Progress in overcoming the chain association issue in bispecific heterodimeric IgG antibodies

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Abbreviations: CDR, complementarity-determining region; DAF, dual acting Fab; DVD, dual variable domain; KiH, knobs-into-holes

The development of bispecific antibodies has attracted substantial interest, and many different formats have been described. Those specifically containing an Fc part are mostly tetravalent, such as stabilized IgG-scFv fusions or dual-variable domain (DVD) IgGs. However, although they exhibit IgG-like properties and technical developability, these formats differ in size and geometry from classical IgG antibodies. Thus, considerable efforts focus on bispecific heterodimeric IgG antibodies that more closely mimic natural IgG molecules. The inherent chain association problem encountered when producing bispecific heterodimeric IgG antibodies can be overcome by several methods. While technologies like knobs-into-holes (KiH) combined with a common light chain or the CrossMab technology enforce the correct chain association, other approaches, e.g., the dual-acting Fab (DAF) IgGs, do not rely on a heterodimeric Fc part. This review discusses the state of the art in bispecific heterodimeric IgG antibodies, with an emphasis on recent progress.

Introduction

In the field of recombinant antibody technology, bispecific antibodies have attracted substantial interest¹⁻⁴ due to potential advantages summarized in Table 1. From the perspective of their development as biopharmaceuticals, bispecific antibodies should clearly differentiate from the respective monotherapies and combination therapies. It can be envisioned that this is the case for all applications where targeting or specificity for a specific tissue or broad neutralization, e.g., of viruses such as HIV,¹ is required. Similarly, bispecific antibodies are required to mediate transport phenomena, e.g., through the blood-brain-barrier,⁵ or effector cell recruitment.⁶ Furthermore, considerations such as pharmacoeconomics, convenience or an advantageous clinical development path may favor the choice of a bispecific antibody over the respective combination or co-formulation; in particular, in the likely case that future treatment regimens require combination of more than two targeted biologics at the same time.

Preliminary efforts to design and engineer bispecific antibodies focused primarily on non-Fc-containing bispecific antibody formats such as scFv-based or diabody formats. Concomitant advances in antibody engineering and, importantly, antibody expression and purification greatly diminished the technical challenges associated with the production of Fc-containing bispecific antibodies. Encouraging clinical data, including the recent approval of the EpCAM/CD3 mouse/rat chimeric bispecific antibody catumaxomab (Trion)⁸ and promising clinical data from studies of the CD19/CD3 scFv bispecific T cell engager (BiTE) blinatumomab (Amgen) in B-lineage acute lymphoblastic leukemia (ALL)⁹ further fostered a renewed interest.¹⁰ Until recently, bispecific antibodies in clinical trials almost exclusively belonged to the class of effector cell recruiters. The first dual-targeting bispecific non-effector cell recruiters have recently entered clinical trials, e.g., (1) MM-111, a half-life enhanced bispecific human serum albumin/human epidermal growth factor receptor EGFR-HER3 scFv fusion (Merrimack);¹⁰ (2) SAR156597, a bispecific IL-4/IL-14 DVD-IgG (Sanofi); (3) CVX-241, a bispecific tetravalent CovX body against vascular endothelial growth factor (VEGF) and Angiopoietin-2 (CovX/Pfizer).¹¹ As judged by publications and patents, numerous bispecific antibodies are likely to enter clinical trials during the coming years.

In the field of Fc-containing tetravalent bispecific antibodies, several antibody formats such as stabilized IgG-scFv fusion⁵⁻¹⁶ or dual-variable domain DVD-Ig⁶⁻¹⁸ and others have been described. However, although these bispecific antibody formats exhibit IgG-like properties and can be technically developed, they differ in size and geometry from conventional IgG antibodies. Thus, there is continued interest in bispecific antibodies that are as close as possible to the natural IgG design.

This review focuses on the status and recent progress in the field of bispecific heterodimeric IgG antibodies. Several approaches to overcome the chain association problem in the generation of these antibodies, including the KiH technology that allows the generation of defined bispecific heterodimeric IgG...
antibodies when combined with a common light chain approach or the CrossMab technology, and alternative approaches that do not rely on heterodimeric Fc, such as dual-acting Fab (DAF)-IgGs, are discussed in detail.

**The Chain Association Issue and the Quadroma Approach**

Bispecific Fab2 fragments can be made from two different Fab2 fragments using biochemical techniques such as reduction and selective re-oxidation. Similarly, full-length bispecific IgG antibodies can be generated from two IgG antibodies by reduction/oxidation followed by affinity chromatography using the respective antigens. However, these approaches do not allow the large scale supply of bispecific antibodies in qualities required for clinical trials; thus, ideally, the desired bispecific heterodimeric IgG antibody is produced in one cell line. Figure 1 illustrates the basic challenge in generating bispecific heterodimeric IgG antibodies from 4 antibody chains (2 different heavy and 2 different light chains) in one expression cell line, the so-called chain association issue. In Figure 1A, the homo- and heterodimerization interfaces between the individual antibody chains are schematically shown. Use of different chains for the left and the right arm of the antibody will lead to mixtures; the two heavy chains are able to associate in four different combinations, and each of those can associate in a stochastic manner with the light chains, resulting in 2^4 (total of 16) possible chain combinations, or 10 different antibodies of which only one corresponds to the desired functional bispecific antibody. The difficulties in isolating this desired bispecific antibody out of complex mixtures and the inherent poor yield of a maximum of 12.5% (Fig. 1B) make the production of a bispecific antibody in one expression cell line extremely challenging and disadvantageous. Nevertheless, bispecific antibodies have been generated via this approach where two hybridomas of different specificities are fused together to form a “quadroma” cell line that secretes the two antibodies in a mixture that includes the desired bispecific antibody.

Lindhofer et al. successfully managed the challenge of isolating the desired bispecific quadroma-derived antibody from this mixture with specifications allowing sufficient supply for marketing. For this purpose, they selected the expression of mouse IgG2a and rat IgG2b chimeric antibodies from chimeric mouse/rat quadroma cells (Fig. 2A). Aside from the strong immunogenicity, which is a major drawback, the use of mouse and rat antibodies has three major technical advantages: (1) Preferential intra-species heavy-light chain pairing resulting in a reduction of mispairing; (2) efficient heterologous heavy chain pairing; and (3) different affinities of the selected mouse and rat IgG isotypes for protein A, which allows a relatively straightforward isolation of the bispecific antibody product by a combination of protein A affinity and ion exchange chromatography. Using this technology, catumaxomab, a bispecific antibody recognizing EpCAM and CD3, was produced and clinically developed. Due to the retained effector function of the murine/rat Fc part, these antibodies are also termed trifunctional antibodies or Triomabs®. Catumaxomab was approved in 2009 in the European Union for the intraperitoneal treatment of patients with malignant ascites, and thereby became the first approved bispecific antibody. Due to severe infusion reactions as a consequence of FcγR co-activation, catumaxomab cannot be administered via the systemic intravenous route. Furthermore, repeated dosing is impossible because of the highly immunogenic nature of the murine/rat chimeric antibody, and manufacturing from quadromas does not allow large scale supply and may not fulfill specifications for diseases other than late-stage cancer. Taken together, the quadroma/Triomab approach may be feasible for special applications, but suffers from several drawbacks requiring significant optimization by employing state-of-the-art antibody engineering technologies such as humanization and others explained below.

**Enforcing Correct Heavy Chain Heterodimerization**

To overcome the chain association issue and enforce the correct association of the two different heavy chains, in the late 1990s Carter et al. from Genentech invented an elegant approach termed “knobs-into-holes” (KiH). Basically, the concept relies on modifications of the interface between the two CH3 domains where most interactions occur. A bulky residue is introduced into the CH3 domain of one antibody heavy chain...
and acts similarly to a key. In the other heavy chain, a “hole” is formed that is able to accommodate this bulky residue, mimicking a lock. The resulting heterodimeric Fc-part can be further stabilized by artificial disulfide bridges. During the process of optimizing the heterodimerization interface, various rational designs, including steric complementarity, KiH, disulfide bonds and salt bridges juxtaposing oppositely charged residues on either side of the CH3 domain, were evaluated and ultimately optimized using a phage display library.27,28,30,31 Correct heavy chain association with heterodimerization yields above 97% can be achieved by introducing six mutations: S354C, T366W in the “knob” heavy chain and Y349C, T366S, L368A, Y407V in the “hole” heavy chain (Fig. 3A).27 While hole-hole homodimers may occur, knob-knob homodimers typically are not observed; however, approximately equal expression of both chains is a prerequisite to obtain a homogeneous bispecific antibody product. Thus, good product quality with low hole-hole dimer content can be enhanced by selecting appropriate and stable expression clones. In addition, hole-hole dimers can either be depleted by selective purification procedures or by methods explained below. Notably, all KiH mutations are buried within the CH3 domains and not “visible” to the immune system. In line with this observation, MetMab, a one-armed antibody against cMet produced in E. coli carrying the KiH mutations described above32,33 has recently successfully passed Phase 2 clinical trials with promising signs of efficacy in patients with high cMet expression without evidence of immunogenicity different from classical IgG antibodies.34 In addition, properties of antibodies with KiH mutations such as (thermal) stability, FcγR binding and effector functions (e.g., ADCC, FcRn binding) and pharmacokinetic (PK) behavior are not affected. Similar approaches based on charged residues with ionic interactions (compare Fig. 3B) or steric complementarity (Fig. 3C) have recently been described.

Igawa and Tsunoda from Chugai and Gunasekaran et al. from Amgen chose to alter the charge polarity in the CH3 interface so that co-expression of electrostatically matched Fc domains support favorable attractive interactions and heterodimer formation while retaining the hydrophobic core, whereas unfavorable repulsive charge interactions suppress homodimerization.35,36 In 2006, Igawa and Tsunoda identified 3 negatively charged residues in the CH3 domain of one chain that pair with three positively charged residues in the CH3 domain of the other chain. These specific charged residue pairs are: E356-K439, E357-K370, D399-K409 and vice versa. By introducing at least two of the following three mutations in chain A: E356K, E357K and D399K, as well as K370E, K409D, K439E in chain B, alone or in combination with newly identified disulfide bridges, they were able to favor very efficient heterodimerization while suppressing homodimerization at the same time (see Figure 27 in ref. 33). This work was not broadly recognized because it was published in a Japanese patent application, and only recently Gunasekaran et al. described the use of these species conserved pairs of oppositely charged

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**Figure 1.** (A) Schematic depiction of the homo- and heterodimerization interfaces between light- and heavy-chain domains leading to mixtures when expressed simultaneously. (B) Chain association issue when co-expressing two different antibody heavy and light chains in one cell line, assuming random chain association (Quadroma). In total, \(2^4 = 16\) combinations are possible. Of those, 6 are identical; thus, a purely statistical association leads to 6 tetramers that occur twice (each 12.5% yield) and 4 tetramers that occur once (each 6.25%). The desired bispecific antibody makes up statistically 12.5% of the total yield. (C) Light chain association issue when co-expressing two different antibody light chains in one cell line, assuming random chain association. Heavy chain heterodimerization is enforced using KiH technology. The desired bispecific antibody makes up statistically 25% of the total yield.
residues in the CH3-CH3 interface as rationalized on available crystal structures: E356–K439, E357–K370, D399–K409 and K392–D399. Of these, the K409–D399 pair in particular is structurally conserved and buried. Subsequently, they introduced respective mutations switching the charged residue polarity in chain A from K409 to D409 and in chain B from D399 to K399. Taking the symmetry of the homodimeric CH3-CH3 domain into consideration, this results in repulsive interactions in the case of homodimerization and a stabilizing ionic interaction for the heterodimer.\textsuperscript{36} This approach theoretically suppresses formation of both possible homodimers (Fig. 3D), whereas in the case of KIh, an excess of the hole chain may still lead to the observation of hole-hole dimers. Using a suitable combination of mutations, they were ultimately able to achieve a high degree of heterodimerization by introduction of K409D–K392D in chain A and D399K–E356K in chain B with nearly the same expression yields.\textsuperscript{36} However, although theoretically advantageous, the use of charged residue pairs did not appear to result in higher heterodimerization yield and purity of the respective heterodimeric proteins/antibodies compared with the KiH approach combined with disulfide bridge, and can also result in significant decrease of antibody productivity.\textsuperscript{35,36} Clearly, combining the two approaches may result in an even higher rate of heterodimerization, but may not be desirable in terms of production yields and in efforts to minimize the number of “non-human” mutations in therapeutic antibodies.

Moore et al. from Xencor defined 41 variant pairs based on combining structural calculations and sequence information that were subsequently screened for maximal heterodimerization.\textsuperscript{37} Here, the combination of S364H, F405A (HA) on chain A and Y349T, T394F on chain B (TF) (Fig. 3C) resulted in heterodimer formation up to 89% and was comparable to the KIh approach without disulfide stabilization and electrostatic steering approach in their hands.\textsuperscript{37}

Davis et al. from Merck Serono followed an approach that is based on the fact that the CH3 domains of human IgG and IgA do not bind to each other. Structurally-related β-strand segments were exchanged to yield asymmetric CH3 domains that were used to generate heterodimeric IgG-like antibodies.\textsuperscript{38} They termed the resulting bispecific antibodies strand-exchange engineered domain (SEEDbodies; Fig. 2E) and demonstrated that the properties of a native Fc part are retained while gaining almost only heterodimeric products if one of the chains is expressed in slight excess. The concept can be exploited for various IgG-like formats and was assessed in more detail for derivatives of C225, a chimeric anti-epidermal growth factor receptor antibody that was clinically developed as cetuximab (Erbitux\textsuperscript{®}).\textsuperscript{39} The aforementioned approaches of generating bispecific antibodies by introducing changes in the Fc interface region were also addressed by Zymeworks. The company developed a modeling platform called Azymetric\textsuperscript{TM} to generate heterodimeric IgG1 antibodies, but details of this platform are not accessible yet.

In the early 1990s, generation of bispecific antibodies using leucine zippers was described.\textsuperscript{40,41} Those constructs did not include an Fc, and only recently this concept was adapted by Christensen, Wranik et al. from Genentech by fusion of heterodimeric coiled-coil regions at the C-terminus of antibody heavy chains to allow for specific generation of heterodimeric bispecific antibodies (Fig. 2F).\textsuperscript{42} The heterodimerization moiety can be proteolytically cleaved either during the antibody secretion process or as part of the antibody purification process with proteases such as furin.\textsuperscript{42} An overview of approaches to enforce correct heavy chain association is given in Table 2.

**Enforcing Correct Light Chain Association**

While the issue of random heavy chain association is addressed by the methods described above, it is also essential to ensure and enforce correct light chain association. Otherwise, even in the presence of KiH, a mixture of undesired antibodies and the desired bispecific antibody is obtained because of random light chain association (Fig. 1C). Much progress in achieving correct heavy chain association has been made over the recent years, but progress in enforcing correct light chain association has actually hampered progress in the field of bispecific heterodimeric IgG antibodies. This is partly due to the fact that, from a structural point of view, modification of the heterodimeric VL-CL/
generating bispecific antibodies was recently described. In this work, Jackman et al. from Genentech generated bispecific antibodies using a common light chain together with separate expression of half antibodies in *E. coli* (see below) that cross-link FcεRI with the inhibitory receptor FcγRIIb to inhibit the high affinity IgE receptor FcεRI on mast cells and basophils. This approach may be useful for the therapy of asthma and other allergic diseases. Although the common light chain concept is straightforward, few examples have been described so far, and no common light chain antibody has progressed toward the clinic. This may have to do with the fact that one cannot easily rely on pre-existing and validated antibodies, but rather one must identify novel

VH-CH1 interface to enforce correct association in a bispecific antibody is more difficult to achieve and requires more modifications of the interface than for the homodimeric CH3/CH3 interaction (Fig. 1A). Typically, the specificity of antibodies is governed by the complementarity-determining regions (CDRs) residing in the heavy chain, particularly by the heavy chain CDR3; thus the most obvious approach to circumvent the chain association issue is the use of a common light chain (Fig. 2C). This approach requires identification of two antibodies that recognize their target using the same light chain by phage display or immunization of common light chain transgenic animals. A notable example of applying the common light chain approach to generating bispecific antibodies was recently described. In this work, Jackman et al. from Genentech generated bispecific antibodies using a common light chain together with separate expression of half antibodies in *E. coli* (see below) that cross-link FcεRI with the inhibitory receptor FcγRIIb to inhibit the high affinity IgE receptor FcεRI on mast cells and basophils. This approach may be useful for the therapy of asthma and other allergic diseases. Although the common light chain concept is straightforward, few examples have been described so far, and no common light chain antibody has progressed toward the clinic. This may have to do with the fact that one cannot easily rely on pre-existing and validated antibodies, but rather one must identify novel

| Table 2. Overview of approaches to enforce correct heavy chain association in the heavy chain CH3 domains of heavy chain 1 (HC1) and heavy chain 2 (HC2) |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| **HC1**         | **HC2**         | **Type**        | **Reference**   |
| Y349C, T366S, L368A, Y407V | S354C, T366W | KiH + S-S stabilization SAV-W | 25 |
| E356K, E357K, D399K | K370E, K409D, K439E | Ionic, electrostatic | 33 |
| K392D, K409D | E356K, D399K | Ionic, electrostatic DD-KK | 34 |
| S364H, F405A | Y349T, T394F | Mixed HA-TF | 35 |
| Fusion of leucine zipper A to C-terminus of CH3 | Fusion of leucine zipper A to C-terminus of CH3 | LUZ-Y | 40 |
| IgG CH3 | IgA CH3 | SEEDbody | 36 |
antibody pairs that allow a common light chain approach. It is unclear whether this is possible for any arbitrary pair of antigens because the diversity found in natural antibodies is reduced when a common light chain is being used.

Therefore, development of novel approaches allowing the generic conversion of pre-existing antibodies into bispecific heterodimeric IgG antibodies with correct light chain association are still of high importance. Similar to the KiH CH3 domain approach, efforts have been undertaken to investigate asymmetric light chain-heavy chain interactions that might ultimately lead to full bispecific IgGs. Zhu et al. introduced several stericly complementary mutations, as well as disulfide bridges, in the two VL/VH interfaces of diabody variants. When the mutations VL Y87A:F98M and VH V37F:L45W were introduced into the anti-p185HER2 VL/VH interface, a heterodimeric diabody was recovered with > 90% yield while maintaining overall yield and affinity compared with the parental diabody.29 Researchers from Chugai have similarly designed bispecific diabodies by introduction of mutations into the VH-VL interfaces (mainly conversion of Q39 in VH and Q38 in VL to charged residues) to foster correct light chain association.35,46 Although these approaches were able to induce correct heavy-light chain pairing in diabody systems, it is unclear whether they are sufficient to abolish or sufficiently inhibit mispairing to a degree required for bispecific heterodimeric IgG antibodies.

We recently developed the CrossMab approach at Roche as a possibility to enforce correct light chain pairing in bispecific heterodimeric IgG antibodies when combined with the KiH technology47,48 (Fig. 2D) and generate bispecific antibodies from existing antibodies in a generic fashion. In this format, one arm of the intended bispecific antibody is left untouched. In the second arm, the whole Fab region, the VH-VL or the CH1-CL domains are exchanged by domain crossover between the heavy and light chain Fab domains (Fig. 4). As a consequence, the newly formed “crossed” light chain does not associate with the heavy chain Fab region of the the other arm of the bispecific antibody any longer. Thus, the correct “light chain” association can be enforced by this minimal change in domain arrangement.47 Early theoretical considerations predicted certain side products for the CrossMabFab and the CrossMabVH-VL, whereas no theoretical side products were expected for the CrossMabCH1-CL. Indeed, for the CrossMabFab, a non-functional heavy chain dimer as well as an unproductive Fab fragment could be observed, whereas for the CrossMabVH-VL a Bence-Jones-like side product was formed.

As predicted, the CrossMabCH1-CL showed the best side product profile and purity after transient expression.45 The sequences

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**Figure 4.** CrossMab principle. Starting from a conventional IgG antibody correct chain association in a bispecific heterodimeric antibody can be achieved using the KiH technology to enforce correct heavy chain heterodimerization in combination with domain crossover of light chain domains to enforce correct light chain association. The three possible light chain domain crossovers are depicted: Fab domain crossover on the left, VH-VL domain crossover on the right and CH1-CL crossover on the top.
were designed to ensure a Fab domain with domain crossover (CrossFab) with unaltered stability and three-dimensional structure. In line with this, stability and expression yields for monospecific CrossMabs were comparable to those of the parental unmodified IgG antibodies (unpublished data). We also did not see differences in expression rates, binding, or stability due to the different linkers tested. Furthermore, retrospective analysis predicts that the chosen linkers do not contain T cell neo-epitopes, so that no immunogenic potential of the CrossMab approach is expected. Using this approach, the three possible bispecific CrossMab variants against Angiopoietin-2 and VEGF-A were generated based on two existing antibodies, bevacizumab and the Ang-2 selective antibody LC06, with the goal of blocking the two angiogenic factors VEGF-A and Ang-2 simultaneously. Surface plasmon resonance and cellular studies showed that the two different arms of all three Ang-2/VEGF CrossMabs retained their antigen binding affinity for VEGF-A and Ang-2, and interfered with the respective receptor interaction, VEGF-induced human umbilical vein endothelial cell proliferation and Ang-2-induced Tie2-phosphorylation similarly compared with the parental antibodies.57

Based on its superior theoretical and effective side product profile, the Ang-2/VEGF CrossMab CH1-CL was selected for subsequent experiments. This molecule showed potent tumor growth inhibition and anti-angiogenic activity in subcutaneous Colo205 xenograft tumors and superior efficacy compared with bevacizumab treatment.57 In the VEGF-induced cornea pocket assay, the Ang-2/VEGF CrossMab CH1-CL resulted in complete shutdown of angiogenesis. A similar effect regarding superiority to bevacizumab treatment was observed in this model.57 Furthermore, the Ang-2/VEGF CrossMab CH1-CL shows retained Fcγ receptor interaction, VEGF-induced human umbilical vein endothelial cell proliferation and Ang-2-induced Tie2-phosphorylation similarly compared with the parental antibodies.57

The charm of the CrossMab approach is that it can be applied in a more or less generic manner to pairs of existing antibodies without changing the basic antibody architecture and without the need for identification of a common light chain as shown by the inclusion of bevacizumab and LC06 in the Ang-2/VEGF CrossMab. It should also be mentioned that the domain crossover principle is not limited to the 1+1 format; it can equally well be applied to other formats such as tetravalent IgG-Fab fusions in which correct light chain association is desired.

**Bispecific Heterodimeric IgG Antibodies Without Enforced Heavy Chain Heterodimerization**

In the previous section, we summarized methods that foster heterodimerization of heavy chains by systematic modification of the two CH3 domain interfaces. Enrichment of the desired heterodimeric product can also be achieved by protein engineering that does not affect the heavy chain dimerization per se, but relies on established purification methods. In analogy to the Triomab approach in which different affinities of mouse and rat IgG isotypes for protein A are used to isolate the desired heterodimer, residues derived from IgG3, which is known not to bind protein A, can be inserted into one of the IgG1 CH3 domains in order to abrogate protein A interaction. This results in a mixture of bispecific antibodies with two, one or no protein A binding heavy chains that can be subsequently chromatographically separated. Davis et al. from Regeneron chose these known mutations combined with a common light chain approach to generate bispecific heterodimeric antibodies (Fig. 5A).44,50 A similar approach was applied by Igawa and Tsunoda from Chugai who, by mutation in the variable region of the heavy chains, introduced a difference in the isoelectric point between the two chains of the two antibodies that can be used for the chromatographic isolation of the desired bispecific antibody by ion exchange chromatography.51

**Bispecific Antibody Production by In Vitro Assembly of IgG Heterodimers**

Recently, an alternative, more or less generally applicable, approach that combines the KiH methodology with separate expression of half antibodies was described by Jackman et al. from Genentech.49 Here, the two heavy chains with either knob or hole mutation(s) are expressed with their corresponding light chains as half antibodies in separate E. coli cultures and isolated. The half antibodies are subsequently isolated from E. coli periplasm and the bispecific antibody is then formed from the two half antibodies by in vitro assembly (Fig. 2B). This approach allows the correct light chain association during expression of the half antibody without need for crossover or other approaches by avoiding co-expression of the four different antibody chains in one cell line. The subsequent correct heavy chain heterodimerization is achieved in vitro via the KiH technology. One obvious restriction of this approach is the subsequent lack of glycosylation due to expression in E. coli, so that this approach cannot be applied in a fully generic manner, e.g., to IgG1 antibodies where effector function such as ADCC is desired. Furthermore, not all
antibodies are compatible with *E. coli* expression. Nevertheless, using this approach Yu et al. designed a bispecific antibody that binds with low affinity to the transferrin receptor (TfR) and with high affinity to the enzyme β-secretase (BACE1). They could show that the bispecific TfR/BACE1 antibody showed enhanced penetration of the blood-brain-barrier and accumulated in the mouse brain. In an accompanying paper, Atwal et al. showed that treatment with anti-BACE1 antibodies resulted in sustained reduction of amyloid-β peptide plaques in the peripheral and central nervous system, making the bispecific TfR/BACE1 antibody a promising candidate to improve antibody uptake into the brain for Alzheimer disease therapy, which cannot be achieved with a conventional classical monospecific BACE1 antibody.

A different technology to obtain bispecific heterodimeric antibodies is based on the fact that IgG4 undergoes a “Fab arm exchange” in vitro and in vivo. The two arms of an immunoglobulin are held together by hydrophobic and electrostatic forces between the two CH3 domains and two disulfide bonds in the hinge region. In the case of IgG4, the affinity between the CH3 domains is reduced compared with IgG1. As a consequence, reduction and subsequent re-oxidation of a mixture of two different individual IgG4 antibodies leads to separation and recombination of half antibodies, which results in a mixture of the two original homodimeric antibodies (statistically 25% each) and a heterodimeric bispecific molecule (50%). The intermediate half antibodies consist of a heavy chain and its correct light chain partner; the light chain mispairing issue does not occur. Schuurman and colleagues from Genmab combine this IgG4-based bispecific format with a single point mutation in each of the CH3 domains in order to shift the equilibrium in an in vitro process based on two individually produced and isolated monospecific antibodies toward the desired heterodimeric Duobody (Fig. 5B). Similarly, Strop and colleagues from Rinat/Pfizer have combined mutations in the hinge region as well as in the CH3 domain (K409R and L368E) of human antibodies of the IgG1 and IgG2 isotype to form the desired full-length bispecific antibodies when mixed together under appropriate redox conditions with high yields. Specifically, they expressed two different antibodies, e.g., directed against CD20 and CD3, one containing L368E and the hinge mutations 221E, 228E for IgG1 or 223E, 225E, 228E for IgG2 (EEE) and the second containing K409R and the hinge mutations 221R, 228R for IgG1 or 223R, 225R, 228R for IgG2 (RRR) (Fig. 5C). Subsequently, the two antibodies were purified and mixed together in equimolar ratio in the presence of a mild reducing agent. Subsequent experiments demonstrated high and IgG-like stability, unaffected FcγR and FcRn affinity, as well as retained PK properties, the anticipated cytotoxic effects in vitro and potent in vivo B cell depletion.

**Pan-Specific and Dual Acting Fab (DAF) IgG Antibodies**

The terms dual-, multi- or pan-specific refer to monoclonal antibodies that specifically recognize more than one target without being unspecific (Fig. 5D). In fact, promiscuous binding and broad specificity with high affinity, as opposed to the typically-assumed high specificity and selectivity of monoclonal antibodies, is known in inflammatory diseases from natural proteins such as viral chemokine inhibitors vCCI or the smallpox cytokine response modifier CrmD. M. Kosco-Vilbois, N. Fischer et al. from Novimmune explored this observation for the generation of so-called “pan-specific” antibodies that recognize multiple mediators of inflammation and to address redundancy of biological systems. Using extensive phage display selection and affinity maturation campaigns, they were able to identify antibodies that recognize and inhibit, for example, both CXCR3 ligands CXCL9 and CXCL10, while not interfering with CXCL11. Epitope mapping confirmed that the pan-specificity is mediated by structural mimicry.

In this context, it is important to distinguish between (1) antibodies recognizing a common conserved sequence or an identical structural epitope that is found in several members of a protein family such as the HER proteins or angiopoietins (structural
identity); (2) antibodies recognizing two different sequences, e.g., on chemokine receptor ligands that have similar conformations (structural mimicry); and (3) antibodies that can recognize two distinct epitopes in a different binding conformation and orientation with separate parts of their CDRs.58 Bostrom, Fuh et al. from Genentech have generated antibodies that fulfill the latter definition. Based on a rational approach, they produced antibodies with dual specificity, called dual-acting-Fab (DAF)-IgGs or two-in-one antibodies.62,63 The DAF concept requires antibodies that primarily bind antigens via their heavy chain CDRs as starting material. Such antibodies can be identified by screening antigen A against a heavy chain CDR-restricted library. In a second step, antibodies specific for the second antigen B with retained binding affinity for antigen A are selected from these candidates with constant heavy chain(s) by light chain shuffling. The obtained candidates are then subjected to several rounds of affinity maturation until the antibody fulfills the specifications. Bostrom et al. initially described the methodology for a proof-of-concept HER2 and VEGF-A binding DAF that was based on a trastuzumab backbone.62-64 MEHD7945A, an EGFR/HER3 VEGF-A binding DAF that was based on a trastuzumab backbone.62-64 MEHD7945A, an EGFR/HER3 specific DAF IgG1 antibody is currently in Phase 1 clinical studies.65 In both cases, structures of the corresponding Fabs in complex with each of the antigens have been established.63,65 They nicely illustrate how the CDRs of the DAF-IgG can accommodate binding sites for two completely different antigens (Fig. 6). In contrast to the other formats, the DAF approach combines bispecificity with all typical advantages of classical IgG antibodies such as low risk of immunogenicity. However, whether such a DAF can exist at all depends strongly on the structural properties of the complex between the intended antibody and the two antigens. It may thus be impossible to identify an ideal dual specific candidate that exhibits all desired properties such as target binding, epitope recognition, signaling inhibition; more experience is required to judge the general applicability. Since recognition of both antigens can occur on each arm of the antibody in a random fashion, five potential binding modes (A-no binding, A-A, A-B, B-B, and B-no binding) are conceivable for the DAF. In contrast to other bispecific formats, this directly affects the active antibody dose for the less prominent antigen. It can therefore be estimated that DAF antibodies have more complex pharmacodynamics. Depending on the therapeutic application, whether a DAF or a conventional bispecific antibody may be the preferred format must be considered on a case by case basis. For example, the ambiguity of mono- or bivalent antigen binding precludes the possibility of using combinations that require monovalency. For example, an EpCAM/CD3 or CD19/CD3 DAF would not be feasible as it would very likely result in cross-linking of CD3 and subsequent unspecific T cell activation. Based on these structural and therapeutic limitations, dual acting Fab antibodies contribute to the tool set of bispecific antibodies, but this format is not the solution to all problems.

Figure 6. HER2- and VEGF-binding dual acting antibody (DAF)62,63 shown as superposition of the complex structures with HER2 (PDB:3bdy) and with VEGF-A (PDB:3bet). The picture illustrates that HER2 (red) interacts mainly with the heavy chain of the antibody (dark blue), whereas VEGF-A (orange) interacts almost exclusively with the light chain (light blue). There are no significant interactions within the unrelated pairs HC-VEGF and LC-HER2. The Fab of 3be1 has been omitted for clarity since both Fabs exhibit an almost identical structure.

Bispecific Tetravalent IgG Fusions/Conjugates

Although this review focuses on bispecific heterodimeric antibodies, we introduce here two more concepts that rely on a generic IgG format. The Fcab (Fc antigen binding) approach exploits three loop regions in the IgG CH3 domain for generation of an antibody-like library. The overall structural integrity of the Ig-fold is retained and the concept bears similarity to the CDRs found in the IgG variable region. Each CH3 domain is able to bind one antigen, thereby yielding bivalency. Yeast display was used by Rücker et al. to generate HER2 specific Fcabs that retain all properties of a regular Fc domain.66 Bispecificity is obtained by the integration of Fcab within a regular IgG format, which results in antibodies that are termed Mab2 (Fig. 5E).

An alternative approach developed by Doppalpaudi et al. from CovX Research uses a general catalytic IgG antibody as a scaffold for attachment of various binding peptides identified by phage display.11 The scaffold antibody catalyzes an aldolase reaction that leads to a covalent modification of a nucleophilic heavy chain lysine located in a hydrophobic pocket on each of the two Fab arms. Although the PK properties do not match those of conventional IgG antibodies (e.g., an Ang-2 CovX body has a β half-life of 72 to 110 h)67 this approach contributes favorable PK properties to peptides. Obviously this approach can be used to attach mono- and bispecific peptides, allowing development of several CovX bodies including CVX-241, which can bind...
simultaneously to Ang-2 and VEGF-A through a bispecific binding peptide. As the conjugated peptides recognize both proteins, it results in a bispecific “tetravalent” peptide-antibody fusion (Fig. 5F). It should be emphasized that the CovX body, although a bispecific molecule, is based on a monospecific IgG-peptide fusion that differs systematically from the other formats described in this article. CVX-241 was evaluated in Phase 1 clinical studies, but recently discontinued as the molecule’s half-life was found to be shorter than expected, most likely due to instability, or potential immunogenicity of the incorporated peptides.

Outlook

In recent years, the field of antibody engineering has made great progress. It benefits from advancements in antibody selection methods such as phage and ribosome display, implementation of rapid sequencing, cloning and gene synthesis methods, superior transient and stable expression systems, as well as advanced purification and analytical methods. The vast “zoo” of published and patented bispecific antibody formats (Figs. 2 and 5) resulting from these efforts impressively illustrate the high degree of modularity of recombinant antibodies. It is surprising even to people familiar with the field how many novel and ingenious approaches have been designed or are still unexplored. It is somewhat surprising that these efforts have largely been undertaken by researchers from biopharmaceutical companies, and it will be interesting to see these technologies being exploited in an academic setting. However, the impressive zoo of bispecific antibody formats whether bivalent or tetravalent, also raises a number of questions. Which features are required for an ideal bispecific antibody? How will these bispecific antibodies and formats differentiate and perform in clinical trials? Will there be a kind of dominant format in the future, or will we have 20 different bispecific antibody platforms in clinical trials, and ultimately on the market, in some years from now? We are convinced that bispecific heterodimeric IgG antibodies will play an important role throughout the coming years because they mimic the evolutionary design of classical bivalent IgG antibodies with a functional Fc-part. Nevertheless, custom-made bispecific antibodies with additional features not covered by the classical IgG format will still be required for specific purposes. Obviously, bispecific antibodies are ready to progress to multiple clinical trials and ultimately advance medical science within the next 5–10 y.

Disclosure of Potential Conflicts of Interest

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