartley, National Institutes of Health, Bethesda, Maryland, USA) followed by a goat anti-rabbit IgG peroxidase conjugate (Sigma) for detection. The blots were visualized with chemiluminescent Pierce Supersignal ECL reagent (Pierce, USA). The same membrane was reprobed after stripping for immunodetection with the rabbit anti-barnase serum (a gift of R.W. Hartley). Stripping was performed in 100 mM 2-mercaptoethanol, 2% SDS, 65 mM Tris-HCl, pH 7.0, at 60 °C for 1 h.

**$^{99m}$Tc radiolabeling conditions and stability studies.** All constructs were concentrated to 5–10 µM protein concentration by centrifugation with a 10 kDa cutoff membrane (Ultrafree-MC, Millipore). Constructs were mixed 1:1 with freshly synthesized $^{99m}$Tc-tricarbonyl trihydrate, as described[26]. Labeling efficiency was >95%. Labeled constructs were incubated with human sera 1:10 at 37 °C and analyzed on a gel filtration column (Superdex-75), connected to a UV-monitor and a high-performance liquid chromatography (HPLC) radioactivity monitor (LB 508, Berthold). The elution profiles were compared to those of the initial constructs.

**Immunoreactivity and biodistribution studies.** For immunoreactivity studies, recombinant p185$^{HER2-ECD}$ antigen (10 µg) was mixed with $5 \times 10^4$ Bq (30 ng) of labeled construct for 30 min at 25 °C and separated on a Superdex-200 gel filtration column. The bioreactivity was defined as percentage of counts eluting with the higher molecular weight compared to those not shifting in molecular weight (analytical gel-shift assay). Blood clearance studies were performed in xenografted nu/nu mice. They were injected with 10 µg (22 MBq) of $^{99m}$Tc-labeled constructs in 100 µl PBS. Blood samples were taken at 7.5, 15, 30, 60,

120 and 240 min after injection. For organ distribution, animals were killed at 24 h and 48 h after injection, and tissues were collected and measured in a gamma counter. For the tumor targeting study, groups of three mice each were xenografted with $10^7$ SK-OV-3 cells (no. HTB77, American Type Culture Collection (ATCC)) on the left side and with $10^7$ SK-BR-3 cells (no. 3HTB30, ATCC) on the right side and were injected with the radiolabeled constructs 2 weeks later, when the tumors had reached a size of about 40 mg.

*Note: Supplementary information is available on the Nature Biotechnology website.*

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**COMPETING INTERESTS STATEMENT**

The authors declare that they have no competing financial interests.

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