Chemically Generated Bispecific Antibodies – A New Dawn

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Abstract | A bispecific antibody (bsAb, antibody that targets two different epitopes) is an up-and-coming type of construct among biologics, with two such therapeutics FDA approved (emicizumab and blinatumomab) and on the market, and many more in clinical trials. While the first reported bsAbs were constructed by chemical methods, this approach has fallen out of favour with the advent of modern genetic engineering techniques, and nowadays the vast majority of bsAbs are produced by protein engineering. However, in recent years, relying on innovations in the fields of bioconjugation and biorthogonal click chemistry, new chemical methods have appeared which have the strong potential to be competitive with protein engineering techniques, and indeed hold some advantages. These approaches offer modularity, reproducibility and batch-to-batch consistency, as well as the integration of handles whereby additional cargo molecules can be attached easily, e.g. to generate bispecific antibody–drug conjugates. The linker between the antibodies/antibody fragments can also be easily varied, and new formats can be generated rapidly. These attributes offer the potential to revolutionize the field. Here, chemical methods for the generation of bsAbs will be reviewed, showing that the newest examples of these techniques are indeed worthy competitors to the industry standard expression-based strategies.

Introduction to Bispecific Antibodies

Bispecific antibodies (bsAbs) are protein constructs that can bind two different antigens or different epitopes of the same antigen. Among other things, this enables a single molecule to have synergistic effects and/or to help circumvent the issue of resistance, benefits that would otherwise require a combination therapy of two different antibodies.[1–4] Additionally, bispecifics can exhibit novel mechanisms of action (MoAs) unavailable to antibodies with a single paratope. Such novel MoAs include recruitment of effector cells to the target,[5] enabling crossing of the blood–brain barrier (BBB),[6,7] selectively targeting a cell type that expresses the two different epitopes (Figure 1A),[8,9] providing a pathway for internalisation of an ADC (Figure 1B),[10] acting as a substitute for a scaffold protein holding two enzymes together if the original biomolecule has been inactivated due to a mutation (Figure 1C),[11] or promoting the heterodimerization of cell-surface receptors.[12,13] The unique MoAs and applications of bispecific antibodies has been reviewed in detail recently.[14] There are currently two bispecific antibodies on the market, emicizumab[15] and blinatumomab[16] for the treatment of haemophilia A, and B cell acute lymphoblastic leukaemia, respectively, with many more in clinical trials.[17] Most methods rely on generating these constructs *via* protein engineering, but improved chemical methods for their synthesis have been reported recently. Chemical methods have the advantage of modularity, as bsAbs can be generated by these methods from commercially available mAbs without the need for protein engineering, which is costly and the development of which often only works on a trial-and-error basis. To our knowledge, no review currently exists focusing on chemical techniques for generating bsAbs. As such reviewing these methods herein will fill a gap in the literature and provide useful information for those interested in the applications or construction of bispecific antibodies.

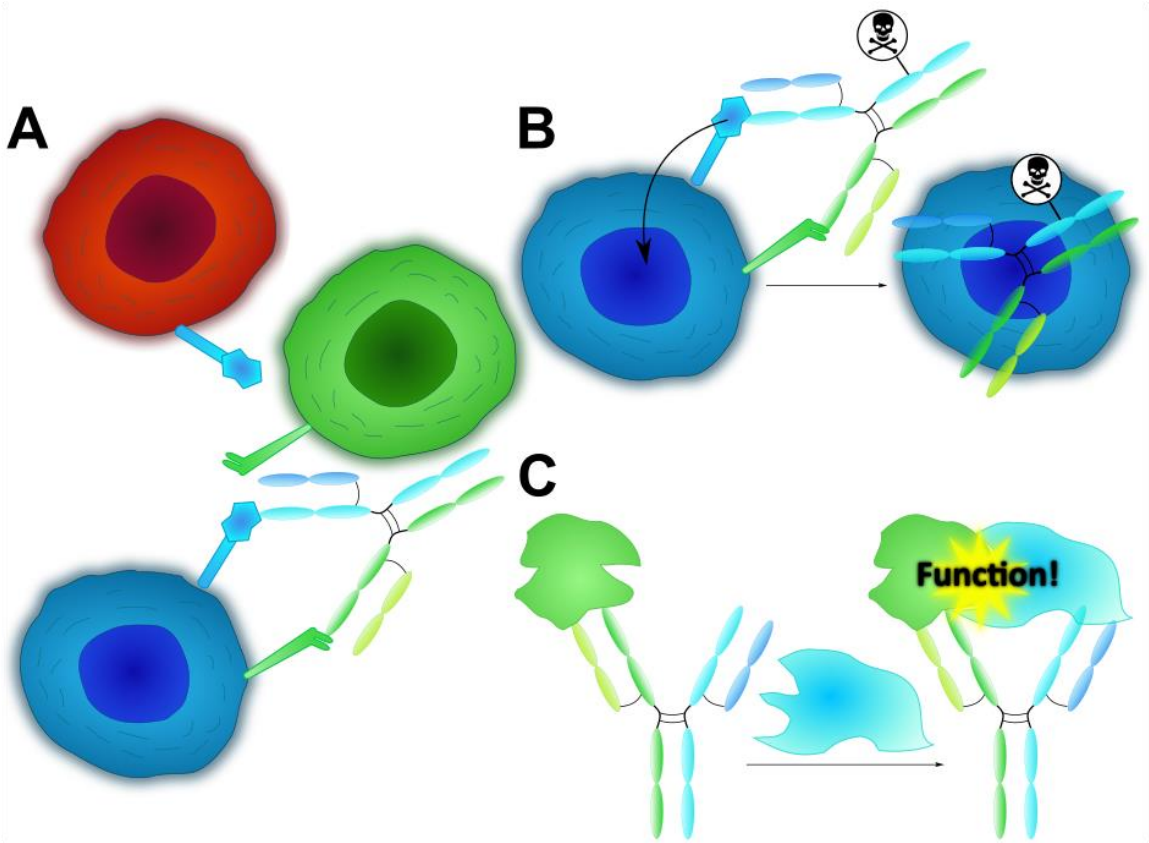


Figure 1 | **Examples of the applications of bispecific antibodies. A** | Selective binding of (pathogenic) cell subset expressing both epitopes.[8,9] **B** | Promoting intracellular delivery of an ADC by having one paratope bind a highly internalizing receptor. The other arm targets an overexpressed disease marker.[10] **C** | Holding two proteins together allowing them to perform their proximity requiring function, if the scaffold protein that should hold them together has been inactivated through mutation (e.g. Factor VIII in haemophilia A.[15])

Early Bispecific Formats

The first bispecific antibodies emerged in the 1960s when reduced $F(ab')_2$ fragments were mixed and re-oxidized.[18] The first in-cell production strategies involved fusion of two different hybridoma cells to create a hybrid-hybridoma (or quadroma).[19] These cells would express the heavy and light chains (HC and LC) of both precursor antibodies and random pairings of the chains would result in 10 different combinations of which one would be the desired bispecific antibody. This led to poor yields and difficulties in purification.[20] Initial clinical trials were also not very successful due to the short plasma half-life and immunogenicity of the constructs and other Fc-related side-effects. Clinical trials on chemically constructed bispecifics had more positive results, but with the methods available at the time they were expensive to make and thus non-scalable.[21] One such early method chemically attached two different full length antibodies with a thiol-to-amine chemical crosslinker to create a T cell re-directing bispecific. Unfortunately, this method led to a heterogeneous mixture due to the lack of basis for promotion of hetero-conjugation over homo-conjugation as well as the random nature of the lysine-conjugation involved.[22] With the advent of modern genetic engineering techniques simplifying expression of engineered sequences, more selective and higher yielding

methods started emerging in the 1990s.[23] One major breakthrough was the "knobs-into-holes" strategy where the constant C_H3 domains of the heavy chains of the antibodies were remodelled to promote heterodimerization based on steric complementarity. When using identical light chains for both antibodies ~95% heterodimerization and bispecific formation could be observed.[24] Another early method, this time relying on a fragment-based approach, was to encode a fused polypeptide consisting of the heavy chain variable fragment (V_HA) of one antibody and the light chain variable fragment (V_LB) of another antibody forming a V_HA-V_LB polypeptide. If the linker connecting the N-terminus of one domain and the C-terminus of the other was short enough, intramolecular dimerization would be disfavoured. Then if paired with a complementary polypeptide, V_LA-V_HB, heterodimerization would occur, leading to a bispecific "diabody".[25] These early methods then evolved into a plethora of approaches partly due to the search for more effective and scalable syntheses, but also due to the need to generate new intellectual property without infringing on the rapidly growing number of patents involving bispecific antibody formats.[14]

Most methods of generating BsAbs currently reported and in use rely on protein engineering and expression systems, and indeed, these have been extensively reviewed.[17,23] This review, however, focuses on chemical methods of generating these constructs as these are much less well known. Indeed, as detailed above, the first bsAbs were generated chemically, but modern genetic engineering techniques caused chemistry-based methods to be superseded. However, with the innovations in bioconjugation and biorthogonal click chemistry laying the groundwork, many novel strategies have been developed recently to address issues with expression-based approaches. The new methods have advantages in modularity, speed of developing new formats, and some offer the option of easily attaching additional cargo to the bsAbs. As extensive protein engineering is not required, development costs can be expected to be lower as well. Thus, the monopoly of engineered bsAbs may now be challenged by chemical techniques, and with increased exposure of the scientific community to these methods, and further improvements thereof, a paradigm shift may follow tipping the balance back towards chemically constructed bispecific antibodies and their conjugates.

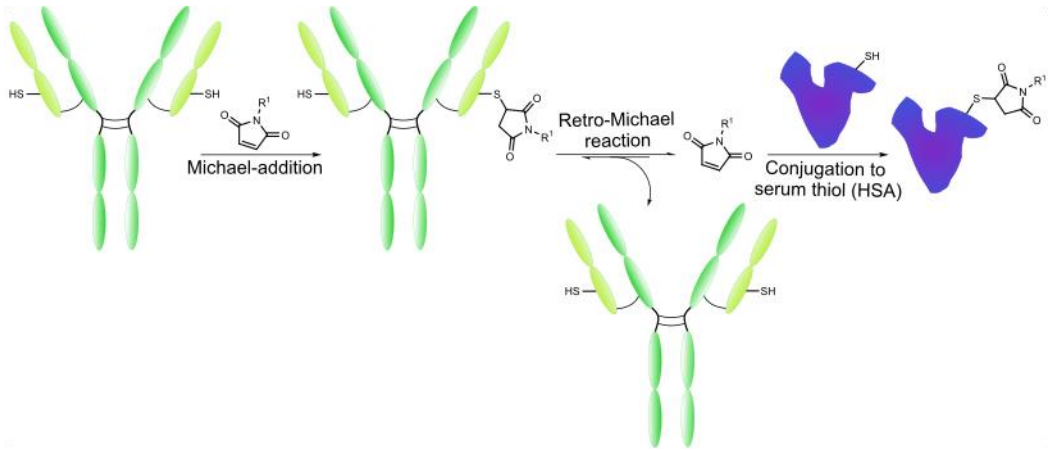
Introduction to Antibody Bioconjugation

To better understand the various methods that have been employed to create bispecific antibodies *via* chemical methods, a brief introduction to chemical antibody modification is in order. Protein bioconjugation – modification of a biomacromolecule with a small molecule or another protein – has a wide variety of applications in biotechnology[26,27] and medicine.[28] One of the main areas in which the chemical modification of antibodies has become fundamental is that of antibody–drug conjugates (ADCs). This field is a good exemplification of the strategies currently available for antibody conjugation.

ADCs are an emergent class of cancer therapy that combine the antigen selectivity of antibodies with the cell killing ability of cytotoxic drugs. An advantage of antibodies is that they can be rapidly raised against a variety of biological targets, including proteins of interest.[29–31] Various methods of antibody modification exist. There are currently eight ADCs on the market: Brentuximab vedotin (Adcetris®), Trastuzumab emtansine (Kadcyla®), Inotuzumab ozogamicin (Besponsa®), Polatuzumab vedotin-PIIQ (Polivy™), Gemtuzumab ozogamicin (Mylotarg®), Enfortumab vedotin (Padcev™), Sacituzumab Govitecan-hziy (Trodelvy™), and Trastuzumab Deruxtecan (Enhertu®) with many more in clinical trials.[32] The lessons learned from the development of the available ADCs highlight the importance of optimising the attachment method of drug to protein.

Three FDA approved ADCs (Kadcyla®, Besponsa®, Mylotarg®) are constructed *via* lysine conjugation. This has the benefit of the conjugates being stable in serum, however, the high natural abundance of lysine residues with similar reactivity profiles makes site-selective targeting very difficult, thus resulting in heterogeneous conjugates.[33–35] Another established strategy relies on partially reducing the four interchain disulfide bonds of a full antibody and modifying the liberated free cysteines with maleimide based compounds (used in Adcetris®, Enhertu®, Padcev™ and Polivy™).[36–40] This method exploits the high nucleophilicity[41] and low natural abundance[35] of cysteine residues in proteins to produce a more homogeneous product (but complete homogeneity is not achieved as the partial reduction is not selective). In the case of Trodelvy™ the antibody is fully reduced before conjugation to a maleimide-bearing molecule leading to a higher drug loading, with up to 8 attachment points per antibody.[42,43] However, such a high loading is not feasible in the case of all cargoes as toxicity or poor pharmacokinetics could be an issue.

One of the most common cysteine modification techniques, used in the assembly of all ADCs on the market that rely on cysteine-modification is based on maleimides. Maleimides are soft electrophiles and react preferentially with the soft nucleophilic thiol group of cysteine over the harder amino group of lysine. However, in antibody conjugation, the structurally important disulfide bonds are reduced to liberate the free cysteines and this may compromise the stability of the protein.[41,44] Another issue with traditional maleimide conjugates is that recently it has been discovered that the thiosuccinimide linkage arising from conjugation of thiols to maleimides is unstable in serum. A retro-Michael deconjugation can occur leading to loss of cargo and re-attachment to serum thiols, such as human serum albumin (Scheme 1). This can then lead to off-site toxicity and a loss in efficacy.[45–47]



Scheme 1 | **The maleimide-stability problem.** | The thiosuccinimide linkage arising from thiol-to-maleimide conjugation is unstable in serum and may undergo retro-Michael deconjugation. The released maleimide can then react with other blood thiols, such as human serum albumin (HSA), and lead to off-site toxicity in the case of antibody-drug conjugates.[45–47]

As this thiol-stability issue was coming to light, methods to circumvent the problem have started being developed. Some groups decided to move away from the maleimide scaffold and use different thiol-reactive moieties.[48–51] Others groups preferred to design improved maleimide molecules; these modifications to the maleimide scaffold involve the addition of leaving groups to the double bond,[52] using exocyclic olefinic maleimides,[53] 3-bromo-5-methylene pyrrolones,[54] or varying the *N*-substituent to increase hydrolysis rate conferring resistance to deconjugation.[55–63]

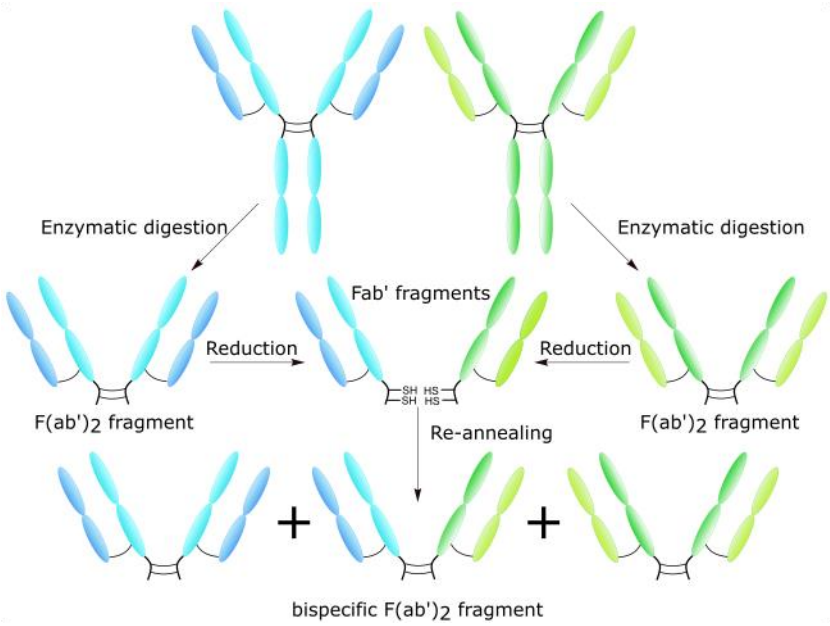
Compounds termed "next generation maleimides" (NGMs) add leaving groups (thiophenyl or halogen) across the double bond of the maleimide, thus altering the mechanism of thiol-reactivity to addition-elimination from Michael addition. Using an NGM with two leaving groups allows re-bridging cysteines that were liberated from a disulfide bond. The final construct is stable to retro-Michael deconjugation. Unfortunately, the conjugate is still susceptible to thiol-transfer in high thiol environments, but forced hydrolysis confers stability to thiols as well. With this method in hand, relatively stable, homogeneous and site-selective conjugates can be synthesized with defined linker-to-antibody ratios.[64,65]

A variation of the NGM method involves the use of pyridazinedione linkers. These moieties expand the maleimide ring by an additional *N*-atom. This eliminates the possibility of hydrolysis and confers an increase in thiol-stability. The possibility to include a second reactive handle is also introduced, enabling dual modification of the antibody, if required.[66–70]

Bispecific Formats Constructed via Chemical Crosslinking

As mentioned before, the first bispecific antibodies synthesized were made through enzymatically digesting two antibodies to their corresponding $F(ab')_2$ fragments. The fragments were then reduced, mixed and allowed to re-oxidize, thus forming a mixture of products, one of which was a bispecific as shown by immunoprecipitation (Scheme 2). Unfortunately, separation of the bispecific molecule from the mixture is difficult and requires sequential adsorption and elution to both targets as immunoadsorbents. The disulfide linkage formed is also potentially labile to thiols

in *in vivo* environments.[18] An improved protocol was reported that used sodium arsenate to complex vicinal thiols and thus prevent intrachain disulfide formation. The Fab' fragments were also activated as the thionitrobenzoate (TNB) derivative with Ellman's reagent. The TNB groups were then removed from one of the Fabs by reduction, and the Fab mixed with the other, TNB-activated, Fab to promote heterodimerization. The bsAb was obtained in 50-70% yield, however as a slightly heterogeneous mixture due to some incomplete re-oxidation of the sulfhydryl groups. The paratopes in this construct are still held together by relatively exposed disulfide bridges and thus could still undergo thiol cleavage in environments containing thiols.[71]

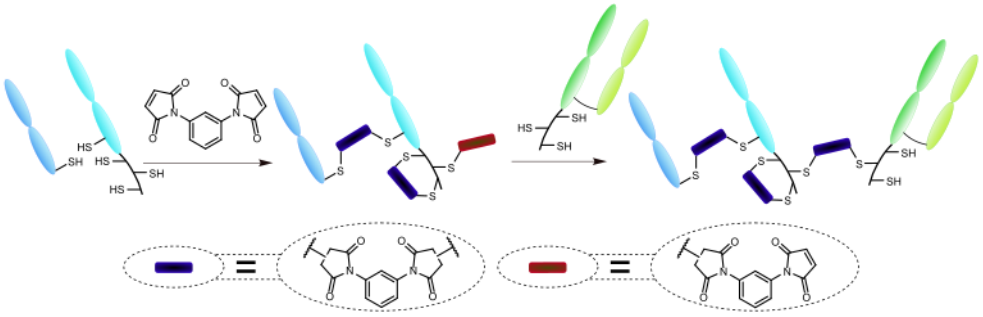


Scheme 2 | **The first published method to chemically create bispecific antibodies.** | This relied on digesting two antibodies to the corresponding F(ab')$_2$ fragments, reducing them, and mixing the reduced Fab' fragments of the two antibodies letting them re-oxidize to form a mixture of products, including the bispecific.[18]

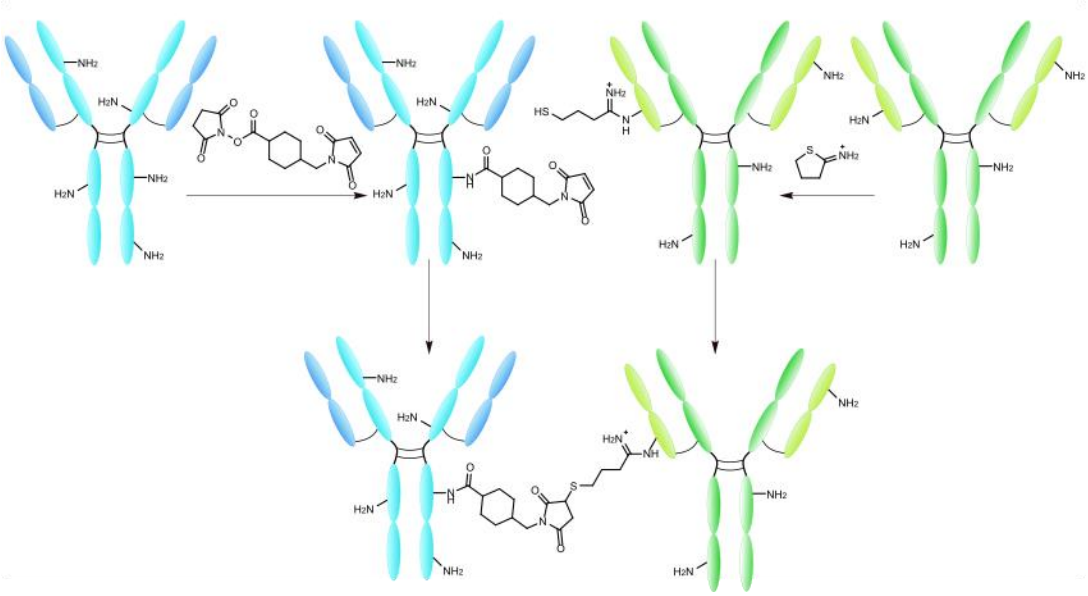
A method was also developed utilising the homobifunctional thiol-thiol crosslinker, *o*-phenylenedimaleimide (*o*-PDM). This molecule has two thiol-reactive maleimide moieties and is thus able to crosslink cysteine residues. The method is restricted to Fab' fragments with an odd number of sulfhydryl groups as the method relies on excess *o*-PDM intrachain crosslinking an even number of thiols while singly modifying the "odd one out". This singly-modified thiol is then available to react with a cysteine residue in another Fab'. As the reduced Fab' has three available thiol groups, multimer formation may occur, reducing yields and necessitating purification (Scheme 3). Unfortunately, human IgGs have even-numbered disulfide bonds in the hinge region, and thus this method is not applicable to them, raising issues with immunogenicity.[72] To address this issue, recombinant Fab' fragments with 1 cysteine residue in the hinge region have been developed and used to generate a T cell redirecting bispecific against HER2 with *o*-PDM.[73] In the case of Fab' fragments with two cysteines, *N*-ethylmaleimide was used to cap one of the cysteines and conditions that preferentially re-oxidize the interchain disulfide were used to remove all other free thiols from the fragment.[74] Recently, an optimization of the Fab' crosslinking was reported, where mild re-oxidation of the Fab' fragments with dehydroascorbic acid promoted re-formation

of disulfide bridges from intramolecular pairs of cysteines, leaving only one sulfhydryl group to react with the bis-maleimide crosslinker. The selective re-oxidation process was ~80% efficient, with <10% Fab–Fab dimer formation and <12% Fab without any re-oxidation as shown by SDS-PAGE, and as such a purification step before bispecific formation was needed. The bispecific bisFabs were formed in 15-30% yields starting from the mAbs, with 65-80% purity after purification.[74] Unfortunately, as detailed above, the thiosuccinimide linkage is unstable and thus these conjugates would have suboptimal serum stability.

Heterobifunctional amine-amine crosslinkers have been used to generate full IgG - full IgG bispecifics. SPDP (succinimidyl 3-(2-pyridyldithio)propionate) has an amine-reactive NHS-ester group on one end and an activated disulfide on the other. Two native antibodies can be reacted with SPDP *via* lysine conjugation. One of the conjugates can then be reduced with DTT (dithiothreitol) at low pH to preferentially liberate a thiol group in the linker as opposed to reduce the interchain disulfides of the antibody. On addition of the other (non-reduced) conjugate, the free thiol will react with the pyridylthiol propionate group to form a disulfide linked bispecific. In addition to relying on lysine modification which leads to heterogeneous mixtures, this linkage still contains a potentially labile disulfide bond.[75] A similar method, but one leading to a linker without a disulfide bond is to use Traut's reagent and SMCC (succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate). Traut's reagent behaves similarly to SPDP in that it is a lysine-reactive group that has a thiol moiety, but in this case, it is unmasked on conjugation and needs no reduction step. SMCC contains an amine-reactive NHS-ester group through which it is attached to lysine residues on an antibody and a thiol-reactive maleimide group, through which it forms a bispecific with the antibody that has had a sulfhydryl group attached with Traut's reagent (Scheme 4). This bsAb unfortunately also contains a labile thiosuccinimide bond and again relies on lysine modification.[76,77]



Scheme 3 | **The ortho-phenyldimaleimide (o-PDM) method of chemically generating bsAbs.** | An odd number of cystines is required as the bis-maleimide re-bridges cysteines in pairs but singly modifies the odd one out. The maleimide functionalized antibody fragment can then be reacted with a reduced Fab' to generate a bispecific.[72] These thiosuccinimide linkages would likely hydrolyse to their thiosuccinamic acid counterparts post-conjugation based on recent research.[63] However, the papers reviewed on the methods here did not investigate this aspect of the reaction. N.B.: On addition of the 2nd (reduced) Fab, any (or all) of the cysteines can react with the maleimide functionalized Fab.



Scheme 4 | **The method relying on succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) and Traut's reagent to chemically generate bsAbs.** | One antibody is lysine modified with SMCC introducing a maleimide functionality. A second antibody is lysine modified with Traut's reagent to introduce a thiol group. The two antibodies are then reacted with each other to form a bispecific.[76,77]

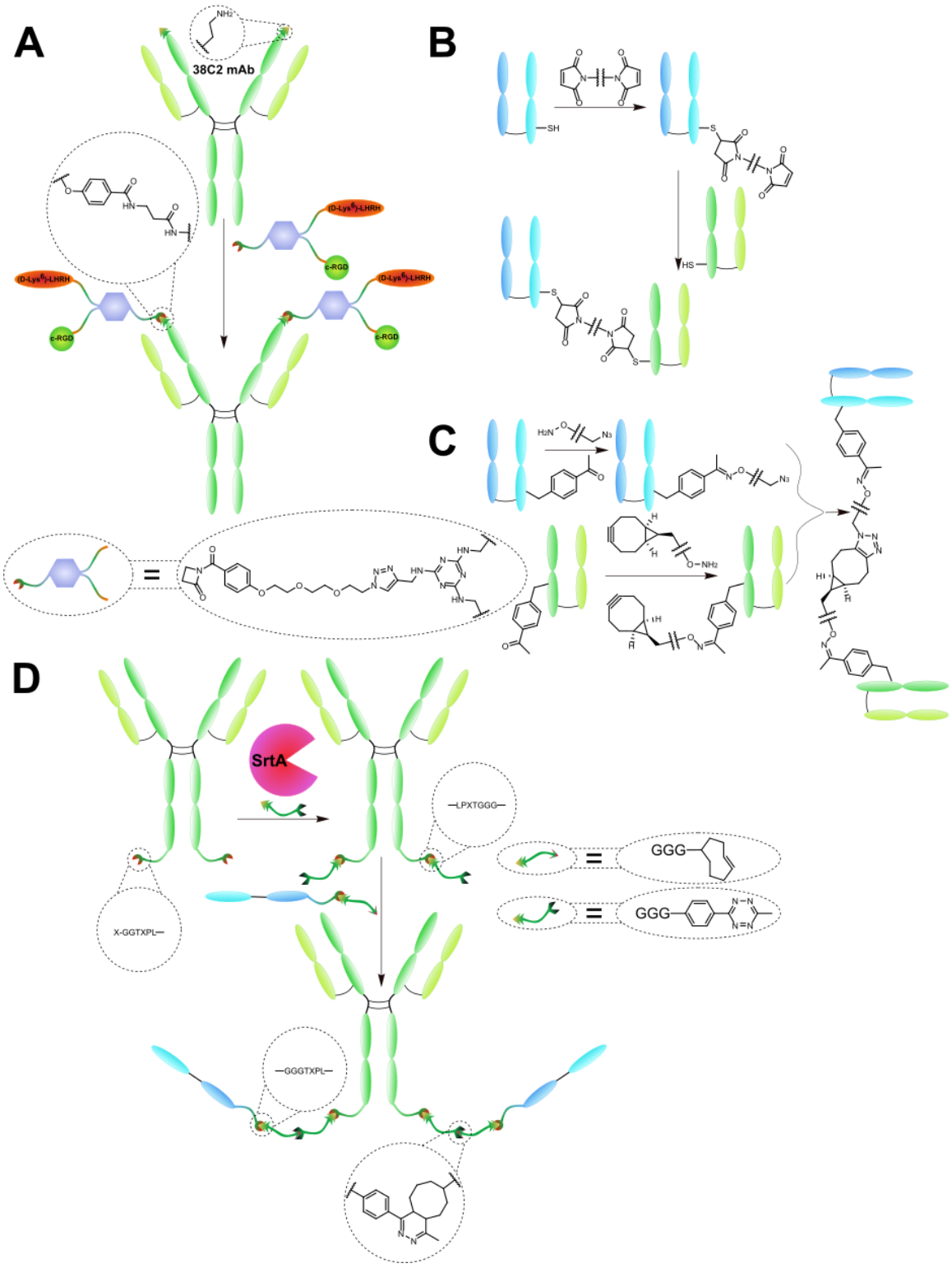
Generating bsAbs with a Combination of Protein Engineering and Chemistry

Some methods employ a combination of chemical tools and protein engineering/expression systems. While these techniques sacrifice some inherent advantages of chemical approaches, the strategies used here, and the conclusions arrived at, can inform purely chemistry-based syntheses. Thus, it would be remiss not to briefly explore this hybrid field.

The antibody 38C2 has catalytic aldolase activity and possesses a highly nucleophilic lysine in the active site, in a hydrophobic pocket, leading it to be unprotonated at neutral pH. The antibody is generated through "reactive immunization" where mice are injected with a 1,3-diketone and antibodies that bind this moiety are selected.[78] This lysine residue can be selectively and reversibly labelled with a diketone or irreversibly conjugated to a β-lactam containing moiety. By conjugating an integrin-binding Arg-Gly-Asp peptidomimetic to a diketone the antibody gained a re-programmed specificity for integrins.[79] In subsequent studies the 38C2 antibody was reprogrammed to target luteinizing hormone releasing hormone (LHRH) receptor as well as integrins with a β-lactam based scaffold that had both LHRH and integrin-targeting peptides attached (Scheme 5A).[80] This method has also been used to express a bispecific DART. These constructs are constructed by co-expressing two polypeptides. Each chain contains two domains fused together; the V_H domain derived from one and the V_L domain derived from another antibody. When expressed, these peptides, V_HA-V_LB and V_HB-V_LA will preferentially heterodimerize due to complementarity forming a bispecific. Importantly, a disulfide bridge between the chains increases the stability of the construct. Here one V_H and V_L were derived from the 38C2 antibody and thus the resulting bispecific was re-programmable by β-lactam based scaffolds.[81]

ThiomAbs are antibodies with one engineered cysteine in either each light or each heavy chain, for a total of 2 cysteines per mAb.[82] Fabs generated with the ThiomAb architecture and thus containing a single solvent accessible free cysteine each have been produced by enzymatic digestion from full ThiomAbs. Two different such Fabs were then conjugated to each other with a bis-maleimide scaffold to create "bisFabs" with ~60% conversion. The product was isolated after 2 rounds of gel-filtration, with no final yield given. The thio-Fab method requires some protein engineering to insert the cysteine residues and is thus less modular, however, the authors also showed how the strategy can be adapted to use native Fab's instead. An additional drawback of the method is that the final construct contains labile thiosuccinimide linkage (Scheme 5B).[83]

A cell-free expression system was reported where incorporation of azide and alkyne containing unnatural amino acids into dihydrofolate reductase (DHFR) and superfolder green fluorescent protein (sfGFP) proceeded in good yields. DHFR and sfGFP were then cross-linked with Cu-catalysed azide-alkyne click chemistry to obtain the conjugate in 43% yield. Unfortunately, the conjugate was shown to lose most of its activity post-reaction. This was attributed to the effect of copper, and indeed homodimerized sfGFP, which lost activity after Cu click and regained activity after the Cu salts were removed by using a desalting column.[84] As such, this method would benefit from incorporation of a strained-alkyne moiety instead of an alkyne group. This was indeed later done, although *via* a slightly more complex route. The unnatural amino acid p-acetylphenylalanine (pAcF) was incorporated an anti-CD3 and an anti-HER2 Fab. These were then reacted *via* oxime-ligation with bifunctional reagents containing an alkoxy-amine and either a strained-alkyne or an azide handle. Fab_{CD3}-BCN and Fab_{HER2}-N_3 were then reacted over 2 days to generate a T cell redirecting antibody against HER2 in 70% yield pre-purification (Scheme 5C).[85] This method was later expanded to generate a wide range of bispecific antibody formats, including some trivalent species (*e.g.* Fab_{HER2}-Fab_{CD3}-Fab_{HER2}), in respectable yields, including an optimized post-purification for Fab_{CD3}-Fab_{HER2} of 75%. The results showcase the power of biorthogonal click chemistry coupled with site-selective conjugation.[86] A drawback of this method is the slow reaction time due to the BCN / azide click pair not being very fast when it comes to overcoming the steric hindrance inherent in attempting to conjugate proteins together. Thus, the technique would benefit from a faster click reaction pair, such as BCN / tetrazine.



Scheme 5 | **Combined protein engineering and chemical methods for the generation of bsAbs. A** | Chemical re-programing of the 38C2 antibody by reacting its very reactive lysine residue with functionalised beta-lactams.[80] **B** | Digesting ThiomAbs into Fabs with a single solvent-accessible cysteine to generate bisFabs with a bis-maleimide crosslinker.[83] **C** | Expressing Fabs

with aldehyde-bearing unnatural amino acids followed by oxime-ligation to introduce click handles. The click-enabled Fabs are then clicked to each other to generate bsAbs.[85] **D:** | Sortase A mediated generation of an antibody-ScFv bispecific. A tetrazine-bearing handle is introduced to the mAb enzymatically and a TCO-bearing handle into the ScFv in the same manner. These are then reacted with each other.[88]

The cysteine transpeptidase sortase A (SrtA) from *Staphylococcus aureus* recognises a short peptide motif (LPXTGG-X) and forms a thioester at the threonine cleaving off the GG-X motif. The enzyme-substrate complex then recognises a triglycine motif, and the N-terminal amine of the GGG reacts with the intermediate forming a stable amide bond. The triglycine motif can be conjugated to many chemical modalities, including azide or strained alkyne (*e.g.* DIBAC) containing moieties. This provides a method to enzymatically install click handles into a protein engineered with a terminal peptide motif with site selectivity. The recombinant protein does not need to express unnatural amino acids and thus higher yields can be achieved with less upfront engineering costs. This chemo-enzymatic method has been used to generate bispecific antibodies and antibody–protein conjugates, by attaching complementary click handles (azide / DIBAC or tetrazine / trans-cyclooctene, TCO) to the proteins of interest. An IgG–IgG conjugate from two different influenza neutralizing antibodies, was prepared by azide – DIBAC click with ~60% conversion as judged by the SEC UV trace, with no reported post-purification yields.[87] The tetrazine / TCO pair was used to conjugate a mAb to an ScFv. The reaction was carried out over 6-18 h with 5 equivalents of ScFv-TCO to drive it to completion. The product was purified by gel filtration, but complete purity was not achieved, as the difference in size between the mAb and the mAb-ScFv conjugate is quite small (150–206 kDa), and thus the product contained unconjugated mAb, mAb+ScFv and mAb+ScFv*2 (Scheme 5D). Yield was not reported. An antibody-cytokine conjugate was also prepared with the same methodology, also using tetrazine – TCO click. Similarly, complete conversion or purification was not achieved, but according to the SEC UV trace the majority of mAb was converted to mAb-IL2.[88]

A similar combined approach was used recently to assay different click partners to identify the optimal pair for protein-protein conjugation.[89] The engineered lipoic acid protein ligase A (LplA) can be used to ligate unnatural lipoic acid analogues to the lipoate acceptor peptide motif, a 13 amino acid long sequence, that can be engineered into recombinant proteins. Multiple tetrazine and strained alkyne (*e.g.* BCN) or strained alkene (*e.g.* TCO, norbornene) containing LplA substrates were prepared and inserted *via* the LAP motif into a flexible loop of the protein EGFP. This was reacted with multiple click-handle functionalized dyes to identify the best reactive partners. Due to the tendency of TCOs to isomerize to the unreactive *cis*-isomer, and the slow reaction rate of other candidates, BCN was identified as the best dienophile partner to react with tetrazines. This is in line with the observations of the Chudasama group on the efficacy of this click pair.[90] Next, the authors explored the suitability of the tetrazine / BCN click pair for the attachment of two proteins to each other. After testing this method to generate an EGFP-mRuby3 protein-protein conjugate, the method was applied to the generation of an antibody-protein construct. Trastuzumab was equipped with the LAP motif at the *C*-termini of each HC. This was reacted with a BCN-bearing LplA substrate to install two strained alkynes per mAb. This was then reacted with 4 equivalents tetrazine-functionalised EGFP over 4 h. Complete conversion of trastuzumab HC to HC-EGFP was observed. This method highlights the power of choosing the optimal click reaction pair to enable efficient protein-protein conjugation.

Chemically Constructed Bispecifics by Disulfide Re-bridging

Chemical strategies for site-selective homogeneous bispecific formation started being explored as ways of re-bridging disulfide bonds emerged. A study published in 2013 utilised a scaffold with two bis-sulfone molecules attached to a large PEG linker (Figure 2B) to create Fab-PEG-Fab monospecifics using Fabs from the same mAb. The Fab-Fab conjugates were shown to retain binding affinity and stability in PBS at 4 °C over 6 months. Yields however were low (~18%).[91] Molecules with two "next generation maleimide" (NGM) moieties (Figure 2A) have also been used to create homo-ScFv-ScFv in 64%, Fab-ScFv in 52%,[92] albumin-ScFv in 57%, albumin-Fab in 49% and ScFv homotrimers in 30% yields after SEC purification. It was found that diiodomaleimide (DIM) was necessary for the generation of the HSA–protein conjugates as the rate of protein–protein conjugation was low due to steric hindrance and dibromomaleimide hydrolysis proved to be a competing reaction reducing yield significantly. DIM, however, was shown to react with thiols faster and hydrolyse slower and thus allow conjugation of sterically bulky proteins. The conjugates were hydrolysed to confer stability.[64] NGMs are an efficient method to generate protein–protein conjugates, with rapid reaction times of 2-5 h and with linkers that are easy to synthesize. There are some drawbacks however; the competing hydrolysis reaction (even with DIM) is unfavourable and as such higher equivalents of the first protein must be used (3 in the case of HSA) to push the reaction towards completion.

The Kauffmann group have reported NGM and click chemistry based methods for the generation of Fab-Fab[93] and full length IgG2-IgG2 bispecifics.[94] Both methods rely on re-bridging disulfide bonds in the antibody or Fab with either a strained-alkyne (DBCO) functionalised or an azide-bearing dibromomaleimide (Figure 2C). To achieve a controlled reaction in the case of the IgG2 bispecific, partial reduction of the interchain disulfides was carried out with 10 equivalents of dithiothreitol (DTT). The azide and DBCO-functionalised proteins were then combined, and strain-promoted azide-alkyne click (SPAAC) formed the bispecifics, which were separated from unreacted starting materials by size-exclusion chromatography (SEC).

An advantage of this approach lies in its modularity as it can be applied to any disulfide-bearing Fab expressed or generated enzymatically from commercially available antibodies, or to any commercially available IgG2. Once it has hydrolysed, post-conjugation, to the thiomaleamic acid the construct is also stable. In the case of the Fab, homogeneity can be achieved as there is only one available disulfide and thus only one possible site of modification on each fragment. In the case of the full antibody, partial reduction is difficult to achieve or control and thus side-reactions can arise from over-reduction and liberation of more than two cysteines per molecule. These by-products can however be removed in subsequent purification steps, but nonetheless have an impact on yield. Furthermore, the reaction takes 48 h in both cases and affords 70% and 56% conversion in the case of Fab-Fab and IgG2-IgG2, respectively. As such a faster click reaction leading to higher conversion with a lower reaction time would be advantageous.

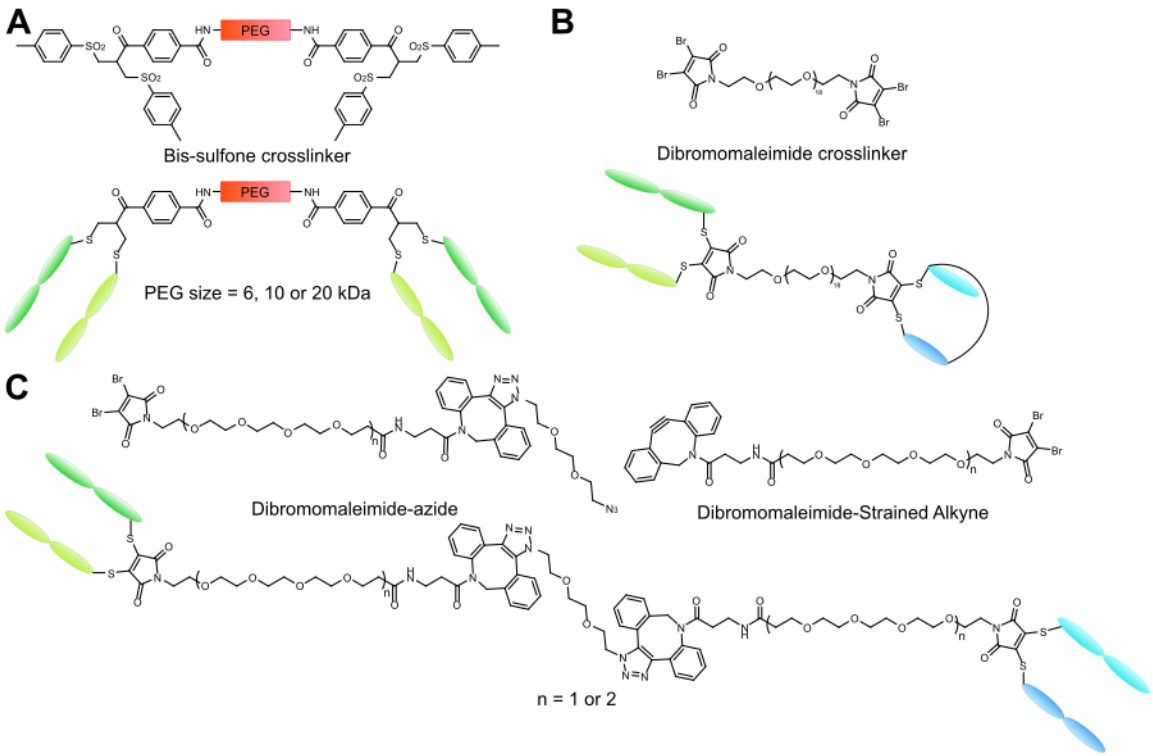
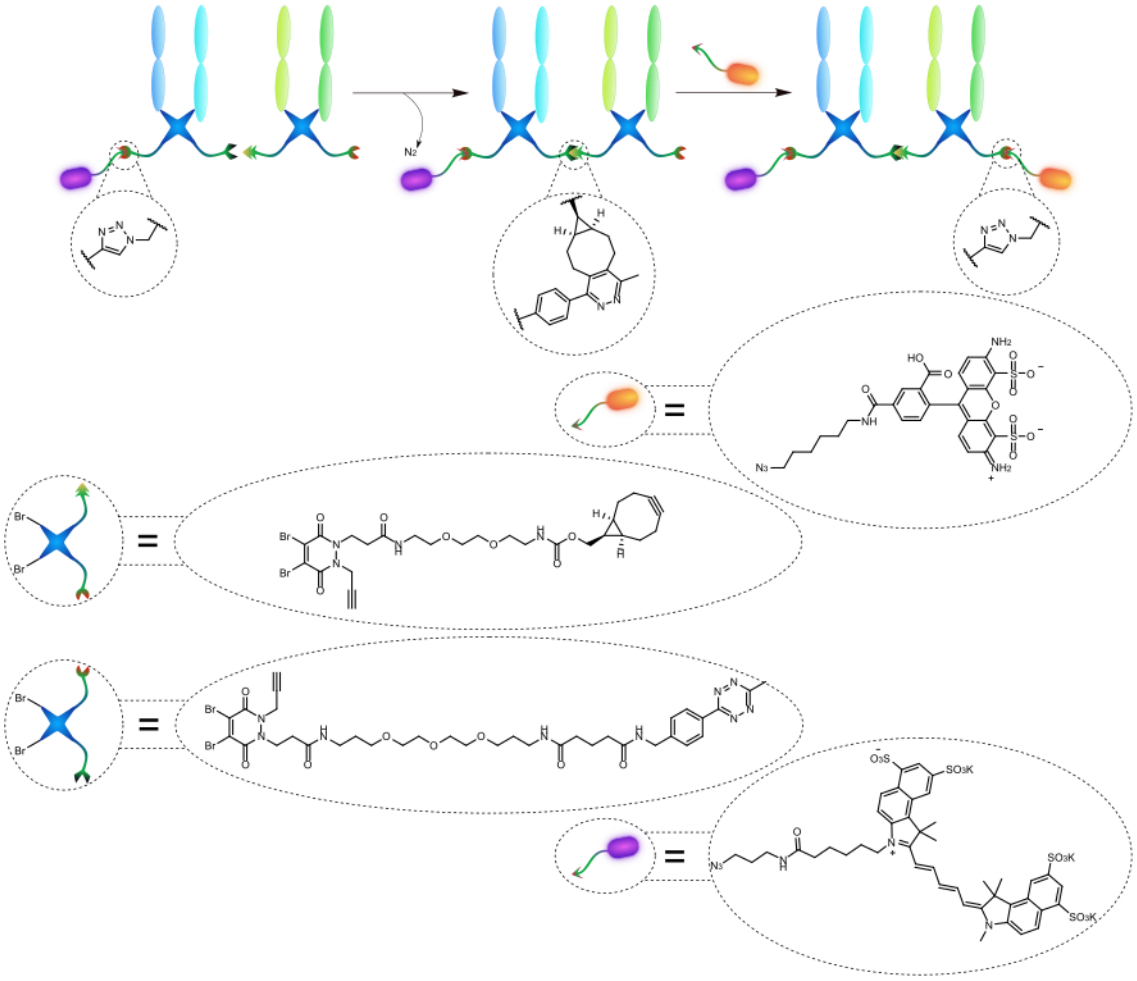


Figure 2 | **Reagents used for the generation of homogeneous bispecific protein–protein conjugates via disulfide re-bridging.** | **A**: Fab-Fab conjugate generated via a bis-sulfone crosslinker.[91] **B** | Fab-ScFv conjugate generated with a dibromomaleimide crosslinker.[92] **C** | Fab-Fab conjugate generated via dibromomaleimides and click chemistry.[93]

The Chudasama and Baker groups have also recently developed a modular, click-chemistry-based method for the construction of homogeneous bispecific antibody-conjugates.[90] The method relies on using the dibromopyridazinedione scaffold to re-bridge the disulfide bonds of enzymatically generated (or expressed) Fabs to introduce click handles. The biorthogonal click handles (BCN strained alkyne and tetrazine) can then undergo strain-promoted inverse electron-demand Diels-Alder click (SPIEDAC) click reaction to generate a bispecific construct comprised of two different Fabs linked by a flexible (PEG-containing) tether. The observation that tetrazine / BCN is an optimal SPIEDAC reaction pair parallels the findings of other groups.[89] With this method bispecifics (Fab_{HER}-Fab_{CET} and Fab_{HER}-Fab_{RIT}) were generated over 12 h in 71% and 78% yields, respectively, without the need for SEC purification, reacting close to stoichiometric quantities of the functionalized Fabs.

As pyridazinediones enable dual modification through the two *N*-atoms in the ring, a second, orthogonal click handle, in this case a terminal alkyne, was introduced on both PD scaffolds. This was then used to introduce two copies of the dye AlexaFluor488 azide *via* CuAAC chemistry in 56% yield for a total dye loading of two per bispecific. If only one PD had the alkyne handle, a bispecific with only one dye attached was generated in 82% yield. And if one of the PD scaffolds was pre-clicked (*i.e.* still as the small molecule, before conjugation) to the dye sulfo-Cy5.5 azide, and then after formation of the bispecific, AF488 azide was clicked to the other available alkyne handle, a bispecific with two different dyes was synthesized in 55% yield (Scheme 6). Thus, it was

demonstrated that with this technique, loadings of 1, 1+1 and 2 could all be achieved. This method relies on a stable, site-selective cysteine modification technique (pyridazinediones) and rapidly produces homogeneous bispecifics with the possibility of attaching up to two different cargoes (*e.g.* dyes or drugs) in moderate to high yields, often without the need for purification steps.



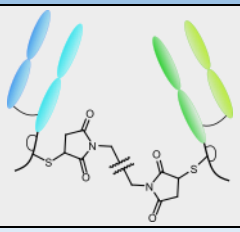
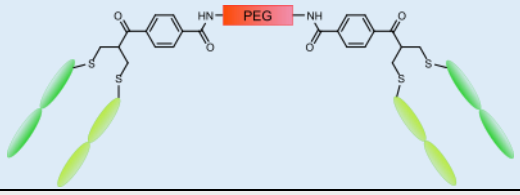
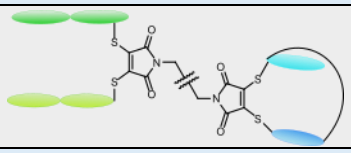
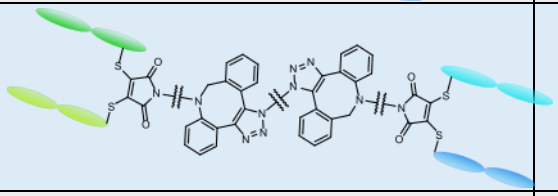
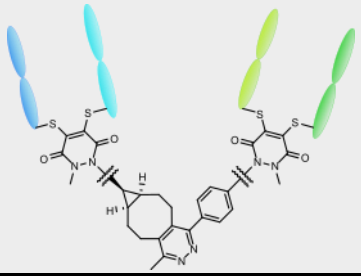
Scheme 6 | **Pyridazinedione-based method for the generation of a dually functionalized bsAb.** | One Fab is site-selectively and homogenously modified with a strained alkyne-bearing pyridazinedione (PD) while the other with a tetrazine-containing PD. These are reacted together to form a bispecific. If the PDs each also contain an alkyne handle, additional cargo molecules (such as dyes shown here) can be attached by copper catalysed azide-alkyne click. If one PD is pre copper clicked to the cargo, and the other PDs is copper clicked after conjugation, two different cargoes can be attached to the bispecific.[90]

Conclusion and Future Perspectives

In conclusion, with the development of new bioconjugation and click chemistry-based methods, chemical ways of constructing bispecific antibodies are becoming competitive with more traditional protein engineering/expression-based techniques. The newest methods have good yields and produce stable, homogeneous and well-defined bsAbs rapidly, with the whole process starting from the corresponding mAbs taking 2-4 days, assuming the small molecules needed are available

(Table *1*). Starting from the relevant Fabs, the generation of the bsAb can take as little as 2 days, including purification if necessary.[74,90,92,93] The modularity of the approaches also offers potential for reactions to be performed in a combinatorial manner, rapidly and simultaneously generating a library of bsAbs from available Fabs (*e.g.* in a 96-well plate). These methods are highly modular as the chemistry is designed to work on any standard mAb platform from which enzymatic digestion yields Fabs with one interchain disulfide bond. Scalability also does not seem to be an issue, with the potential need for purification, typically size-exclusion chromatography (SEC), being the only real limitation (other than costs). However, SEC tends to have higher yields on higher reaction scales and provides excellent purity. An additional advantage of these chemical platforms is that they can provide a direct way of adding additional functionalities in well-defined ratios, site-selectively to generate homogeneous bsAb conjugates. Cargo could entail fluorescent dyes for imaging/diagnostic purposes or cytotoxic drugs to easily generate bispecific ADCs, without a need for a separate methodology. A combination of drug and dye could also be used for theranostic purposes (diagnosis and therapy combined). And the fields of bsAbs and ADCs have much to benefit from each other as a bsADC can be greater than the sum of its parts, benefitting from synergies between the drug and the bispecificity.[21]

Table 1 | **Comparison of Methods for the Chemical Synthesis of Homogeneous bsAbs from native antibody fragments.** | Abbreviations in order of appearance: O-PDM (ortho-phenyldimaleimide), Fab (fragment antigen binding), PEG (polyethylene glycol), DBM (dibromomaleimide), ScFv (single-chain variable fragment), NGM (next-generation maleimide), PD (pyridazinedione).

Method	Structure of Product	Yield from Fab #1	Reaction Time	Eq. of protein #2	Additional comments
O-PDM Fab-Fab		20-39%	Overnight	1 eq. Fab #2	65-80% purity
Bis-sulfones Fab-PEG-Fab		18%	3.5 h	N/A	Homodimer, PEG size = 6, 10 or 20 kDa
DBM Fab-ScFv		52%	2 h	2 eq. ScFv	ScFv homotrimers were also produced
NGM click Fab-Fab		60-68%*	Overnight + 24-48 h	1 eq. Fab #2	*Conversion, not purified yield
PD click Fab-Fab		43-80%	15 h	1-1.1 eq. Fab #2	Up to 2 additional cargo molecules can be attached

As a future perspective, these methods could be further improved by eliminating the need for the attachment of the additional cargo by CuAAC by introducing Cu-free click handles, by adapting these methods to allow attachment of multiple proteins to generate multispecific antibodies or complex antibody-protein conjugates and further exploring what advantages the attachment of cargo small molecules can offer. Chemical bispecifics incorporating Fc fragments would also be interesting to explore. Such an artificially reconstructed antibody could have beneficial Fc-mediated effects such as prolonging serum half-life or directing an immune-response against the target (e.g. antibody-mediated cellular cytotoxicity or opsonization). The scalability of these

techniques would also need to be further investigated to assess suitability for large scale industrial applications.

The field of chemically generated bispecific antibodies, with the development of new bioconjugation techniques, is catching up to expression-based approaches. The advantages of these methods are starting to be felt – modularity, reproducibility and flexibility in attaching other functionalities, whether protein or small molecule – paving the way for a wider adoption of these approaches. We expect that chemical strategies of making these highly versatile artificial biomacromolecules will gain increased traction in the scientific community in the years to come.

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Author contributions

P.S. and V.C. co-wrote the review and co-analysed the literature.

Competing interests statement

V.C. is a co-founder and director of the company ThioLogics.

Display Items

Items are placed in appropriate locations in the text.

ToC blurb

Bispecific antibodies are an up-and-coming type of construct among biologics. They are currently being produced by genetic engineering and expression. This Review highlights recently developed chemical methods for their construction.