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Bispecific and bifunctional single chain recombinant antibodies

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Abstract

Bispecific and bifunctional monoclonal antibodies as second generation monoclonals, produced by conventional chemical or somatic methods, have proved useful in the immunodiagnosis and immunotherapy of cancer and other diseases Recombinant antibodies produced by genetic engineering techniques have also become available for use in preclinical and clinical studies Furthermore, through genetic engineering, it is possible to remove or add on key protein domains in order to create designer antibody molecules with two or more desired functions This review summarizes the strategies for development of single chain variable fragment (scFv) bifunctional and bispecific antibodies The advantages and disadvantages as well as the problems of generating the various bispecific and bifunctional antibody constructs are reported and discussed. Since conventionally prepared bispecific and bifunctional monoclonal antibodies have already shown promise in clinical trials and results from preclinical studies of recombinant bispecific antibodies are encouraging, clinical trials in humans of recombinant bispecific and bifunctional antibodies, as a new generation of biologicals, are likely to be the thrust in the next decade and beyond

Keywords. Bispecific antibodies, Immunotherapy, Bifunctional, scFv, Recombinant antibodies

1. Introduction

Rapid progress in the area of immunodetection and immunotherapy of cancer and infectious diseases has been made in the past 25 years, since monoclonal antibodies were first described [1] Despite great successes having been reported in the in vitro application of monoclonal antibodies, in vivo uses of murine monoclonal antibodies in humans have certain limitations. The xenogeneic nature of murine antibodies stimulates human anti-mouse antibody (HAMA) responses when repetitively injected into patients. Furthermore, nonspecific binding of the Fc portion of antibody molecules to patients' cells generates undesirable side effects. To avoid the latter problem, Fc deficient antibody fragments such as Fab and $F(ab)'_2$, prepared by enzymatic digestion of immunoglobulins, were used as alternatives to whole antibody In the last two decades, with the advent of recombinant DNA technology, various forms of recombinant antibodies have been generated [2,3]. These include chimeric and humanized antibodies as well as antibody fragments such as Fab, $F(ab)'_2$ and single chain variable fragment antibodies (scFv) In the latter construct, variable domains of heavy and light chains are joined together with a linker to form a single polypeptide chain. Recombinant antibodies have been further altered by genetic fusion with ligands or biologically active molecules resulting in the formation of bifunctional antibodies Alternatively, fusion of two different antibodies gives rise to the formation of bispecific antibodies.

Bispecific antibody is a man-made antibody that can bind two different epitopes Bispecific antibodies that cross link tumors and immune cells have been shown in numerous studies to retarget cytotoxicity of killer cells, resulting in the destruction of tumor targets and im-

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proved survival rate of treated animals. Its effective dose is also very low, making bispecific antibody an attractive immunotherapeutic for cancer treatment The use of bispecific antibody for clinical studies, however, was hampered by the difficulties of production in sufficient amounts Early approach exploited conventional chemical conjugation that is less stable with some batch to batch variation [4] Subsequently, bispecific antibody produced by the somatic fusion of two hybridomas, i e. quadroma, provides more stable bispecific antibody but variable yield because up to 10 different variants of antibodies may be generated if the two heavy and light chains are randomly associated [5] More recently, bispecific antibody was produced by genetic engineering at high yield [3] Various engineering designs have been used to put together the two-antibody specificities. Current investigations focus on the use of Fv domains in forms of scFv or similar structures, such as diabody and disulfide linked Fv (dsFv) as a building block. This seems to be preferred to Fab or $F(ab)'_2$ fragments due to the smaller size, less immunogenicity, improved tumor penetration and increased clearance rates [6]. An argument that scFvs could have reduced binding affinity compared with Fab, which in turn reduces the quality of bispecific antibody, could now be overcome by the selection of high binding scFv using phage display technology. Moreover, Fc portion of immunoglobulins may not be necessary for the primary function of bispecific antibodies The moderate size of bispecific scFv also makes it more convenient to produce in the presently available expression systems

Two main approaches have been used for engineering scFv based bispecific antibodies as summarized in Fig 1. In the non-colinear approach, bispecific scFv are generated by two or more chains capable of heterodimerization to generate a bivalent or multivalent bispecific antibody These include diabodies and the combining of two specificities with the aid of naturally formed dimerization structure In the second colinear approach, two different scFv or a scFv and a biological active protein are fused together as a single polypeptide chain. An important distinction is made here between a bispecific antibody and a bifunctional antibody. A bispecific antibody incorporates two distinct paratopes, which are capable of binding two different epitopes or antigens non-covalently A bifunctional antibody, in this review, generally refers to the colinear molecule that has one or more paratopes linked to a diagnostic or therapeutic molecule Thus, in this context, bispecific antibody is distinct from bifunctional antibody both in

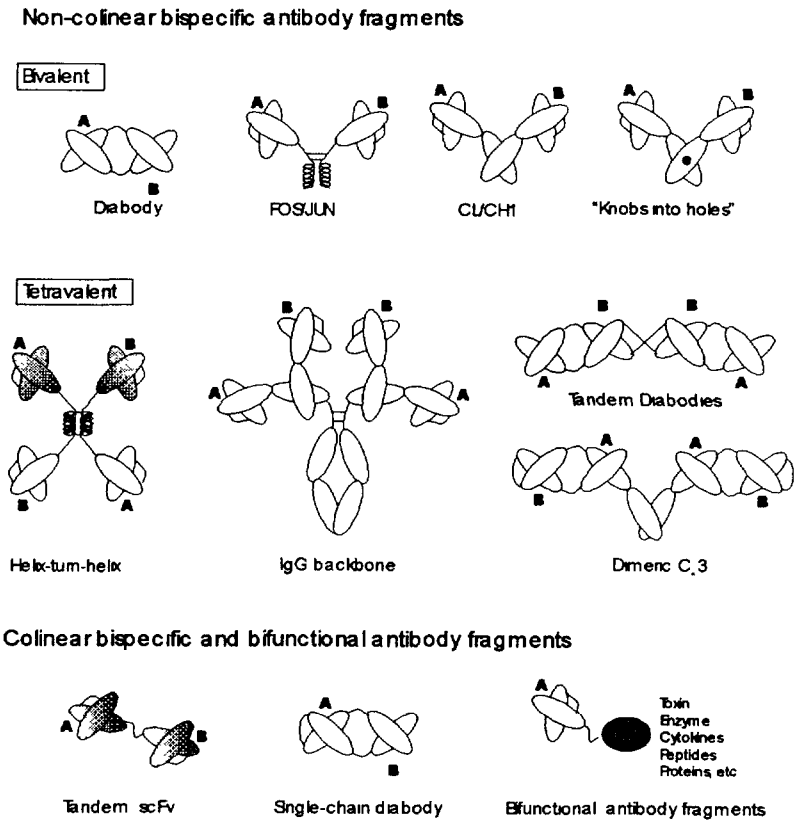

Fig 1 Structure of genetically engineered bispecific and bifunctional antibodies, non-colinear and co-linear constructs A and B refer to two different target epitopes

terms of structure and function. For example, covalent linking of a signal-generating moiety may be advantageous to avoid dissociation in diagnostic applications. However, in some in vivo therapeutic applications, non-covalent bispecific antibody based interactions may be desirable to modulate the therapeutic entity by varying the dose or timing of administration of a second antigen. In the latter application, the bispecific antibody is pretargeted to the desired site followed by the administration of a second therapeutic or diagnostically relevant molecule.

This review describes the recent development and application of various bispecific and bifunctional scFv constructs. The advantages and disadvantages of the construction design, as well as their potential in vivo applications, are addressed.

2. Non-colinear bispecific scFv and diabodies

Two general strategies have been used to generate non-colinear bispecific constructs that are not genetically linked as a single polypeptide In the first method, variable domains of heavy and light chains are joined together with a linker that is too short to allow scFv formation (3–12 amino acids). As a result, two polypeptide chains laterally associate to form a dimeric structure, termed a diabody [7]. Bispecific diabodies generated by combining V_H1-V_L2 and V_H2-V_L1 polypeptide chains have been shown to carry both functional paratopes that are capable of crosslinking the target molecules on two different cells and mediating redirected cytotoxicity [8]. The short linker of diabody is predictably rigid, which raises a concern whether diabody can cross-link two adjacent epitopes on the same cell An interesting study of a bispecific diabody, specific against two different epitopes on vascular endothelial growth factor receptor 2, showed that diabody was flexible enough to cross link the two epitopes on the same receptor, which resulted in acquired antagonistic activity [9]. Diabody structures are currently the focus of extensive research for their therapeutic roles in vitro and in vivo [10,11].

The second approach exploits the natural ability of certain protein domains to associate as heterodimers A modular design of bispecific antibodies has been made by the construction of a leucine zipper based dimerization cassette In transcription factor AP-1, FOS and JUN form a heterodimer via the leucine zipper domain FOS/JUN heterodimerization was preferred and its protein interaction was more stable than FOS/FOS or JUN/JUN homodimers [12]. Thus, leucine zipper sequences adopted from FOS and JUN were selected and fused to recombinant antibodies to facilitate the formation of bispecific antibodies This strategy was successfully used for developing bispecific Fab' [13] and, subsequently, bispecific scFv [14]. In the latter study, scFv was fused to a truncated IgG3 hinge region and a FOS or JUN leucine zipper motif. Stable FOS–FOS or JUN–JUN homodimers composed of bivalent, monospecific scFv were first produced in *E. coli* Bispecific scFv of FOS/JUN heterodimers could be generated by mixing the two reduced halves of the homodimers. This modular design provides a convenient way to produce various bispecific antibodies by preparing individual FOS and JUN linked antibodies, then mixing and matching them, as desired.

The Fab fragment of immunoglobulin is composed of two polypeptide chains with variable and constant domains generating a stable protein structure, that may be used as a platform for constructing bispecific antibodies. Muller et al. [15] has replaced variable domains of Fab with two different scFvs As a result, a miniantibody with dual specificity was generated. A design using adjacent dicistronic gene arrangement driven by a single promoter has been used for this miniantibody production in *E. coli*

An impressive design of bispecific antibody formation utilizes 'knobs into holes' strategy [16]. Amino acids in the interface of C_H3 domains, which form homodimers naturally, are mutated at sites where two chains interact, such that one of them has a small side chain (hole), while the other has a large side chain (knob). The more favorable protein interaction between knobs and holes led to the almost exclusive heterodimerization of two different C_H3 domains. The studies showed that up to 90% of heterodimeric IgG heavy chains may be formed using knobs into holes strategy [16]. This method should be able to combine two different scFvs when fused to the modified C_H3 domains to create bispecific scFv Knobs into holes approach has also been used in an attempt to enhance correct assembly of bispecific diabody but it has proved difficult to enhance heterodimerization while simultaneously maintaining the binding affinity of diabody [17]. Several multivalent non-colinear bispecific constructs have also been described (Fig. 1) and they are discussed in a separate section

3. Colinear bispecific scFv

Joining the two scFvs tandemly with a linker is possibly the simplest way to keep two scFv together as bispecific molecules, which will avoid promiscuous or undesired associations. Several colinear bispecific antibodies have been successfully produced and have been shown to mediate redirected targeted cytotoxicity, as well as tumor specific immune activation (Table 1). Despite the functionality of these molecules, the wrong domain association between the two scFvs and the constraint between the two paratopes, created by the

Table 1
Examples of colinear bispecific scFv molecules

Name	Effector	Target	Tumor type	References
TCRxFITC	TCR	FITC	FITC conj human lymphobalstoid cell line	[23]
C6 5xNM3E2	CD16/NK	HER2/*neu*	Ovarian	[55]
17 1AxCD3	Hu CD3	17-1A	Colorectal	[18]
B1x2C11	Mu CD3	Idiotype BCL1	BCL Lymphoma	[19]
CD3xCD19	CD19	CD3	B cell lymphoma	[56]
2C11xOKT9	Mu CD3	hTfR	Various tumors	[25]
2C11xB43 13	Mu CD3	CA125	Ovarian	[57]

additional oligopeptide linking of the two scFvs, are the concerns for this type of construct Hence, the length and sequence of the linker may determine the flexibility and correct folding of the molecule Several linker sequences have been used to join the two scFvs. Mack et al [18] joined anti-epithelial 17-1A scFv and anti-CD3 scFv with a (gly_4ser) or $(gly4ser)_3$ linker and showed that there was no significant difference in terms of their abilities to redirect cellular cytotoxicity, activate cytokine production and upregulate IL-2 receptor expression The short (gly_4ser) linker seems to provide sufficient flexibility, which may closely resemble a diabody. The $(gly4ser)_3$ sequence has also been used as inter-scFv linker by other groups of investigators regardless of scFv orientation [19]

In addition to the gly-ser linker, colinear bsscFv has been produced using CBH1 linker, a 24 amino acid sequence derived from a fungal cellulase protein (*Trichoderma reesi* cellobiohydrolase I; PGGNRGTTRPATSGSSPGPTNSHY) [20]. A bsscFv specific against anti-ssDNA and anti-fluorescein generated by joining together the two scFv with CBH1 linker was used as a model for the study of antigen binding [21], as well as the analysis of interactive site distance and solution dynamics [22]. The latter study showed that bsscFv joined together with a linker could display considerable conformational dynamics in solution since the actual measurement of distance between the two scFv was three to four times shorter than the calculated distance of the extended conformation. This is likely due to the flexible linker that permits independent motions of the two scFvs. Other linkers of the similar length are 205C' (ASADDAKKDAAKKDDAKKDDKKDL) [23], which has been successfully used in the *E coli* expression system; a 27 amino acid residue helical peptide linker (DQSNSEEAKKEEAKKEEAKKSNSLESL) which has been used for an antibody expression in COS cells [24]; and a linker that primarily derived from N-terminus of the C_H1 domain (EFAKTTAPSVYPLAPVLESSGSG) [25]. The last linker sequence has been used in our laboratory for the construction of several bsscFv (Table 2)

We have observed that the linker sequence plays a critical role in determining the successful production of bsscFv. In the study of an anti-murine CD3 x anti human ovarian (2C11xB43 13) bsscFv, a stable product of 65 kDa could be produced from mammalian cells (COS and NSO cells), while the identical protein produced in *P. pastoris* underwent significant proteolytic cleavage (Fig. 2A). Expression in yeast, driven by the inducible AOX1 promoter, allows the production in methanol buffered media of bsscFv 2C11xB43.13, which gradually degraded into smaller subfragments (32 kDa) Since expression of individual 2C11 scFv or B43.13 scFv in the same expression vector did not indicate the instability of scFv (unpublished data), it is likely that the proteolysis occured in the inter-scFv linker. Expression of 2C11xB43.13 in *P. pastoris*, using a constitutive promoter, did not yield any useful product because of the similar breakage of bsscFv in the linker region followed by the total degradation of protein in the acidic pH of culture medium Hence, it appears that the design of the linker and selection of appropriate host platform for expression are some issues that require careful consideration

Another observation was made in regard to the level of antibody expression and cleavage of linker We have recently developed three different bsscFv with specificity against two alphaviruses, namely, Venezuelan equine encephalitis virus (VEE, with two scFv, 5B4D6 [26] and A4A1) and western equine encephalitis virus (WEE, scFv 11D2 [27]) (Table 2). All three constructs are similar to 2C11xB43 13 bsscFv, in that the VEE or WEE scFv is fused to the C-terminus of anti-murine CD3 scFv (2C11), flanking with the inter-scFv linker A comparative study was done in a mammalian expression vector to determine the level of antibody production and the cleavage of the linker, if any Transient transfection was done in COS cells and the culture supernatant was analyzed 3 days post-transfection The results showed that two bsscFv, 2C11xB43.13 and 2C11x11D2, which had a V_L-V_H-V_L-V_H were expressed in similar quantities and had no indication of protein degradation. However, 2C11xA4A1 and 2C11x5B4D6, which had a V_L-V_H-V_H-V_L were expressed in relatively low amount and/or underwent some level of proteolytic degradation (Fig. 2B). Hence, in order to obtain a useful quantity of product, each antibody-expression system combination will require its own optimization

A new design of single-chain bispecific construct was generated by joining the two chains of bispecific diabody together with a linker. A 27 amino acid residue linker from the N-terminal part of a murine C_H1 domain joined to Gly-Ser motif sequence promoted the formation of single chain bispecific diabody, while a short linker (12 amino acids) promoted the formation of dimerized tetravalent bispecific antibodies (Fig. 1). Interestingly, the monomer or dimer formation largely depends on the expression methods, i.e. inclusion bodies versus periplasmic extracts. The study also showed that single chain bispecific diabody improved the in vitro stability when compared with conventional bispecific diabody structure [28]

4. Multivalent recombinant bispecific antibodies

Conventional bispecific antibodies produced have univalent binding properties to a given epitope. Hence, strong binding affinity of each arm dictates the quality of the particular bispecific antibody produced Increasing the valency of a bispecific antibody is one way to improve its overall binding avidity This may be done in several ways using genetic engineering. Muller et al has used a helix-turn-helix motif derived from DNA binding proteins to generate a Di–Bi miniantibody [29]. Two scFvs of different specificity were fused to the N- and C- terminus of the helix-turn-helix motif. Spontaneous homodimerization of polypeptide chains via dimerization motif resulted in tetravalent scFvs with dual specificities (Fig. 1) The binding properties of Di–Bi miniantibody have been shown to be superior to the bivalent bispecific scFv. Others have made use of the Fc portion or C_H3 domain of IgG_1 to dimerize single chain bispecific diabody[30]. Yet another group of researchers has replaced the V_H and V_L domains of immunoglobulin with two different scFvs, yielding a tetravalent bispecific scFv on an IgG backbone while retaining the biological properties of IgG [31].

An alternative design to increase the valencies of diabody is to link, tandemly, two bispecific diabodies (Tandab). Four V_H and V_L domains may be joined together in an orientation that prevents intramolecular pairing. The bispecific tetravalent diabody that has a molecular weight of 114 kDa is coexpressed in *E. coli* with the 57 kDa single chain diabody. Tandem diabody specific against human CD3 and CD19 turned out to be better for therapeutics, as compared with diabody or bispecific scFv of the same specificity. Thus, increasing valencies did improve the quality of in vivo efficacy, at least in this particular study with Burkitt's lymphoma in SCID mice [32].

5. Bifunctional scFv

Fusion of a second functional domain to an antibody as a co-linear construct gives rise to a bifunctional antibody. In this design, the high specificity of an antibody to its target antigen provides the means of delivering the second functionality to the specific site Conventionally, antibodies have been joined to drugs, radionuclides, peptides or proteins by chemical conjugation, a procedure that is difficult to control for its ratios and is often not site-directed. Alternatively, genetic engineering approaches to fuse a novel peptide/protein allow site-specific linking and ensure the highest specific activity of the molecule However, the limitation is that the second functionality has to be a protein or a peptide In this instance, the chemical methods provide versatility to link both peptide and non-peptide second functionalities. Table 3 summarizes various bifunctional scFvs that have currently been described in the literature

An example of a simple form of bifunctional antibody is a scFv fused to short oligopeptides, such as a streptavidin tag [33]. Thus, expressed scFv is capable of binding to streptavidin and may be used for detection and purification purposes (Fig 3). Highly purified scFv can be prepared by single step affinity chromatography using streptavidin-agarose and eluted with low pH buffer or biotin and biotin derivatives. The procedure is simple, provided that the culture sample is in biotin-free medium The presence of biotin in the culture medium competed for the binding to streptavidin and resulting in an unsuccessful purification (Fig 3B) Biotin–streptavidin-based assay can be further used for detection and antigen binding studies (Fig. 3A). In addition, streptavidin tag permits the attachment of scFv to yet

Table 2
Bispecific scFv constructs produced in our laboratory

Name	Effector	Target	Orientation
2C11xB43.13	Murine CD3	CA125	$(V_L\text{-L-}V_H)_{2C11}$ -L-$(V_L\text{-L-}V_H)_{B43.13}$
2C11x11D2	Murine CD3	Western equine encephalitis virus	$(V_L\text{-L-}V_H)_{2C11}$-L-$(V_L\text{-L-}V_H)_{11D2}$
2C11xA4A1	Murine CD3	Venezualan equine encephalitis virus	$(V_L\text{-L-}V_H)_{2C11}$-L-$(V_H\text{-L-}V_L)_{A4A1}$
2C11x5B4D6	Murine CD3	Venezualan equine encephalitis virus	$(V_L\text{-L-}V_H)_{2C11}$ -L-$(V_H\text{-L-}V_L)_{5B4D6}$

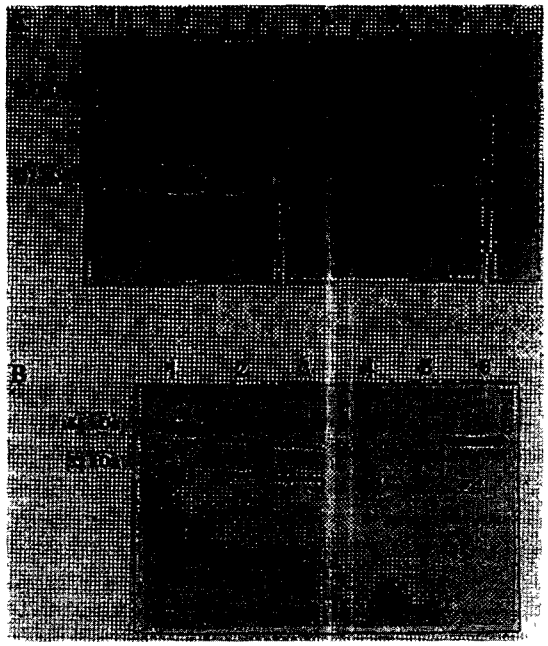

Fig 2 Expression of different colinear bispecific scFv in various systems Western blot analysis detected bispecific scFv at the *c-myc* tag that attached to the end of C-terminus (A) 2C11xB43 13 was expressed in *P pastoris* (lanes 1–6) or mammalian cells (lane 7) Constitutive expression (GAP promoter) in *P pastoris* was analyzed, by growing recombinant clones in YPD media (1% yeast extract, 2% peptone, 2% glucose), and monitored after 1 day (lane 1), 2 days (lane 2), and 3 days (lane 3) in culture Inducible expression (AOX1 promoter induced by methanol) was performed by growing cells in glycerol containing buffered media (1% yeast extract, 2% peptone, 1 37% yeast nitrogen base, 0 1 M phosphate buffer pH 6 0, and 1% glycerol) and induced by changing media to methanol containing buffered media (1% yeast extract, 2% peptone, 1 37% yeast nitrogen base, 0 1 M phosphate buffer pH 6 0, and 0 5% methanol) Product was monitored after 1 day (lane 4), 2 days (lane 5), and 3 days (lane 6) post-induction Mammalian expression of 2C11xB43 13 was produced by NSO transfectomas (lane 7) (B) Transient transfection of COS cells using various bispecific scFv constructs Among the four constructs tested, the N-terminus of 2C11 scFv was joined to the linker and the second scFv inserted to form the C-terminus of the molecule The expression of bispecific scFv was driven by the CMV promoter Culture supernatant was tested 3 days post-transfection Lane 1, 2C11xB43 13, lanes 2 3, 2C11xA4A1, lanes 4–5, 2C11x5B4D6, lane 6, 2C11x11D2

another functionality via streptavidin bridge, providing a convenient way to generate an immunologic reagent with dual functions, which can be used for in vitro and in vivo applications Other simple bifunctional scFv include the addition of glycosylation sites to the C-terminus of scFv for in vivo glycosylation and subsequent site-specific conjugation of polyethelene glycol for increasing the half life of scFv [34] or addition of lipid modification site to scFv for incorporation into liposomes [35] In a more recent study, the commonly used polyhistidine tag was used, instead of the large metal binding protein [36], for attachment of a radionuclide [37] for immunoscintigraphy and radioimmunotherapy applications.

In the area of targeted cytotoxicity and immune activation, a recombinant immunotoxin generated by fusing anti-CD-22 scFv with truncated *Pseudomonas* exotoxin (PE-38) has already entered phase I clinical trial [38] Fusion of scFv to nuclease enzymes, such as DNaseI or RNase, mediate degradation of DNA or RNA upon internalization into the tumor cells [39,40] Antibody-directed enzyme prodrug therapy approach employs antibody–enzyme fusion proteins to activate a prodrug into a cytotoxic agent at the specific tumor site while antibody fused to cytokines and costimulatory molecules targeted to the immune cells can be used to elevate the immune response [41–43]

In the area of infectious diseases, we have fused the human IgG1 constant domain to a scFv 10B5, directed against an envelope protein of WEE. The human heavy chain provides domains for effector function to the scFv, including the binding of complement C1q and the formation of multimer [44]. In the study of HIV infection, anti-p17 scFv genetically fused to cytoplasmic or nucleus retention sequences, expressed intracellularly, was shown to inhibit the replication of the virus [45] More recent studies in the area of DNA vaccines have made use of the scFv fused with the viral envelope to direct the selective gene transfer into the targeted cells [46]. Similarly, anti-adenovirus scFv was fused to the

Table 3
Examples of bifunctional scFv molecules

Application	Ligand/biological active molecule attached to scFv	References
Immunoassay and antibody directed enzyme prodrug therapy (ADEPT)	Alkaline phosphatase, Core streptavidin, Streptavidin tag	[58–62]
Targeted tumor killing	Psuedomonas exotoxin	[38,63]
	Bovine pancreas DNaseI	[39]
	Bovine seminal RNase	[40]
	Carboxypeptidase G2	[64]
Superantigen targeted cytotoxicity	Staphylococcus enterotoxin Ag	[65 66]
Inhibit appositional clot growth	Factor Xa recognition site	[67]
Targeted selective gene transfer	Murine leukemia virus envelope, Epidermal growth factor	[46,47]
Passive immunization	Fc human IgG1	[44]
Targeted cytotoxicity	Fc human IgG1	[68]
Targeted immune activation	IL-2	[42]
	TNF	[43]
	B7-2	[41]
Immunoscintigraphy and radioimmunotherapy	Metallothionein	[36,69]
	Lanthanide binding protein	
	Histidine tag	[37]
Intracellular antibody	Retention signals	[45,70]

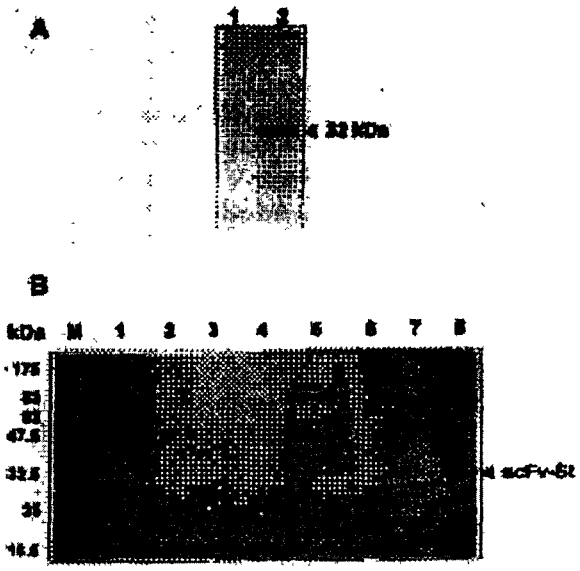

Fig 3 A bifunctional scFv joined to streptavidin tag (PCHPQF-PRCY) for detection and purification purposes (A) Western blot analysis of the scFv A4A1 with streptavidin tag using horseradish peroxidase conjugated streptavidin Lane 1, negative control, lane 2 scFv-strept tag (B) Purification of scFv with streptavidin tag using streptavidin-agarose column chromatography and elution with 0 1 M glycine pH 2 0 Lane 1, scFv in biotin containing yeast culture medium, lanes 2–4, eluates did not yield any scFv product lane 5, scFv in biotin-free yeast culture medium, lanes 6–8, purified scFv eluted with 0 1 M glycine pH 2 0

epidermal growth factor (EGF) to enhance adenoviral gene delivery by interacting with EGF receptor [47].

6. Theoretical considerations

Genetic engineering has generally been accepted as a method of choice for the production of clinical grade antibodies for in vivo applications, as this technology provides for structurally controlled molecules that have a small size and can be produced in large quantities. Various strategies are currently being used to incorporate two antibody fragments to create a bispecific antibody. The non-colinear approach relies on the promotion of heterodimerization of two protein species. While a certain level of success was achieved in enhancing heterodimerization, promiscuous combinations were still a problem. As a result, purification of bispecific antibodies to eliminate unwanted side products would still be one of the key steps in the preparation of bispecific antibody fragments.

The colinear construct, on the other hand, is a single polypeptide chain that should be simpler to produce The homogeneity of the product should also approach 100%, due to the lack of side products This approach also ensures that the two scFvs would always stay together. Conversely, the multi-chain bispecific antibody structures would need to be evaluated for their half-life, tissue penetration and stability when administered for in vivo applications. Further, recombinant bispecific and bifunctional antibodies contain some foreign peptide sequences (linker, heterodimerization domains, etc) that may be immunogenic. It remains to be determined whether these foreign peptides may generate unwanted immune responses, a situation that may have implications for repeat injections of the immunotherapeutic

The search for an ideal bispecific antibody construct may have to wait for comparative in vivo studies of various designs in order to answer this question. So far, only the colinear bispecific scFv construct and diabody have been studied in vivo Bispecific diabody may have advantage over the colinear bsscFv in terms of its paratope formation that should closely resemble the natural immunoglobulin because the V_H and V_L are not artificially linked. The reduced binding affinity of scFv, when compared with Fab, has been reported in many scFv which may reduce their efficacy Fortunately, protein engineering and phage selection of high affinity scFv can overcome this problem. Given that the two recombinant structures have good antigen binding affinity, the assessment of their in vivo stability, half-life and efficacy will be the key factors for future selection of bispecific antibody.

In this line of study, a recent report compared the properties of an anti-sialyl Tn single chain divalent scFv (scFv-linker-scFv) and the corresponding non-covalent dimeric scFv (diabody like structure) [48] The results showed that divalent scFv, in both formats, had similar binding affinity constants. In certain applications, however, single chain divalent scFv may be more advantageous because of their higher tumor to blood ratio and faster clearance. In a study of anti-CD3xCD19 diabody, it was shown that diabody is less stable than CD3 scFv but more stable than CD19 scFv in vitro [49] The same diabody has been shown to have longer blood retention and is more stable than CD3 scFv in vivo [11]. However, this comparison may not be applied to the bsscFv because of the difference in size.

Expression of recombinant antibodies in high yield, in itself, is another challenge that needs to be addressed. Unique sequences in each antibody make each protein expression different. While producing small amounts of recombinant antibodies for pre-clinical studies is reasonably straightforward, achieving a high production yield for eventual clinical studies needs further optimization For in vivo applications, the choice of an expression system can be critical, especially if the glycosylated recombinant antibodies are to be generated. Bacterial expression systems are efficient for scFv expression [50] but produce nonglycosylated antibodies with contamination of toxic lipopolysaccharide that could reduce the yield of functional antibodies during

purification. Glycosylated yeast products with increased mannose content, on the other hand, may be different from mammalian expressed glycoproteins, leading to early removal from the circulation [51]. Under these circumstances, mammalian expression may be more desirable, provided that a stable, high level of expression of the desired construct is achieved. The advantages and disadvantages of choosing different expression systems for recombinant antibody production have been recently reviewed [52,53].

There might be some conceptual differences between the use of bispecific antibodies and bifunctional antibodies in immunotherapy Bispecific antibodies are constructs that have two different paratopes in one molecular entity This allows for the distinct manipulation of the two functions, particularly for in vivo applications where pharmacokinetics, toxicity and clearance of the therapeutic entity need to be controlled This has been elegantly tested in pre-targeting strategies, wherein bispecific monoclonal antibodies are first administered for maximal accumulation in the target, to be followed by administration of the second entity (diagnostic or therapeutic) for subsequent attachment to the second paratope followed by clearance of the unbound fraction. Thus, bispecific constructs allow for separate manipulation of the two paratope reactivities with respect to concentration and time of administration

Bifunctional antibodies, on the other hand, are constructs that have one paratope that is colinearly attached to a therapeutic or diagnostic entity. Linkage of the two components ensures the delivery of both biologicals at the target site However, the fixed ratio between the two component entities may restrict the dose of usage While one component may be required in high dosage to push for efficiency, the other may exceed its maximum tolerable limit. Thus, the dose of administration and the efficacy of treatment would be compromised.

Bispecific antibodies have been shown to be beneficial in the immune cell recruitment for the treatment of cancer Depending on the effector–target interaction, bsAb can induce cytotoxicity, phagocytosis as well as antigen presentation Several of these antibodies are currently evaluated in phase I and II clinical trials. The most extensively studied bsAb are 2B1 and MDX-210 2B1 is produced from hybrid hybridoma with specificities against HER-2/*neu* and FcγRIII (CD16) [71] while MDX-210 is a chemically conjugated hetero $F(ab)'_2$ fragment specific against HER-2/*neu* and FcγRI (CD64) [72] Both antibodies showed therapeutic promise in late stage cancer patients whose tumor was refractory to conventional therapy. In addition, the first humanized bispecific Fab fragment, MDX 447, reactive to epidermal growth factor receptor (EGF-R) and CD64 is also being tested in phase I and II studies [73] Another bispecific antibody, CD3xCD19, was used in combination with anti-CD28 for locoregional treatment of low-grade B-cell lymphoma. Phase I study showed that at least in some patients, lymphoma-specific T cells could be recruited by this immunotherapeutic approach [74] In all these studies, HAMA responses are found in the majority of patients, which points towards the requirement of new formats of bispecific proteins with reduced immunogenecity.

Future candidates, namely, bispecific scFv and diabodies are currently being tested and promising results were obtained in the experimental models Study of a bsscFv by De Jonge et al. has demonstrated, for the first time, that bsscFv was stable in vivo and could destroy tumors in the murine BCL1 lymphoma model [19] Similarly, Adams et al. [54] showed that a human diabody targeting the extracellular domain of human HER2/*neu* was stable and retained binding activity in vivo. Study of a bispecific diabody, CD16xCD30, in mice bearing xenografted Hodgkin's lymphoma showed that bispecific diabody could markedly regress the tumor growth [75] This study demonstrated for the first time the capability of diabody for immune cell recruitment in vivo. The most impressive experimental studies perhaps came from the study of a tetravalent tandem diabody, CD3xCD19 [32] Tandem diabody was shown in this study to have longer blood retention than diabody, when injected into mice Treatment of SCID mice bearing established Burkitt's lymphoma with human peripheral blood lymphocytes and anti-CD28 monoclonal antibody resulted in the complete elimination of tumors in all of the animals within 10 days. This particular study showed that the tetravalent bispecific diabody is superior to the bivalent bispecific diabody. These findings would point towards the future trend in the development of multivalent bispecific antibody or perhaps multivalent, tri or tetra specific reagents.

In conclusion, the improved design of recombinant bispecific and bifunctional antibodies, in combination with improved knowledge in immunology and treatment regimens, make bispecific and bifunctional antibodies a promising therapeutic for the treatment of cancer and infectious diseases.

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