

# Novel tetravalent and bispecific IgG-like antibody molecules combining single-chain diabodies with the immunoglobulin $\gamma$ 1 Fc or CH3 region

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**Abstract** Although bispecific IgG molecules have been successfully applied for antibody-mediated immunotherapy of tumours, applicability is hampered by the difficulties associated with their generation. In the present study, we have used a bispecific single-chain diabody (scDb) directed against carcinoembryonic antigen and *Escherichia coli*  $\beta$ -galactosidase as a model to generate bispecific IgG-like antibody molecules. We show that the fusion of this single-chain diabody to the Fc (scDb-Fc) or CH3 (scDb-CH3) region of the human immunoglobulin  $\gamma$ 1 chain results in the expression of dimeric fusion proteins exhibiting four functional antigen binding sites with increased functional affinity. This strategy represents a new and convenient way to generate IgG-like multivalent and bispecific molecules that are efficiently secreted from mammalian cells.

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**Key words:** Single-chain diabody; Bispecific antibody; Immunoglobulin constant domain; Dimerization; Functional affinity

## 1. Introduction

Bispecific IgG molecules have been successfully used for antibody-mediated immunotherapy of tumours [1]. For example, anti-tumour responses were observed in various clinical trials administering bispecific antibodies directed against a tumour antigen and receptor molecules on cytotoxic or phagocytotic effector cells [2–8].

Bispecific antibodies are routinely generated by the hybrid-hybridoma technology [9]. However, hybrid-hybridomas produce a substantial number of undesired molecules due to random light and heavy chain association [10]. Recently, Merchant and co-workers [11] presented a strategy to generate bispecific IgG molecules by introducing a knob-into-hole structure and engineered cysteine bridges in the CH3 domain of human IgG. Although this led to the preferential formation of heavy chain heterodimers, the light chains of the two antibody specificities can bind to either heavy chain.

Alternatively, bispecific antibody molecules can be produced by recombinant techniques (for review see [12]). For example, small bispecific antibody fragments can be assembled as diabodies from the variable domains of two antibodies expressing two polypeptide chains in the format VHA-VLB and VHB-VLA in the same cell [13]. Since the linkers within these fragments are too short to allow intrachain assembly of the VH and VL domains (single-chain Fv frag-

ments; scFv), two fragments assemble into a dimeric molecule (diabody; Db). The expression can be further simplified by joining these two fragments with an additional linker generating a monomeric single-chain diabody molecule (scDb) with improved stability [14]. These scDb molecules are expressed in functionally active form in bacterial as well as mammalian cells. Furthermore, active scDb can be displayed on the cell surface or expressed as intrabodies in the secretory pathway [15].

In lacking the constant regions, recombinant antibody fragments also lack the associated effector functions and are rapidly cleared from serum due to their small size [16]. However, certain therapeutic applications require an IgG molecule with its Fc-mediated effector functions and its long serum half-life.

We sought to combine the advantages of single-chain diabodies with those of the IgG-Fc portion creating IgG-like molecules. These molecules should possess the immune response-mediating and pharmacokinetic properties of immunoglobulin molecules as well as four antigen binding sites. Here we demonstrate that fusion of a single-gene-encoded bispecific scDb fragment directed against carcinoembryonic antigen and *Escherichia coli*  $\beta$ -galactosidase to either the CH3 or Fc region of the immunoglobulin  $\gamma$ 1 chain results in the expression of functionally active dimeric IgG-like molecules.

## 2. Materials and methods

### 2.1. Materials

5-Bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-Gal), 6-bromo-2-naphthyl- $\beta$ -D-galactopyranoside (BNG), and fast garnet were purchased from Sigma. Beta-galactosidase was purified as described [17]. Antibody 9E10 was purchased from Genosys and Cy3-labelled goat anti-mouse antibody from Dianova. HRP-conjugated goat anti-human Fc antibodies and HRP-conjugated protein G were purchased from Sigma.

### 2.2. Oligonucleotides

HingebackNot: 5'-AGC TCA GCG GCC GCA GAC AAA ACT CAC ACA TGC CCA CCG TGC CCA-3'; CH3backNot: 5'-AGC TCA GCG GCC GCA GGG GGA GGG CAG CCC CGA GAA CCA CAG GTG TAC ACC-3'; CH3forXba: 5'-CGA GCT TCT AGA TTT ACC CGG AGA CAG GGA GAG GCT CTT CTG-3'.

### 2.3. Construction of scDb-Fc and scDb-CH3

A previously constructed bispecific single-chain diabody directed against carcinoembryonic antigen and *E. coli*  $\beta$ -galactosidase (scDb CEAGal) [14] cloned into mammalian expression vector pSecTagA (Invitrogen) was used to construct scDb-Fc and scDb-CH3 fusion proteins. The Fc region of human IgG1 was PCR-amplified from plasmid pVL1393-scFv-Fc with primers HingebackNot and CH3forXba, digested with *NotI* and *XbaI*, and cloned into plasmid pSecTagA-scDb CEAGal digested with *NotI* and *XbaI*. The CH3 region was amplified with primers CH3backNot and CH3forXba and cloned as described for the Fc fragment. Both fusion proteins contain a C-terminal Myc tag and hexahistidyl tag. Constructs were sequenced with primers pcDNA-Seq1 and pSecSeq2 [15].

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#### 2.4. Transfection of mammalian cells

HEK293 cells grown in DMEM Nut-Mix F12 medium were transfected with plasmid DNA using Lipofectamine (Gibco BRL) following the manufacturer's protocol. Stable clones were selected in the presence of 200 µg/ml zeocin. Positive clones were identified by immunofluorescence with antibody 9E10 and Cy3-labelled goat anti-mouse antibody [15].

#### 2.5. Purification of antibody fragments

The His-tagged antibody fragments were purified by immobilised metal affinity chromatography (IMAC) from cell culture supernatant of stably transfected clones. Supernatant was adjusted to IMAC loading buffer conditions (50 mM Na-phosphate, 500 mM NaCl, 20 mM imidazole, pH 7.5) and loaded onto Ni-NTA resin (Qiagen). After washing with IMAC wash buffer (50 mM Na-phosphate, 500 mM NaCl, 35 mM imidazole, pH 7.5) bound proteins were eluted with IMAC elution buffer (50 mM Na-phosphate, 500 mM NaCl, 100 mM imidazole, pH 7.5). For large-scale purifications (0.5–1 l supernatant), proteins present in the cell culture supernatant were precipitated with 50% saturated ammonium sulphate. After centrifugation at 10 000 × *g* for 30 min, the pellet was resuspended in 20 ml IMAC loading buffer and purified by IMAC as described above. For purification with protein A, cell culture supernatant was directly loaded onto 1 ml protein A Sepharose (Pharmacia) equilibrated with PBS. Bound proteins were eluted with 100 µM glycine buffer pH 2.8 and dialysed against PBS overnight. Concentrations of purified antibody fragments were calculated spectrophotometrically using the calculated molar extinction coefficients. IMAC-purified antibody fragments were size-fractionated by gel filtration on a Superose 12 column (Pharmacia). Two hundred microlitres of purified material in PBS were loaded onto the column and separated with a flow rate of 0.3 ml/min.

#### 2.6. SDS-PAGE and immunoblot

Proteins were analysed on 8% SDS-PAGE under non-reducing or reducing conditions and either stained with Coomassie R-250 or transferred onto nitrocellulose by semidry blotting. Immunoblot analysis was performed with anti-myc tag antibody 9E10 diluted 1/1000 and subsequent incubation with HRP-conjugated goat anti-mouse antibody diluted 1/5000. Bound antibodies were detected by chemiluminescence (Amersham).

#### 2.7. ELISA and recruitment experiments

Proteins (β-galactosidase, CEA, BSA) were coated onto microtitre plates overnight at a concentration of 10 µg/ml in PBS. Remaining binding sites were blocked with PBS, 2% skimmed milk powder (MPBS). Plates were incubated for 1 h with purified antibody fragments diluted in MPBS. Binding of antibody fragments was detected indirectly with monoclonal antibody 9E10 and HRP-conjugated goat anti-mouse antibodies (Sigma), or directly with HRP-conjugated anti-human Fc antibodies (Sigma). Recruitment experiments in microtitre plates were performed as described [17]. In brief, CEA-coated microtitre plates were incubated with the antibody fragments at varying concentrations and subsequently incubated with *E. coli* β-galactosidase at 10 µg/ml. Bound enzyme was detected using *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as substrate.

### 3. Results

#### 3.1. Secretion of scDb-Fc and scDb-CH3 from mammalian cells

The Fc region or the CH3 domain of human immunoglobulin γ1 was fused to the C-terminus of bispecific single-chain diabody CEAGal (Fig. 1) and cloned into mammalian expression vector pSecTagA containing the Igκ leader sequence and the CMV promoter. Secreted scDb-Fc and scDb-CH3 were purified by IMAC from cell culture supernatant of stably transfected 293 cells. SDS-PAGE analysis showed single bands of 73 kDa for scDb-CH3 and 87 kDa for scDb-Fc under reducing conditions, corresponding approximately to the calculated molecular mass ( $M_r$ ) of the monomeric polypeptides (taking into account the presence of *N*-linked carbohydrate moieties in the CH2 domain). Under non-reducing

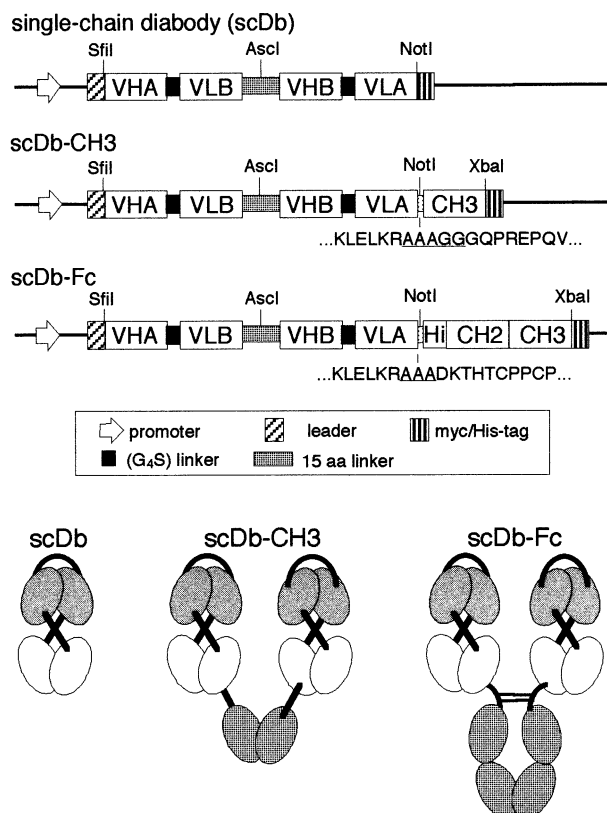


Fig. 1. Structure of scDb-CH3 and scDb-Fc constructs. The amino acid sequences joining the scDb fragment and the CH3 or Fc region as well as restriction sites used for cloning are indicated.

conditions, scDb-CH3 migrated with an apparent  $M_r$  of 65 kDa, while scDb-Fc possessed a  $M_r$  of approximately 200 kDa indicating that scDb-Fc formed disulphide-linked dimers (Fig. 2). Immunoblot experiments with antibody 9E10 recognising the C-terminal myc tag confirmed the identity of the purified proteins. These experiments also revealed minor bands with lower  $M_r$  in the scDb-Fc preparation run under non-reducing conditions not visible in the Coomassie-stained gel with one band corresponding to monomeric scDb-Fc (Fig. 2). Approximately 200–250 µg of scDb-CH3 or scDb-Fc were

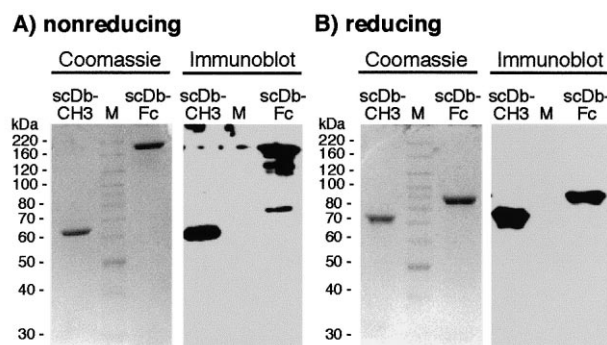


Fig. 2. SDS-PAGE analysis of purified scDb-CH3 and scDb-Fc. Approximately 5 µg of IMAC-purified samples were analysed under non-reducing (A) or reducing (B) conditions on an 8% polyacrylamide gel. Gels were either stained with Coomassie brilliant blue or blotted onto nitrocellulose. Recombinant proteins were detected by immunoblotting with anti-myc tag antibody.

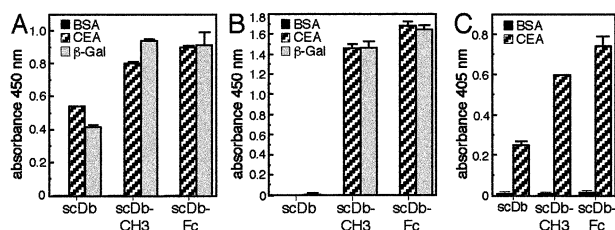


Fig. 3. Antigen binding and enzyme recruitment with scDb-CH3 and scDb-Fc. CEA, β-galactosidase (β-Gal), as well as BSA as negative control were coated onto microtitre plates and incubated with purified antibody fragments scDb, scDb-CH3, or scDb-Fc, respectively. Bound antibodies were detected with either antibody 9E10 recognising the C-terminal myc tag (A) or horseradish peroxidase-conjugated anti-human Fc antibodies (B). Recruitment of β-galactosidase to CEA but not to BSA coated onto microtitre plates as shown by enzymatic activity demonstrated bispecificity of the antibody fragments (C).

purified from 100 ml of cell culture supernatant using IMAC. These yields were approximately 5-fold higher than that observed for scDb CEAGal expressed and purified under identical conditions. Yields of approximately 450–500 μg per 100 ml were obtained for scDb-Fc using protein A chromatography (not shown).

### 3.2. Antigen binding of scDb-Fc and scDb-CH3

The antigen binding sites of scDb-CH3 and scDb-Fc were functionally assembled as shown by ELISA with coated CEA or β-galactosidase (Fig. 3A). ScDb-Fc as well as scDb-CH3 could not only be detected with antibody 9E10 but also with horseradish peroxidase-conjugated anti-human Fc antibodies (Fig. 3B). In contrast, binding of purified scDb CEAGal to CEA or β-galactosidase could only be detected with 9E10. Furthermore, the bispecific binding properties of the antibody fragments were demonstrated directly by recruitment experiments. ScDb-Fc and scDb-CH3 were both able to specifically recruit β-galactosidase to plastic-bound CEA (Fig. 3C).

### 3.3. Dimeric assembly of scDb-Fc and scDb-CH3

In order to demonstrate dimeric assembly of scDb-Fc and scDb-CH3, IMAC-purified antibody fragments were analysed by gel filtration on a Superose 12 column. ScDb-Fc eluted at one major peak with a  $M_r$  greater than that of human IgG (150 kDa) and scDb-CH3 at one major peak corresponding to

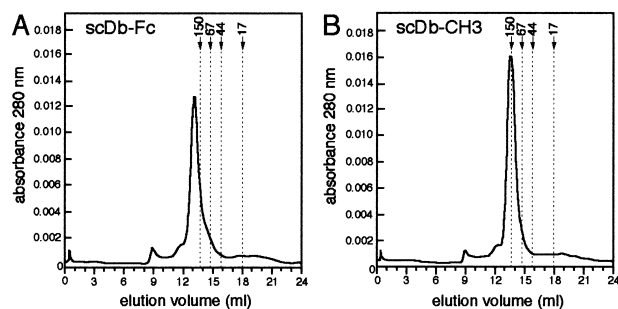


Fig. 4. Gel filtration of scDb-CH3 and scDb-Fc. Approximately 40 μg of IMAC-purified scDb-Fc (A) or scDb-CH3 (B) in 200 μl volume were separated on a Superose 12 column. The elution volumes of human IgG (150 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), and hen egg lysozyme (14 kDa) used as molecular weight standards are indicated.

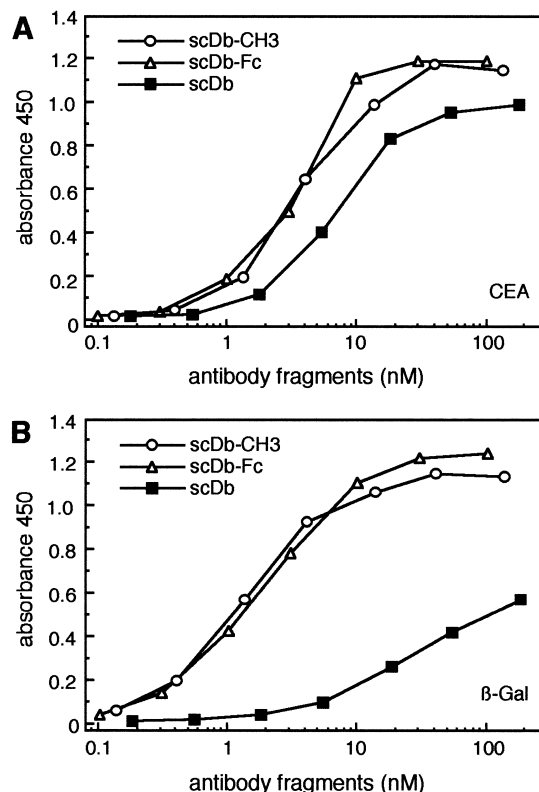


Fig. 5. Functional affinity in ELISA. Binding of varying concentrations of scDb, scDb-CH3, or scDb-Fc to CEA (A) or β-galactosidase (B) in ELISA using an anti-myc tag antibody for detection of bound antibody fragments.

150 kDa. No peaks corresponding to the size of monomeric fragments were observed (Fig. 4). Thus, both scDb-Fc and scDb-CH3 are present as dimers in the preparation. The presence of functionally active antibodies in the peak fractions was confirmed by enzyme recruitment experiments with CEA coated onto microtitre plates (not shown).

### 3.4. Comparison of functional affinity

In further experiments we compared the antigen binding of varying concentrations of scDb-CH3 and scDb-Fc with scDb purified under identical conditions from stably transfected HEK293 cells [14]. ScDb-CH3 and scDb-Fc showed an increased reactivity with CEA and β-galactosidase in ELISA with plastic-bound antigen, in particular with β-galactosidase as the antigen, when compared at an equimolar concentration of monomeric fragments (Fig. 5A,B). In these experiments, similar values were obtained for scDb-CH3 and scDb-Fc. An approximately 10-fold increased sensitivity of scDb-CH3 and scDb-Fc compared to scDb was found in recruitment experiments with CEA coated at 10 μg/ml onto microtitre plates and incubation with β-galactosidase at concentrations of 50 or 1 μg/ml, respectively (Fig. 6A,B). Signals observed with scDb-CH3 and scDb-Fc were approximately twice as strong as those seen with scDb under the applied assay conditions and compared at an equimolar concentration of monomeric fragments. Similar effects were found in recruitment experiments with CEA coated at varying concentrations onto microtitre plates (Fig. 6C,D). This experiment showed that the strength of antigen binding depended on the concen-

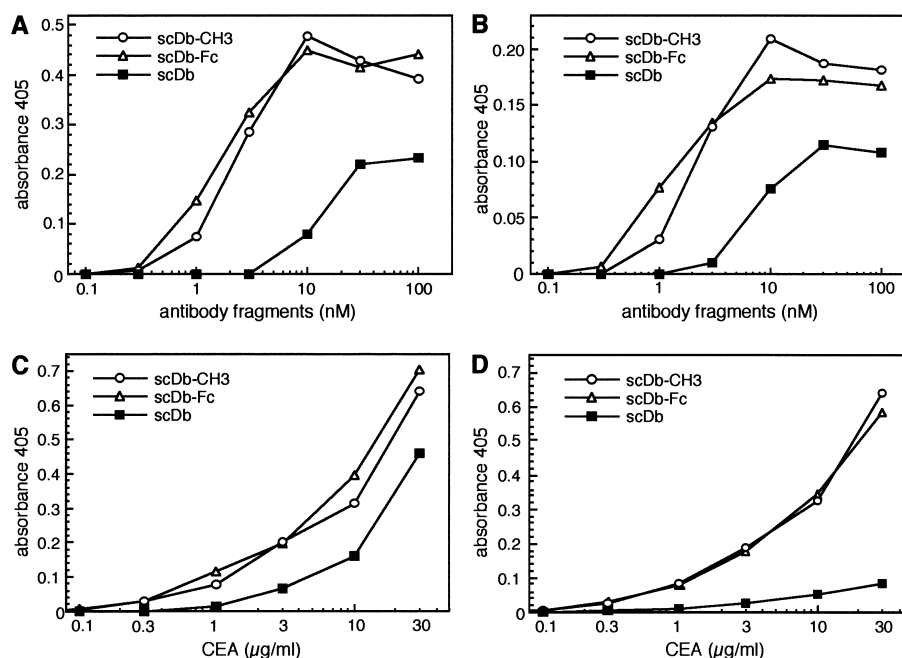


Fig. 6. Functional affinity in recruitment experiments. Recruitment experiments were performed with plastic-bound CEA immobilised at a concentration of 10  $\mu\text{g/ml}$  and varying concentrations of antibody fragments. Bound antibody fragments were detected by incubation with  $\beta$ -galactosidase at 50  $\mu\text{g/ml}$  (A) or 1  $\mu\text{g/ml}$  (B) and subsequent incubation with substrate. In a second set of experiments, CEA was immobilised at varying concentrations and incubated with antibody fragments at 100 nM (C) or 10 nM (D). Bound antibody fragments were detected by incubation with  $\beta$ -galactosidase at 50  $\mu\text{g/ml}$ .

tration of antibody used. While at 100 nM the difference in signal strength was approximately two-fold between scDb and scDb-CH3 or scDb-Fc, at 10 nM this difference increased to up to 10-fold.

#### 4. Discussion

Here, we present a simple route to generate bispecific IgG-like molecules exhibiting four antigen binding sites by fusing single-chain diabodies to the CH3 or Fc region of IgG. As demonstrated by ELISA and recruitment experiments, the binding sites for both antigens are functionally active and accessible in these constructs. This finding indicates that the fusion of a scDb fragment to the hinge region or the CH3 domain does not interfere with antigen binding. In addition, scDb-CH3 as well as scDb-Fc formed dimers. Therefore, folding and correct homodimerisation of the constant domains are not hindered by the presence of single-chain diabodies.

A stronger reactivity and increased sensitivity of scDb-CH3 and scDb-Fc compared to scDb was observed in ELISA with immobilised antigens as well as in recruitment experiments. These findings indicate an increased functional affinity of the dimeric scDb fusion proteins, which can be attributed to the presence of two functional binding sites for each antigen per antibody molecule. Similar effects were also described for antibody fragments di- or multimerised by the use of short peptide sequences [18,19] or protein domains like the tetramerisation domains of p53 [20] or streptavidin [21]. Compared to small monovalent antibody fragments, the larger dimeric and divalent fragments showed an improved tumour targeting as a result of an increased serum half-life and a higher functional affinity [22,23]. The functional affinity of the scDb fusion proteins presented in this study might be further in-

creased by fusion of divalent scDb to the CH3 or Fc region, creating molecules with four identical binding sites.

Dimerisation and functional assembly of antigen binding sites was also described for scFv fragments fused to the Fc region [24–27] or the CH3 domain of IgG1 [28,29]. In the latter case, covalently linked dimers were generated by linking the scFv to the CH3 domain by a cysteine-containing hinge region (minibodies) [28]. This approach might also be applicable to the generation of covalently linked dimeric scDb-CH3 fusion proteins.

Bispecific IgG-like molecules can be easily generated by the strategy described in the present study using bispecific single-chain diabodies as starting point. One of the advantages of single-chain diabodies over the original dimeric diabody format is that the construct is encoded by a single polypeptide sequence, which facilitates cloning and assembly and improves stability [14]. Furthermore, since the two binding sites are present in one single-chain diabody molecule, bispecific IgG-like molecules are formed by simple Fc-mediated homodimerisation giving rise to a homogeneous population of molecules. A similar approach to generate bispecific IgG-like molecules has recently been described fusing two scFv fragments arranged in tandem to the IgG1 Fc region [30]. These bispecific antibody molecules recognising two epitopes on CD2 exhibited increased mitogenic properties compared to the parental monoclonal antibodies.

Merchant and coworkers [11] recently presented a new strategy to generate bispecific immunoglobulins by introducing a knob-into-hole structure and additional cysteine residues into the CH3 domain favouring formation of heterodimeric heavy chains. However, the light chains can bind to either heavy chain resulting also in non-functional antibodies. As discussed by the authors, this problem might be overcome

by using antibodies with identical variable light chains, a phenomenon frequently seen with antibody fragments isolated from phage display antibody libraries. However, this might be a severe limitation when antibodies with diverse light chains, e.g. obtained from established monoclonal antibodies, are used as the starting material.

In another approach, tetravalent bispecific IgG-like molecules were generated fusing a scFv fragment encoding a first specificity to the C-terminus of the CH3 domain of an antibody encoding a second specificity [31]. However, these constructs were unable to carry out complement-mediated haemolysis and showed a reduced affinity for the Fcγ receptor I.

Fusion of a single-chain diabody to the IgG Fc region combines multiple binding sites with the effector functions mediated by the Fc region of immunoglobulins. In contrast, the scDb-CH3 format generates tetravalent antibody fragments of the size of IgG lacking the effector functions associated with the Fc region. These constructs might be useful for therapeutic applications where a long serum half-life is desired but where Fc-mediated side effects are observed [32].

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