# Antigen binding and stability properties of non-covalently linked anti-CD22 single-chain Fv dimers

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**Abstract** **By varying linker length and domain orientation three multivalent derivatives of a monovalent anti-CD22 single-chain fragment variable (scFv) antibody were generated. Shortening the linker of the $V_H$–$V_L$ oriented scFv to 5 or 0 residues resulted in the formation of diabodies or a mixture of tetramers and trimers, respectively. Unexpectedly, a $V_L$–0–$V_H$ scFv assembled to homogenous dimers, remained substantially more stable than the $V_H$–5–$V_L$ diabody when incubated in human serum at 37 °C, and retained its dimeric state when concentrated up to 4 mg/ml. These properties suggest the $V_L$–0–$V_H$ scFv could become an attractive vehicle for the selective delivery of multiple effector molecules to $CD22^+$ tumor cells.**


*Keywords:* CD22; Single chain Fv; Diabody; Dimer; Stability

## 1. Introduction

Single-chain fragment variable (scFv) antibodies have become a popular format of engineered antibodies. They have gained substantial clinical interest by their use as vehicles to deliver diagnostic and therapeutic agents such as radionuclides, enzymes, or toxins, selectively to tumor target cells [1–3]. Although monovalent scFvs can penetrate solid tumor tissue more efficiently than whole antibodies [4] they are cleared from the circulation within minutes after administration and are poorly retained at tumor sites [4–7]. These properties particularly limit therapeutic applications of such reagents.

To increase the size and apparent binding affinity of monovalent scFvs, they can easily be engineered into oligomeric species through manipulation of the linker peptide connecting the variable domains. Holliger and colleagues pioneered the construction of a bivalent molecule by shortening the linker peptide to five residues, which prevents pairing of the variable domains on the same polypeptide chain but promotes association of two molecules to a bivalent diabody [8]. Further shortening the linker peptide below three residues was shown to result in the formation of trimeric, tetrameric, or larger aggregate species [9–12]. More recently, the formation of $V_H$–$V_L$ oriented scFv antibodies with <3 residue linkers to predominantly dimeric molecules has been described [13,14]. Several studies have shown that in comparison with monovalent counterparts, multivalent scFv fragments exhibited increased functional antigen binding affinity [12,15], favorable in vivo retention within tumor tissue [7,16,17], and markedly slower clearance from the bloodstream [6,18]. These properties suggest multivalent scFv fragments to be excellent candidates for the fusion with small antineoplastic effector molecules.

The success of scFv antibodies in clinical applications further relies on sufficient thermal stability, because it was shown that only stable scFv molecules enriched at xenografted tumors in severe combined immunodeficiency mice [19]. We recently described the generation of the highly stable scFv fragment MJ-7 ($V_H$–15–$V_L$) [20] that derived from the clinically established, internalizing anti-CD22 monoclonal antibody (mAb) LL2 [21]. This construct, obtained after rational mutagenesis of three destabilizing residues within the variable region from antibody heavy chain ($V_H$) domain core structure, retained similar antigen binding properties as the wild-type scFv but exhibited several orders of magnitude greater stability when incubated in human serum at 37 °C.

To exploit the described advantages of using multimeric scFvs as therapeutic targeting molecules, we aimed this study at the construction of stable multimeric derivatives of the monovalent anti-CD22 scFv MJ-7.

## 2. Material and methods

### 2.1. Construction of multivalent anti-CD22 antibodies

The cDNA encoding the previously constructed anti-CD22 scFv MJ-7 [20] was used to generate scFv multimers. A bivalent diabody (MLD-6) was constructed by shortening the 15 amino acid linker between the $V_H$ and variable region from antibody light chain ($V_L$)

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*Abbreviations:* scFv, single-chain Fv antibody; Fv, fragment variable; $V_H$, variable region from antibody heavy chain; $V_L$, variable region from antibody light chain; mAb, monoclonal antibody; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis; $K_D$, binding affinity equilibrium constant; IMAC, immobilized metal affinity chromatography

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domain to a $Gly_4Ser$ five-residue linker. To obtain scFv fragment MLT-1 with the $V_H$ domain directly linked to the $V_L$ domain, the $V_H$ domain of scFv MJ-7 was PCR amplified with flanking restriction sites *Nco*I and *Eco*RV. The *Nco*I/*Eco*RV digested PCR fragment was ligated into cleaved bacterial expression plasmid pHOG21/MJ-7 [20]. For cloning of scFv MLT-7 with variable domains directly joined in the opposite orientation ($V_L$–0–$V_H$), the variable domain encoding genes were PCR amplified in separate reactions and assembled by overlap extension PCR as described in [22]. The resulting PCR fragment was cloned into the *Nco*I/*Bam*HI restricted plasmid pHOG21/MJ-7. Correct sequences of scFv encoding fragments were verified by automated DNA sequencing.

### 2.2. Expression and purification of scFv multimers

ScFv fragments were expressed using the *E. coli* strain TG1 (Stratagene, La Jolla, CA), isolated from the periplasm and purified by immobilized metal affinity chromatography (IMAC) as previously described [20]. Affinity purified scFv fragments were fractionated by size-exclusion chromatography using either a calibrated Superdex 75 HR 10/30 column (Amersham Pharmacia, Piscataway, NJ) or a Superdex 200 HR 10/30 column (Amersham Pharmacia) in phosphate buffered saline and 50 mM imidazole, pH 7.4, with a flow rate of 0.3 ml/min. The purity of size-fractionated antibodies was monitored by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions after staining with Simply Blue™ Safe Stain (Invitrogen, Carlsbad, CA). The specificity of eluted fractions was determined by Western blot analysis using an anti-(c-myc)-peroxidase conjugated mAb (Roche, Indianapolis, IN) followed by chemiluminiscent detection (ECL Plus, Amersham Pharmacia).

### 2.3. Cell binding measurements

Specific binding of the constructs was determined by flow cytometry using the human $CD22^+$ B cell lines Raji and Daudi (American Type Culture Collection, Manassas, VA) and the $CD22^-$ T cell lines Jurkat and HUT102 (American Type Culture Collection) as control. Staining was performed as previously described [20]. Stained cells were analyzed on a FACScan Flow Cytometer (BD Bioscience, San Jose, CA) and the median fluorescence intensity (MFI) was calculated using the CellQuest™ software (BD Bioscience). Binding affinity constants ($K_D$) were measured as previously described [20,23].

### 2.4. Biophysical stability analysis

At a concentration of 20 μg/ml, scFv fragments were incubated in 90% human serum at 37 °C for up to 7 days. Samples were taken at different time points and frozen at −20 °C until the end of the experiment. Samples were subsequently analyzed for binding activity to $CD22^+$ Raji cells by flow cytometry.

## 3. Results

### 3.1. Construction of scFv multimers

To create multivalent scFv molecules, three scFv constructs differing in linker length and orientation of the variable domains were made. The stable LL2-derived scFv MJ-7 [20], in which the $V_H$ domain is separated from the $V_L$ domain by a 15 amino acid residue linker, was used as a template (Fig. 1). In order to produce a bivalent diabody [8], the $V_H$–$V_L$ orientation of the parental scFv MJ-7 was preserved and the linker shortened to five amino acids (MLD6, Fig. 1). To direct scFv association into multimers with more than two binding sites [9–11,24], the variable domains of scFv MJ-7 were directly connected to each other both in $V_H$–0–$V_L$ (scFv MLT-1) and reversed orientation (scFv MLT-7) (Fig. 1). ScFv genes were fused at their C-terminal ends with a c-myc and a hexahistidine epitope tag for detection and purification purposes, respectively.

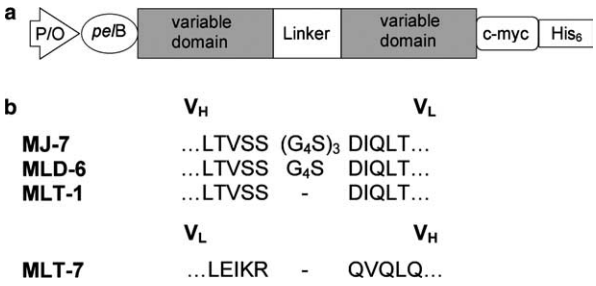


Fig. 1. Schematic illustration of anti-CD22 scFv constructs. (a) ScFv expression cassette with locations of lac promoter/operator (P/O); *pel*B leader sequence (*pel*B); c-myc tag (c-myc) and hexahistidine tag ($His_6$). (b) Variable domain orientation ($V_H$, $V_L$) with linker peptide and amino acid sequences of variable domain junction for each construct.

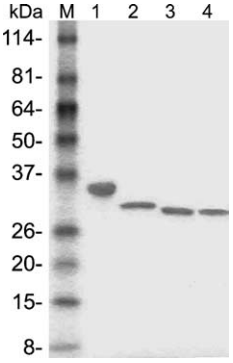


Fig. 2. SDS gel electrophoresis of affinity-purified scFv fragments under reducing conditions. Lanes: M, molecular weight markers; 1, MJ-7 ($V_H$–15–$V_L$); 2, MLD-6 ($V_H$–5–$V_L$); 3, MLT-7 ($V_L$–0–$V_H$) and 4, MLT-1 ($V_H$–0–$V_L$). The gel was stained with Simply Blue™ Safe Stain.

### 3.2. Expression, purification and biophysical analyses

All antibody fragments were produced as soluble proteins in *E. coli* and purified from the bacterial periplasm by IMAC. Affinity purified scFv fragments migrated as single bands with an expected molecular mass (27–30 kDa) on a reducing SDS–PAGE gel (Fig. 2).

To examine the multimerization behavior of the scFv fragments, size exclusion chromatography was performed. ScFvs MJ-7 and MLD-6, containing either 15 or 5 amino acid linkers, respectively, were analyzed on a calibrated Superdex 75 column (Fig. 3(a)). ScFv MJ-7 eluted in two peaks (12.5 ml and 10.9 ml) corresponding to monomers (29 kDa; 63%) and dimers (54 kDa; 37%), respectively (Fig. 3(a), Table 1). MLD-6 showed a predominant peak at an elution volume of 11 ml corresponding to a diabody (96%) and an additional small peak indicating tetramers (Fig. 3(a), Table 1). Gel filtration on a calibrated Superdex 200 column of the fragments with directly linked variable domains revealed for the $V_H$–0–$V_L$ construct MLT-1 a heterogeneous mixture of molecules with apparent molecular masses corresponding to trimers (84 kDa; 39%) and tetramers (112 kDa; 61%) (Fig. 3(b), Table 1). MLT-7 ($V_L$–0–$V_H$) showed an unexpected elution profile with a major peak (95%) eluting at 15.4 ml corresponding to an apparent molecular mass of a dimeric scFv fragment and only a small proportion (5%) of tetramers eluting at 13.7 ml (Fig. 3(b), Table 1). MLT-1 formed a non-separable equilibrium between trimers and tetramers and was therefore excluded from further investigation.

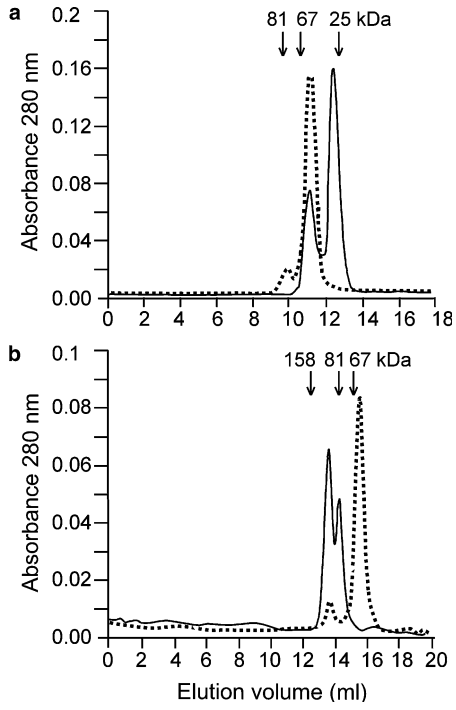


Fig. 3. Size-exclusion chromatography of affinity purified anti-CD22 scFvs. Superimposed are elution profiles from a (a) Superdex 75 gel filtration column of scFv MJ-7 (solid line) and diabody MLD-6 (dashed line) and (b) Superdex 200 gel filtration column of zero-linker constructs MLT-1 (solid line) and MLT-7 (dashed line). Elution peaks and molecular weight of calibration reference proteins are indicated.

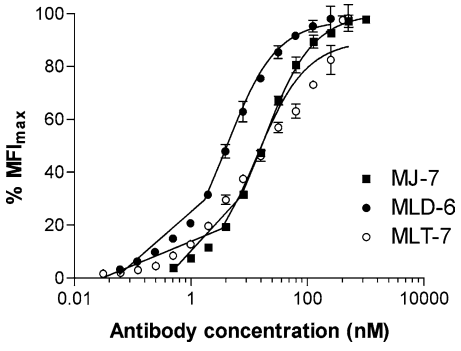


Fig. 4. Equilibrium binding curves of anti-CD22 antibody fragments. Binding activity to $CD22^+$ Raji cells of scFv monomer MJ-7 and dimeric scFvs MLD-6 and MLT-7 was determined by flow cytometry and is shown as median fluorescence intensity (MFI) with subtracted background fluorescence. Bars represent standard deviation.

### 3.3. Immunoreactivity and antigen affinity

Flow cytometry analysis revealed specific binding of all scFv fragments to the $CD22^+$ cell lines Raji and Daudi and no binding to the $CD22^-$ cell lines Jurkat and HUT102 (data not shown). Affinity constants of the antibody fragments for binding to $CD22^+$ Raji cells were determined by flow cytometry. All three scFv fragments showed an antibody concentration-dependent increase in fluorescence intensity (Fig. 4). Fitting the data from the equilibrium-binding curves into the non-linear regression model according to the Levenberg–Marquard method revealed a 3.5-fold higher apparent affinity for the diabody MLD-6 than the parental scFv MJ-7 (Table 1). Diabody MLD-6 retained the same apparent affinity as the murine mAb LL2 [20] ($K_D$ 4.8 nM vs. 4.7 nM). In contrast, the zero-linker dimer MLT-7 bound $CD22^+$ Raji cells with an apparent affinity similar to that of the monovalent scFv MJ-7 (Table 1).

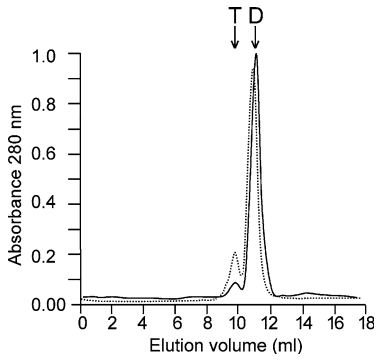


Fig. 5. Size exclusion chromatography of dimeric scFv fragments on a calibrated Superdex 75 column. Superimposed are elution profiles of concentrated MLT-7 (4 mg/ml; solid line) and MLD-6 (2.8 mg/ml: dotted line). (D) dimers, (T) tetramers.

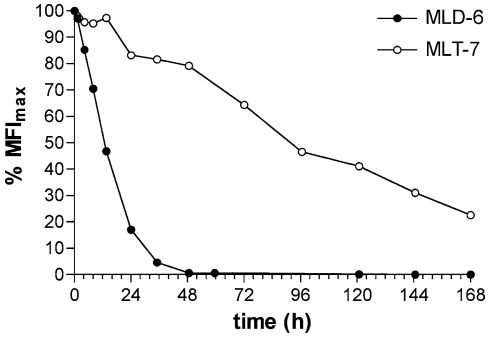


Fig. 6. Serum stability of scFv dimers. Immunoreactivity of dimeric scFv fragments MLD-6 and MLT-7 with $CD22^+$ Raji cells was determined after incubation in human serum at 37 °C at indicated time points.

### 3.4. Stability of dimeric scFvs

The oligomeric status of scFvs can strongly be influenced by the protein concentration. Therefore, following IMAC purification and gel filtration, dimeric MLD-6 and MLT-7 were concentrated to >1 mg/ml and further characterized by size exclusion chromatography using a calibrated Superdex 75 column. About 18% of the purified diabody MLD-6 converted to tetramers when concentrated to 2.8 mg/ml (Fig. 5). In contrast, a defined dimeric status was kept for MLT-7 at protein concentrations >1 mg/ml and only at the highest tested protein concentration of 4 mg/ml a small fraction of tetramers (~4%) was observed (Fig. 5).

The thermostability of the dimeric molecules MLD-6 and MLT-7 was analyzed. Diabody MLD-6 ($V_H$–$G_4S$–$V_L$) lost 50% of its initial binding activity after only 12 h incubation in human serum at 37 °C and completely lost binding activity after 48 h incubation time (Fig. 6, Table 1). In contrast, the dimeric molecule MLT-7 ($V_L$–0–$V_H$) remained fully active after a 12 h incubation period, exhibited an 8-fold greater half life

Table 1

| Variant | Format (%)[a] | | | | $K_D$ (nM)[b] | $t_{1/2}$ (h)[c] |
|---|---|---|---|---|---|---|
| | Monomer | Dimer | Trimer | Tetramer | | |
| MJ7 | 63 | 37 | | | 16.6 | >144 |
| MLD6 | | 96 | | 4 | 4.8 | 12 |
| MLT1 | | | 39 | 61 | n.d. | n.d. |
| MLT7 | | 95 | | 5 | 18.7 | 96 |

[a] Deduced from size exclusion chromatography.
[b] Equilibrium constants ($K_D$) deduced from binding to tumor cells measured by fluorescence cytometry.
[c] Determined from binding activity to tumor cells after incubation in human serum at 37 °C.

than diabody MLD-6 and retained specific binding even after 7 days (Fig. 6, Table 1).

## 4. Discussion

The purpose of this study was to generate and characterize multivalent scFv antibody derivatives from a stable anti-CD22 scFv fragment, previously obtained by rational mutagenesis of three destabilizing $V_H$ common core residues [20]. The most straightforward method to generate a multivalent scFv is to shorten the variable domain connecting linker peptide, thereby allowing for the non-covalent association of multiple polypeptide chains to a dimeric or multimeric molecule [8–12,24,25]. In addition to the linker length, the orientation of the variable domains was shown to impact the multimerization behavior of the polypeptide chains [9,10], an effect largely attributed by the asymmetry of variable light chain and heavy chain domains [26,27]. We therefore made several scFv variants differing in both linker length and domain orientation. Consistent with reports of other scFvs generated in the same domain orientation and with the same 15 amino acid linker, the monovalent scFv MJ-7 exhibited an inherent tendency to form dimers [8,10]. Likewise, shortening the linker of the scFv MJ-7 in this orientation to five residues ($Gly_4Ser$) resulted in the almost exclusive formation of bivalent diabodies [8,10]. The deduced functional affinity of the MLD-6 diabody was similar to that determined for the parent mAb LL2, reflecting the gain in avidity due to dimerization and capability of the molecule for simultaneous binding to two epitopes.

It has been described that further shortening the linker to less than three residues promotes the formation of molecules with three or four antigen binding sites, ''triabodies'' or ''tetrabodies'', respectively [28]. Linker dependent oligomerization behavior of the murine anti-neuraminidase scFv NC10 [29] has been studied in detail. Joining $V_H$S112 to $V_L$D1 of scFv NC10 by 3–0 residues resulted in a precise transition from dimers into trimers [25]. Another study examining the linker-length dependent oligomerization behavior of the HD37 derived anti-CD19 scFv in $V_H$–$V_L$ orientation reported an exclusive trimer formation by fusing $V_H$S112 to $V_L$D1 and an unexpected exclusive tetramer conformation when $V_H$S113 was ligated to $V_L$D1 [12]. When joining $V_H$S113 to $V_L$D1 of an OKT-3 derived scFv, however, formation of 40% dimers and 60% trimers was observed [14]. In the present study we observed an equilibrium between trimers and tetramers when joining $V_H$S113 to $V_L$D1 (scFv MLT-1), indicating that linker dependent oligomerization behavior of scFv fragments may also be dictated by the particular antibody $V_H/V_L$ sequence.

Because the distance between the carboxyl terminus end of $V_L$ and the amino terminus of $V_H$ is greater than that for the opposite orientation, it has been discussed that $V_L$–$V_H$ orientated scFvs are more constrained than $V_H$–$V_L$ oriented fragments when connected by the same linker and therefore tend to exhibit a higher tendency to form higher molecular weight oligomers [30]. Experimental evidence for this assumption has been presented for scFv NC10 by demonstrating that direct linkage of the variable domains in $V_L$–$V_H$ orientation resulted in the predominant formation of tetrabodies [9], whereas in reversed orientation formation of trimers was described [10]. Thus, the association of scFv MLT-7 ($V_L$–0–$V_H$) to homogeneous dimers in the present study was unexpected. To our knowledge, the exclusive formation of a zero linker scFv construct in $V_L$–0–$V_H$ orientation to a dimer has not yet been described, further confirming the notion that linker length dependent scFv multimerization may substantially vary among different antibodies and thus cannot reliably be predicted.

Despite its bivalency, and in contrast to the $V_H$–5–$V_L$ oriented diabody MLD-6, the $V_L$–0–$V_H$ dimer MLT-7 bound to the target antigen with a similar apparent affinity as the monovalent counterpart MJ-7. Possible explanations for this phenomenon could be incorrect association of one pair of the VL/VH domains, inability of the dimeric molecule to span the distance for simultaneously binding to two epitopes, or binding of both antigen binding sites but each with reduced intrinsic affinity due to steric constraints of the molecule.

Despite its weaker binding affinity, the $V_L$–0–$V_H$ dimer MLT-7 exhibited a markedly superior thermostability over the $V_H$–5–$V_L$ oriented diabody MLD-6 and showed no tendency to form higher molecular weight species even at concentrations of up to 4 mg/ml. For clinical applications, this is a most important property because even highly robust monovalent scFvs were shown to multimerize especially at concentrations >1 mg/ml [31]. The formation of stable dimers may indicate that the $V_L$–0–$V_H$ MLT-7 interface may structurally be less constrained than that of the $V_H$–5–$V_L$ diabody MLD-6. To elucidate the structural basis for this phenomenon on a molecular level, the scFv fragment will be crystallized within the near future.

In conclusion, we have generated a scFv without a linker in $V_L$–0–$V_H$ orientation that associates to a highly stable dimeric molecule. We expect that this antibody may become an attractive targeting moiety for the subsequent generation of novel

immunotherapeutic reagents for $CD22^+$ B-cell Non-Hodgkin's lymphoma.

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