Modular chemical construction of IgG-like mono- and bispecific synthetic antibodies (SynAbs)

Fabien Thoreau,[a,†] Peter A. Szijj,[a,†] Michelle K. Greene,[b] Léa N. C. Rochet,[a] Ioanna A. Thanasi,[a] Jaine K. Blayney,[b] Antoine Maruani,[a] James R. Baker,[a*] Christopher J. Scott,[b*] Vijay Chudasama[a*]

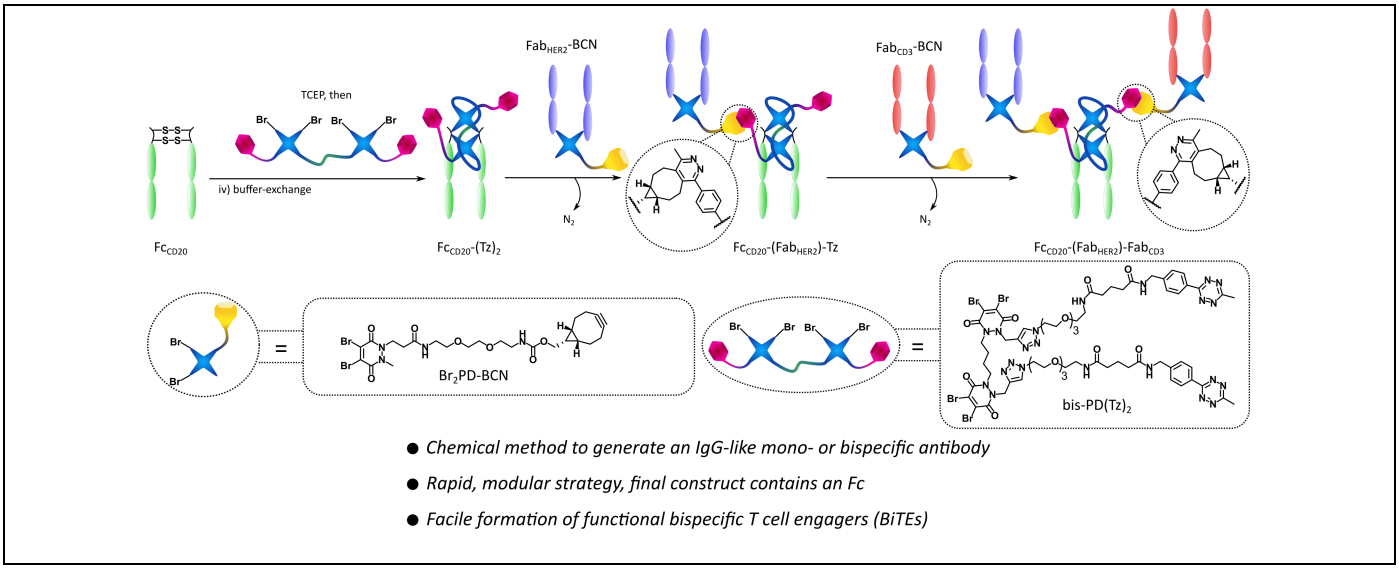
a: Department of Chemistry, University College London, 20 Gordon Street, London, WC1H 0AJ, UK.

b: Patrick G Johnston Centre for Cancer Research, School of Medicine, Dentistry and Biomedical Sciences, Queen's University Belfast, Belfast, UK.

*: Correspondence: v.chudasama@ucl.ac.uk, c.scott@qub.ac.uk, j.r.baker@ucl.ac.uk

†: These authors contributed equally to this work. As F.T. and P.S. contributed equally to this work, they are permitted to list their names as first on the author list on any C.V. or grant and fellowship application, etc. Their names were merely listed in this order arbitrarily, determined by a game of Keyforge.

Graphical Abstract



Abstract

In recent years there has been rising interest in the field of protein–protein conjugation, especially related to bispecific antibodies (bsAbs) and their therapeutic applications. These constructs contain two paratopes capable of binding two distinct epitopes on target molecules and are thus able to perform complex biological functions (mechanisms of action) not available to monospecific mAbs. Traditionally these bsAbs have been constructed through protein engineering, but recently chemical methods for their construction have started to (re-)emerge. While these have been shown to offer increased modularity, speed and for some methods, even the inherent capacity for further functionalization (e.g., with small molecule cargo), most of these approaches lacked the ability to include a fragment crystallizable (Fc) modality. The Fc component of IgG antibodies offers effector function and increased half-life. Here we report a first-in-class disulfide re-bridging and click-chemistry-based method for the generation of Fc-containing, IgG-like mono- and bispecific antibodies. These are in the Fc_Z-(Fab_X)-Fab_Y format, i.e., two distinct Fabs and an Fc, potentially all from different antibodies, attached in a homogeneous and covalent manner. We have dubbed these molecules synthetic antibodies (SynAbs). We have constructed a bispecific T cell-engager (BiTE) SynAb, Fc_{CD20}-(Fab_{HER2})-Fab_{CD3}, and have confirmed that it exhibits the expected biological functions, including the ability to kill $HER2^+$ target cells in a co-culture assay with T cells.

Introduction

Antibodies are symmetrical proteins composed of two identical fragment antigen-binding (Fab) domains responsible for their binding to a specific target, and one fragment crystallisable (Fc) conferring them immune-effector capacity and increased half-life. Bispecific antibodies (bsAbs) are (usually) artificial proteins containing two different binding elements (not necessarily Fab moieties) enabling their interaction with two epitopes of the same target or, in most cases, interaction with epitopes of two different targets.[1] The capacity of bsAbs to simultaneously interact with two targets/receptors offers various applications: 1) redirection of immune cells such as T cells, NK cells or macrophages toward tumour cells in order to trigger or improve immunosuppression of the tumour (such bsAbs are referred to as “immune cell engagers”, and have had a huge impact on the immunotherapy landscape);[2] 2) the simultaneous modulation of two different pathways in pathogenesis;[3] 3) increasing selectivity and/or avidity for a target cell by interacting with two different antigens at the cell surface (or two epitopes of the same antigen);[4] 4) holding effector proteins together as a substitute for an inactivated or faulty scaffold protein.[5,6] The majority of reported bsAbs in literature and clinical trials are T cell engagers, termed “Bispecific T cell Engagers” (BiTEs).[2] These BiTEs are designed to recruit immune cells to the tumour site by combining affinity for a receptor on the surface of T cells (usually CD3, a T cell co-receptor involved in T cell activation) and a tumour associated antigen (TAA). While one side of the bsAb interacts

with a T cell, activating it, the other side can interact with a target cell, bringing them into close vicinity and leading to formation of an immunological synapse which allows target cell destruction by the activated T cell.

Following initial clinical success in the early 2000s, bsAbs have been extensively studied and multiple bispecific antibody formats have been designed — more than a hundred, actually — where the binding elements can either be complete Fab moieties, or only portions of a Fab moiety such as scFv, DART or diabody. This multitude of bispecific antibody formats has been extensively reviewed elsewhere.[7–9] Interestingly, some bsAb formats possess an Fc moiety ("IgG-like bispecific antibodies"), while others do not ("bispecific antibody fragments").

Among the wide variety of bispecific antibody formats developed, fuelled by the search for optimal efficacy, geometry, half-life, stability, solubility, scalability or reproducibility, or the production of new IP, the method employed to produce them is almost exclusively protein bioengineering, generating so called recombinant or fusion proteins.[8,9] Indeed, as early as in the 60's, the first attempts at the chemical production of bispecific formats suffered from poor yields and complicated purification protocols.[10,11] Quickly, the production of bsAbs evolved to make use of quadroma cell line technology. Later on, several technologies improved the bioengineered production of bsAbs, including the "knobs-into-holes" (KIH),[12] the CrossMab,[13] and the FORCE ("Format Chain Exchange")[14] approaches. Despite obvious benefits, notably in terms of efficiency and high scalability, the use of bioengineering for bsAb production suffers from a lack of modularity — each new bsAb requires creation of new recombinant DNA sequences and expression of the related recombinant protein. The process can thus be time- and cost-intensive.

With recent improvements in the field, chemical and bioconjugation-based methods for the production of bsAbs have started appearing as valuable complementary approaches and potential alternatives to genetic engineering.[15] Indeed, organic chemistry/bioconjugation allows for the introduction of complementary chemical handles on two or more distinct proteins (native or recombinant) to enable their covalent assembly and thus generate a new protein construct, such as a bsAb. This strategy has been empowered by recent progress in selective protein modification,[16,17] and the development of ultra-fast, metal-free, and bioorthogonal click reactions,[18] including strain-promoted azide–alkyne cycloaddition (SPAAC),[19] strain-promoted alkyne-nitrone cycloaddition (SPANC),[20] or inverse electron-demand Diels–Alder (iEDDA) reactions involving partners such as tetrazine with trans-cyclooctene or strained alkynes, or strained alkyne with fluorosydnone.[21–23] The possibility to modify individual proteins and quickly assemble them into a bsAb offers great benefits over bioengineering regarding the modularity and speed of production. Furthermore, the chemical tools employed for protein modification and assembly also offer

the opportunity to introduce additional functionality on the protein construct (e.g., toxins, fluorophores, sensitizers for bsAb-conjugates, or masking moieties) similarly to how ADCs or probodies are produced.[16,24–26] This approach could also allow for varying of the nature, length, flexibility and stability of the linkers between the protein fragments. If the "chemical approach" was to become more mainstream, it could also facilitate access to these constructs for chemistry research teams without ready access to bioengineering. Nonetheless, the bioengineering and chemical methods to generate bsAbs and other protein constructs are not necessarily meant to compete. While the high scalability of bioengineering is well optimized, this aspect of chemical methods still needs to be investigated. However, chemical approaches applied at milligram to gram scales have demonstrated high speed and modularity, which are beneficial traits for high throughput screening processes not afforded by protein engineering. This makes chemical strategies a valuable complementary tool to recombinant technologies, e.g., by rapid chemical hit candidate-identification before an efficient fusion-based scale up.

The most modular methods in the field of bioconjugation strive to selectively introduce a chemical handle into the protein of interest — allowing subsequent modification. This handle can then be functionalized with an effector of choice (drug, fluorophore, or other protein) through a bioorthogonal click reaction. Due to the site-selective nature of the initial protein modification and the specificity of the click reaction employed, homogeneous protein–conjugates can be generated. Several tools have been developed for the selective chemical modification of proteins, exploiting solvent accessible (usually nucleophilic) amino acid side-chains (e.g., lysine, tyrosine, or cysteine) for functionalization.[17,24,27,28] A subset of cysteine-reactive modalities, disulfide re-bridging reagents, rely on the reduction of accessible disulfide bridges followed by their covalent reconnection *via* a small molecule — indeed these strategies have been used for the generation of protein–protein conjugates.[29,30] For this purpose, the Chudasama and Baker groups developed the pyridazinedione (PD) scaffold,[31–34] a chemical platform bearing: 1) two leaving groups across the double bond (generally bromine atoms) capable of reacting with the two liberated sulfhydryl groups generated *via* disulfide reduction, allowing for the covalent re-bridging of the disulfide; 2) up to two chemical handles for orthogonal click reactions, allowing selective dual-modification of the protein. In a three-step protocol (reduction, re-bridging and click reaction), the PD platform allows for the generation of protein-conjugates with controlled and homogenous conjugate/protein ratios. As an example, Maruani *et al.* could generate a dually functionalized antibody,[35] and a dually functionalized Fab_X-Fab_Y bispecific antibody construct (note the lack of an Fc fragment in this case).[25] PD platforms, combined with efficient click reactions, have thus proved to be valuable tools for protein modification and protein construct assembly.

In this paper we attempted a first-in-class purely chemical construction of full antibody-like constructs, dubbed SynAbs (synthetic antibodies) from the Fab and Fc fragments of commercially available antibodies. We described the generation of both a monospecific and bispecific SynAb in this manner. All the fragments were obtained by enzymatic digestion from commercially available native mAbs and the process relied completely on PD-mediated bioconjugation and Cu-free click chemistry with no protein engineering required. The SynAb constructs were evaluated for their biological activity. The Fc-containing bispecific SynAb was the first strategy described for chemically generating a bsAb with potential access to the biological functionality that could be provided by an Fc such as half-life extension or effector function.[36,37] Thus, this work represents a major contribution to the field of chemical bsAb-production.

Results

Prompted by our recent advances developing a pyridazinedione-based chemical method to produce a Fab-Fab bsAb format,[25] we decided to evolve the method further and adapt it to the production of Fc-containing IgG-like Abs and bsAbs, dubbed SynAbs (synthetic antibodies). The combination of two Fab targeting modules and an Fc moiety in the same antibody construct is appealing in many situations due to increased half-life and/or Fc-mediated effector function it can provide.[36,37] This approach is far more challenging than the previously reported Fab-Fab format as it involves the separate selective chemical modification, followed by assembly, of three individual protein modules. We theorized that this would be best achieved with pyridazinedione-based methods as they offer high site-selectivity in addition to excellent modularity due to the two possible functional handles they can bear. Our strategy to make IgG-like mono- and bispecific SynAbs was to 1) enzymatically generate and isolate various protein fragments (Fab and Fc) from their parent monoclonal antibodies, and 2) individually modify the isolated fragments with pyridazinedione (PD) molecular platforms bearing different click handles *via* disulfide re-bridging. Importantly, the handle incorporated in the Fab moieties had to be reactive toward the ones introduced on the Fc. 3) These preparations would then culminate in the covalent assembly of the three individual protein fragments into a mono- or bispecific SynAb through selective click ligation.

As demonstrated before in our group,[32,34] a crucial advantage of the Br_2PD-based method is the selective re-bridging of solvent-accessible disulfides in proteins. This leads to the controlled introduction of only one PD per Fab fragment, which presents only one accessible interchain disulfide; and two PDs on the Fc moiety, which has two accessible disulfides located in its hinge region (in case of an IgG1 parent isoform such as rituximab). Thus, if the introduced Br_2PD motifs each contain one click handle, they confer only one modification site for the Fab, and two for the Fc, upon click reaction. With this methodology, controlled modification of Fab or Fc fragments with various small molecules (drug, fluorophore, etc.) is

possible.[35,38] However, here, we intended to connect two "mono-clickable Fab" moieties to the "dually-clickable Fc" moiety. If two identical Fabs (two Fab_X) are chemically connected to Fc_Z, a monospecific Fc_Z-$(Fab_X)_2$ SynAb would be generated, while two different Fabs (Fab_X and Fab_Y) chemically connected to the Fc would yield a bispecific Fc_Z-(Fab_X)-Fab_Y SynAb.

1. Generation of HER2/HER2 SynAb **1** and **2**

1.1 Mono-PD method

As a proof-of-concept, we first attempted to produce a monospecific SynAb (Fc_Z-$(Fab_X)_2$). The process consisted of enzymatic digestion of commercially available native anti-HER2 (trastuzumab) and anti-CD20 (rituximab) antibodies to isolate the Fab_{HER2} and Fc_{CD20} fragments, respectively, following previously described procedures (see ESI for details).[25] After site-selective modification of the Fab and Fc fragments with Br_2PD molecules bearing complementary click handles, a click reaction between the mono clickable-Fab and dually clickable-Fc would enable the construction of an IgG-like SynAb construct, Fc_{CD20}-$(Fab_{HER2})_2$ **1**. The click reactions used to connect proteins have to be both bioorthogonal to avoid undesired cross-linking, and ultra-fast to counteract the steric hinderance of the protein partners that makes protein–protein cross-linking slow. For this purpose, we chose to work with the Tetrazine–BCN (bicyclononyne) click ligation to chemically produce the SynAb, as it is fast (up to 10^4 $M^{-1}.s^{-1}$ in MeOH/H_2O solution),[39] compatible with aqueous media, and the resulting pyridazine linkage is stable.[15,40] This strategy required the synthesis of the corresponding Br_2PD-tetrazine **3** and Br_2PD-BCN **4** to be incorporated in the Fc and Fab fragments. These molecules were synthesized as before in our group (see ESI for details).[25]

Next, we proceeded with the selective modification of Fab_{HER2} and Fc_{CD20}. For both, a two-step procedure was employed, consisting of: 1) reduction of accessible disulfide bridge(s) with excess TCEP over 1-2 h, followed by removal of the remaining TCEP through ultrafiltration/buffer-exchange; and 2) disulfide re-bridging with excess of Br_2PD-Tz **3** or Br_2PD-BCN **4** (10 to 20 eq. for 2-4 h) before removal of unreacted Br_2PD *via* buffer exchange/ultrafiltration.

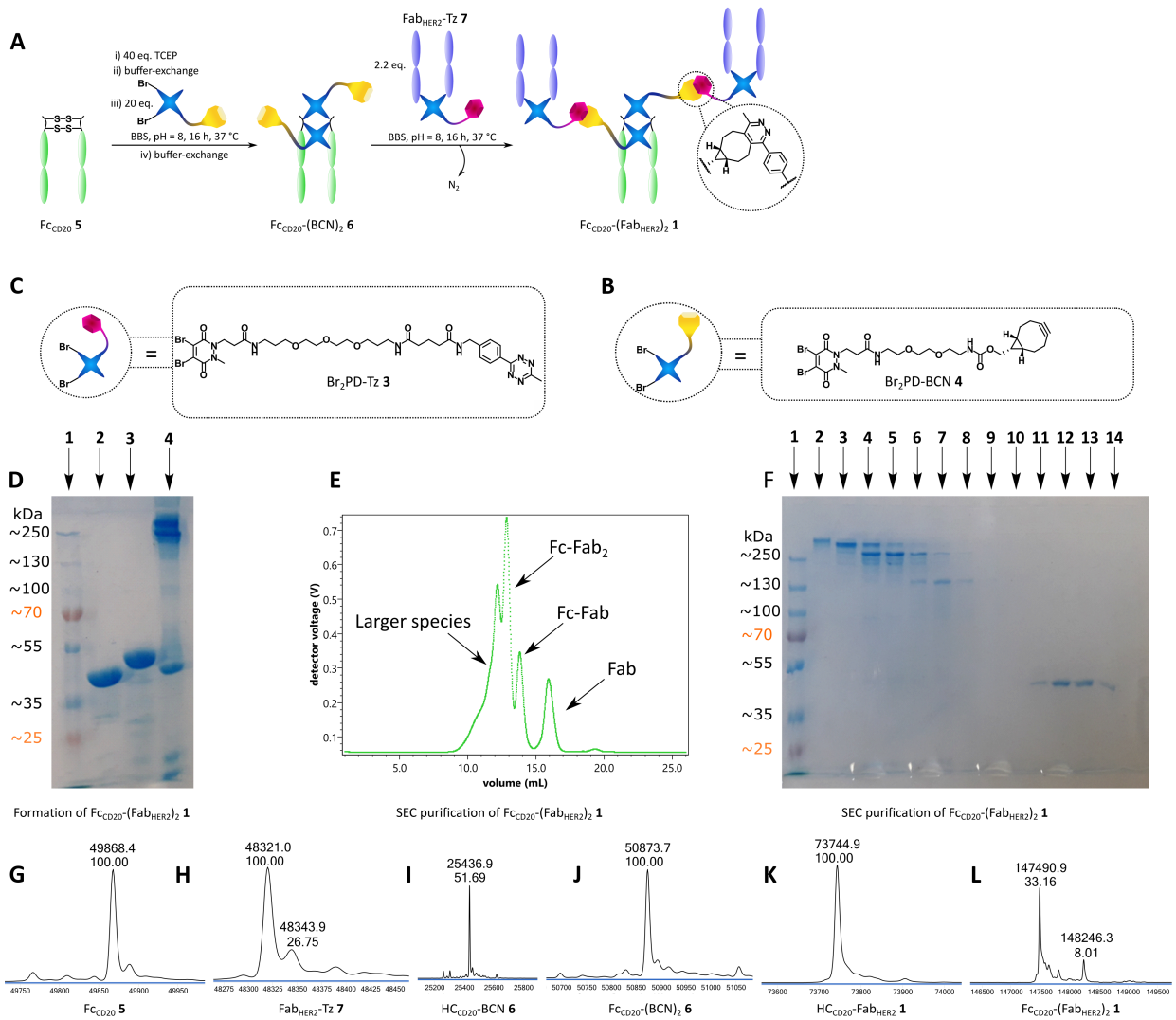


Figure 1 | **Chemical construction of a full antibody to generate Fc_{CD20}-$(Fab_{HER2})_2$ SynAb 1.** **A |** Mono-PD method for the construction of a full antibody Fc_{CD20}-$(Fab_{HER2})_2$ SynAb **1**. Fc_{CD20} **5** is sequentially reduced and re-bridged with Br_2PD-BCN **4**. The resulting Fc_{CD20}-$(BCN)_2$ **6** is then reacted with Fab_{HER2}-Tz **7** to generate Fc_{CD20}-$(Fab_{HER2})_2$ SynAb **1** after SEC purification. **B |** Br_2PD-Tz **3** used for SynAb synthesis. **C |** Br_2PD-BCN **4** used for SynAb synthesis. **D |** SDS-PAGE analysis of Fc_{CD20}-$(Fab_{HER2})_2$ SynAb **1** formation. Lane 1: Ladder. Lane 2: Fab_{HER2} **8**. Lane 3: Fc_{CD20} **5**. Lane 4: Crude Fc_{CD20}-$(Fab_{HER2})_2$ SynAb **1**. **E |** UV trace of SEC purification of Fc_{CD20}-$(Fab_{HER2})_2$ SynAb **1**. **F |** SDS-PAGE analysis of SEC purification of SynAb **1**. Lane 1: Ladder. Lane 2-3: Aggregates. Lane 4-6: SynAb **1**. Lane 7-8: BCN-Fc_{CD20}-Fab_{HER2}. Lane 11-14: Fab_{HER2} **8**. **G |** LC-MS analysis of Fc_{CD20} **5**. Observed mass: 49868 Da. **H |** LC-MS analysis of Fab_{HER2}-Tz **7**. Expected mass: 48334 Da. Observed mass: 48321 Da. **I,J |** LC-MS analysis of Fc_{CD20}-$(BCN)_2$ **6**. Expected mass: 50873 Da (Fc_{CD20}-$(BCN)_2$ **6**) and 25438 Da (disulfide scrambled Fc_{CD20}-$(BCN)_2$ **6**). Observed mass: 50874 Da and 25437 Da. **K,L |** LC-MS analysis of Fc_{CD20}-$(Fab_{HER2})_2$ SynAb **1**. Expected mass: 147486 Da (Fc_{CD20}-$(Fab_{HER2})_2$ **1**) and 73744 Da (disulfide scrambled Fc_{CD20}-$(Fab_{HER2})_2$ **1**). Observed mass: 147491 Da, 148246 Da (Δ = 755 Da) and 73745 Da.

Successful incorporation of one Br_2PD-Tz **3** molecule into Fab_{HER2} **8** or two Br_2PD-BCN **4** into the Fc_{CD20} **5** to yield Fab_{HER2}-Tz **7** and Fc_{CD20}-$(BCN)_2$ **6**, respectively, could be confirmed by LC-MS analysis (Figure 1/H-J). However, as expected, we also observed that re-bridging of the Fc with two Br_2PD-BCN **4** molecules led to some, albeit minimal, disulfide scrambling when each Br_2-PD-BCN **4** connects the two –SH of the same heavy chain (intra-chain re-bridging) rather than those of the two heavy chains (inter-chain re-bridging). This phenomenon is well known in the case of mAb re-bridging, and in that case leads to so-called "half antibody" formation. Non-covalent interactions will hold the two heavy chains together in solution, and it has been shown that disulfide scrambling has no impact on antigen binding and minimal impact on Fc-mediated function in the case of an IgG1.[41] However, under denaturing analytical conditions (SDS-PAGE or LC-MS), the disulfide scrambled species, HC_{CD20}-BCN in the case of Fc_{CD20}-$(BCN)_2$ **6**, can be observed (Figure 1/I). It is important to note, that while these scrambled species appeared in the LC-MS spectra, this was a major overrepresentation of their actual abundance — SDS-PAGE (Figure 1/D and F) clearly shows that this was a minor species in the case of both Fc_{CD20}-$(BCN)_2$ **6** and Fc_{CD20}-$(Fab_{HER2})_2$ SynAb **1**. But even if this species was more abundant, as detailed before, it would not be expected to affect the biological function of the construct.[41]

The next step consisted of reacting 2.2 eq. of Fab_{HER2}-Tz **7** with the Fc_{CD20}-$(BCN)_2$ **6** to generate Fc_{CD20}-$(Fab_{HER2})_2$ SynAb **1**. SDS-PAGE analysis showed that after 16 h, all Fc had been consumed in the crude reaction (Figure 1/D). Satisfyingly, Fc_{CD20}-$(Fab_{HER2})_2$ SynAb **1** was isolated after SEC purification (Figure 1/E) with purity confirmed by SDS-PAGE (Figure 1/F) and LC-MS analysis (Figure 1/K,L). We highlight that, to the best of our knowledge, this is the very first time that an IgG-like antibody construct has been generated exclusively *via* chemical methods.

1.2 Bis-PD method

With these initial promising results in hand regarding the production of SynAb **1**, we set about developing a more elegant method that eliminated the (admittedly minor) issues with disulfide scrambling. It was proposed that using a bis-PD linker (where two Br_2PD functionalities are covalently linked) to install click handles onto the Fc could be the solution. While disulfide scrambling would still occur, the covalent linkage between the two heavy chains of the Fc would be maintained regardless. An additional benefit to this approach is making LC-MS analysis simpler as there would be only one expected product mass. Hence, a new synthetic route was developed to generate an appropriate click-enabled bis-PD. The click handle chosen for the bis-PD was tetrazine, as in our experience it is quite stable under the reaction conditions (Figure 2/B, see ESI for details on synthesis).

New trials of SynAb production were carried out with similar conditions to those described above, except that 7.5 eq. of bis-PD(Tz)$_2$ **9** were employed to re-bridge Fc$_{CD20}$ **5**, and Fab$_{HER2}$ **8** was correspondingly functionalized with Br$_2$PD-BCN **4** (Figure 2/A). To our delight, excellent purity for both Fc$_{CD20}$-(Tz)$_2$ **10** and Fab$_{HER2}$-BCN **11** was obtained as confirmed by LC-MS analysis (Figure 2/G,H). In addition, SDS-PAGE gel analysis revealed only one band corresponding to Fc$_{CD20}$-(Tz)$_2$ **10**, confirming that the bis-PD compound prevents formation of half-Fc by cross-linking the two heavy chains regardless of the way the disulfides are re-bridged by the compound (Figure 2/D). The freshly prepared Fc$_{CD20}$-(Tz)$_2$ **10** (1 eq.) and Fab$_{HER2}$-BCN **11** (3.4 eq.) were then mixed together overnight at 37°C in BBS buffer pH 8 to generate the Fc$_{CD20}$-(Fab$_{HER2}$)$_2$ SynAb construct **2** *via* SPIEDAC reaction. According to SDS-PAGE (Figure 2/D) and SEC UV (Figure 2/E) analysis, complete consumption of Fc$_{CD20}$-(Tz)$_2$ **10** was achieved after this time. However, more Fc$_{CD20}$-(Fab$_{HER2}$)-Tz **12** mono-adduct was observed than with the previous mono-PD strategy, suggesting worse conversion from the mono-adduct to the di-adduct than previously, even with higher equivalents of Fab$_{HER2}$ used in this case. In any event, LC-MS analysis confirmed the formation and isolation of Fc$_{CD20}$-(Fab$_{HER2}$)$_2$ SynAb **2** with good purity (Figure 2/J). Crucially, this time no “half-antibody” type species was observed, with the product represented by a single peak in LC-MS analysis, validating the use of a bis-PD strategy for SynAb assembly.

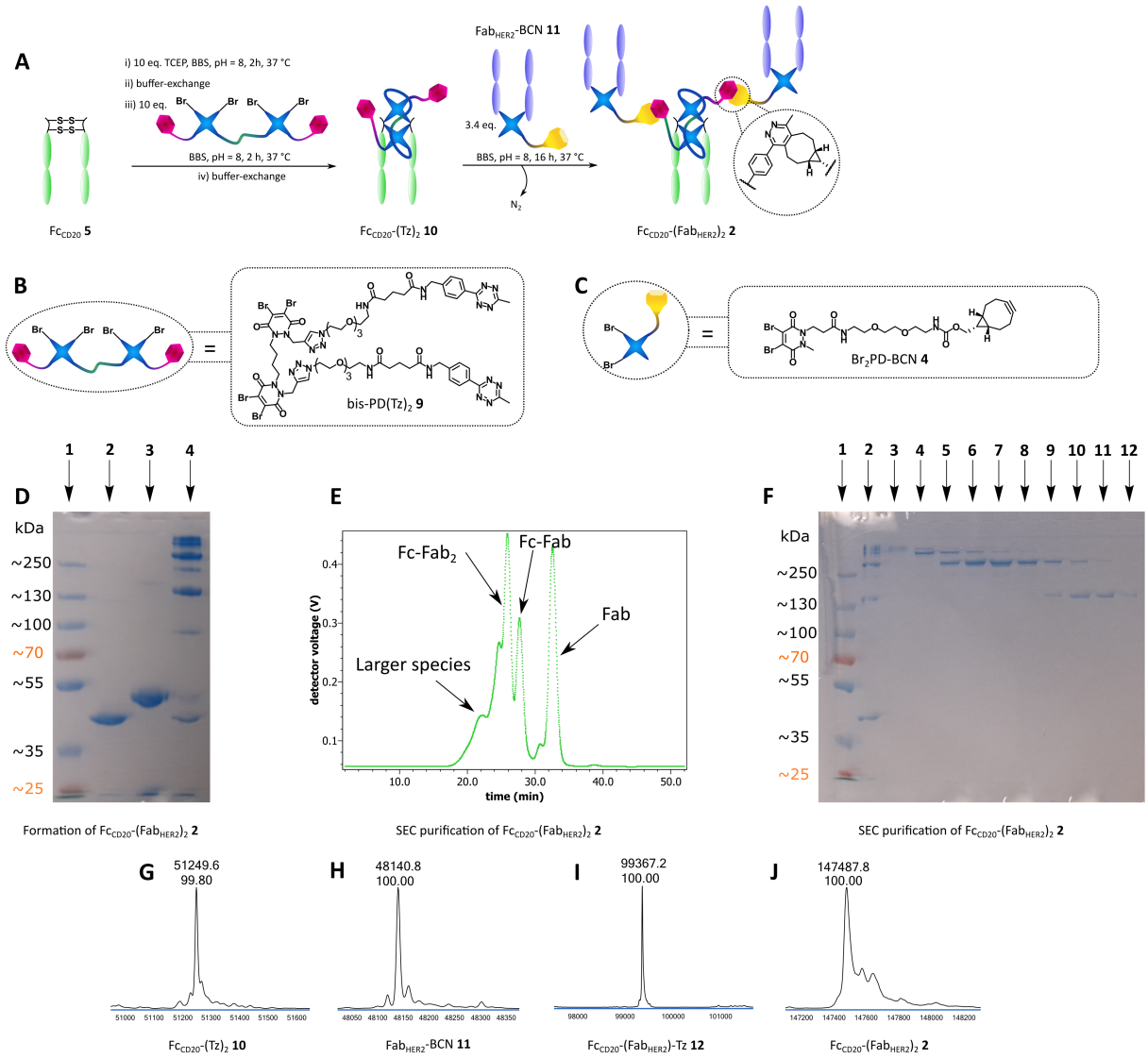


Figure 2 **| Chemical construction of a full antibody to generate Fc$_{CD20}$-(Fab$_{HER2}$)$_2$ SynAb 2.**
A | Bis-PD method for the construction of a full antibody Fc$_{CD20}$-(Fab$_{HER2}$)$_2$ SynAb **2**. Fc$_{CD20}$ **5** is sequentially reduced and re-bridged with bis-PD(Tz)$_2$ **9**. The resulting Fc$_{CD20}$-(Tz)$_2$ **10** is then reacted with Fab$_{HER2}$-BCN **11** to generate Fc$_{CD20}$-(Fab$_{HER2}$)$_2$ SynAb **2** after SEC purification. **B |** Bis-PD(Tz)$_2$ **9** used for SynAb synthesis. **C |** Br$_2$PD-BCN **4** used for SynAb synthesis. **D |** SDS-PAGE analysis of Fc$_{CD20}$-(Fab$_{HER2}$)$_2$ SynAb **2** formation. Lane 1: Ladder. Lane 2: Fab$_{HER2}$-BCN **11**. Lane 3: Fc$_{CD20}$-(Tz)$_2$ **10**. Lane 4: crude Fc$_{CD20}$-(Fab$_{HER2}$)$_2$ SynAb **2**. **E |** UV trace of SEC purification of Fc$_{CD20}$-(Fab$_{HER2}$)$_2$ SynAb **2**. **F |** SDS-PAGE analysis of SEC purification of Fc$_{CD20}$-(Fab$_{HER2}$)$_2$ SynAb **2**. Lane 1: Ladder. Lane 2-5: Large species. Lane 6-9: Fc$_{CD20}$-(Fab$_{HER2}$)$_2$ SynAb **2**. Lane 10-12: Fc$_{CD20}$-(Fab$_{HER2}$)-Tz **12**. **G |** LC-MS analysis of Fc$_{CD20}$-(Tz)$_2$ **10**. Expected mass: 51246 Da. Observed mass: 51250 Da. **H |** LC-MS analysis of Fab$_{HER2}$-BCN **11**. Expected mass: 48141 Da. Observed mass: 48141 Da. **I |** LC-MS analysis of Fc$_{CD20}$-(Fab$_{HER2}$)-Tz **12**. Expected mass: 99359 Da. Observed mass: 99367 Da. **J |** LC-MS analysis of Fc$_{CD20}$-(Fab$_{HER2}$)$_2$ SynAb **2**. Expected mass: 147472 Da. Observed mass: 147488 Da.

2. Generation of the HER2/CD3 bispecific Fc_{CD20}-(Fab_{HER2})-Fab_{CD3} SynAb **13**

The production of the Fc_{CD20}-(Fab_{HER2})$_2$ SynAbs **1** and **2** was a great success. However, the utility of this IgG-like antibody construct is moderate and was merely envisaged as a proof-of-concept. Granted, the ability to vary the Fc compared to the Fab modules can offer some benefits, as the Fc can be extensively engineered to extend or reduce half-life and/or effector function, or to otherwise modulate the bioavailability of the antibody — thus any targeting modality could be coupled to an Fc of choice (chosen from a library of various engineered Fc modalities for instance) based on these parameters.[42–44] But to unlock the full potential of the method, we moved on to our main goal — the chemical production of an IgG-like bispecific SynAb. Since bispecific T cell engagers (BiTEs) are perhaps the most therapeutically relevant class of bsAbs, we chose to attempt the grafting of a Fab_{HER2} and a Fab_{CD3} on Fc_{CD20}, with the aim to produce an "IgG-like BiTE", able to recruit T cells (through CD3-binding) to HER2$^+$ tumour cells. Granted, the presence of an Fc with immune-effector function is not required in the context of a T cell engagement strategy, and it can even be considered detrimental due to decreased tumour penetration resulting from the higher molecular weight of the bsAb, or especially the undesired depletion of engaged T cells. On the other hand, incorporating an Fc moiety into the construct can be crucial for half-life extension.[45–47] For other applications where the dual targeting of a bsAb is exploited, the effector function of an Fc (ADCC, ADCP, etc.)[42,48] can be beneficial as well. In either case, the Fc can also be a platform for further modification (e.g., sugar/glutamine modification).[49,50] For example, Hemlibra® (emicizumab), is an IgG-like bsAb approved by the FDA in 2017 for patients with haemophilia A, replacing the function of coagulation factor VIII, the protein deficient in this condition.

We thus attempted the production of an IgG-like BiTE to exemplify the potential of our method. The strategy was based on the bis-PD method described above for monospecific SynAb production, albeit with some slight modifications. A symmetric bis-PD, with two equivalent tetrazine handles, was used to functionalize Fc_{CD20} **5**. Thus Fab_{HER2} **8** and Fab_{CD3} **14** had to be introduced sequentially for maximum homogeneity. This strategy necessitated an intermediate SEC purification step after the addition of Fab_{HER2}-BCN **11** to Fc_{CD20}-(Tz)$_2$ **10**, to ensure isolation of pure Fc_{CD20}-(Fab_{HER2})-Tz **12** (Figure 3/A).

Digestion of anti-CD3 antibody was carried out successfully to generate Fab_{CD3} **14** (see ESI for details). With the fragments in hand, optimisation of the previous SynAb procedure allowed reduction (10 eq. of TCEP) and re-bridging of Fc_{CD20} **5** with 7.5 eq. of bis-PD(Tz)$_2$ **9** in a two-step procedure (4 h overall). Both Fab_{HER2} **8** and Fab_{CD3} **14** were reduced (10 eq. of TCEP) and re-bridged with 10 eq. of Br_2PD-BCN **4** in a two-

step procedure (4 h overall). Important to note that both these species were used fresh to avoid oxidation of the BCN moiety, thus Fab_{CD3}-BCN **15** was only prepared after the intermediate purification step. These smaller equivalents and shorter reaction times still allowed the generation of the corresponding Fc_{CD20}-$(Tz)_2$ **10**, Fab_{HER2}-BCN **11** and Fab_{CD3}-BCN **15** species with full conversion and high purity as confirmed by LC-MS (Figure 3/G-I). The initial click reaction was carried out by mixing 50 nmol of Fc_{CD20}-$(Tz)_2$ **10** with 30 nmol of Fab_{HER2}-BCN **11** (0.6 eq.), which was introduced in sub-stoichiometric quantity in order to favour the mono-addition product Fc_{CD20}-(Fab_{HER2})-Tz **12**. After 16 h of incubation at 37 °C, SDS-PAGE analysis confirmed the generation of a protein of the expected size of Fc_{CD20}-(Fab_{HER2})-Tz **12** but also some unwanted presence of Fc_{CD20}-$(Fab_{HER2})_2$ **2**, as well as left over Fc_{CD20}-$(Tz)_2$ **10** and Fab_{HER2}-BCN **11** (Figure 3/E).

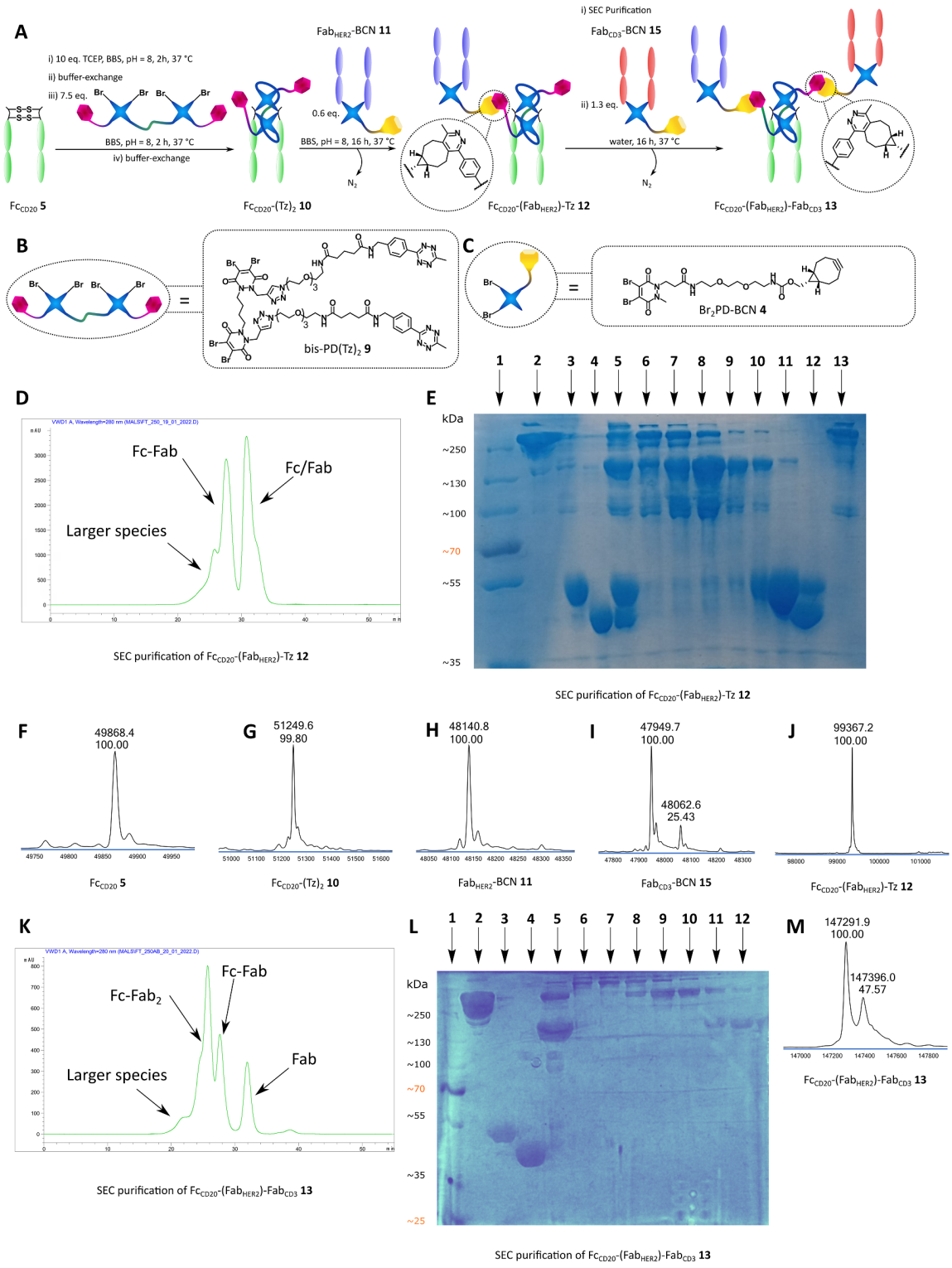


Figure 3 **| Chemical generation of bispecific Fc_{CD20}-(Fab_{HER2})-Fab_{CD3} SynAb 13.**

A | Bis-PD method for the construction of bispecific Fc_{CD20}-(Fab_{HER2})-Fab_{CD3} SynAb **13**. Fc_{CD20} **5** is sequentially reduced and re-bridged with bis-PD$(Tz)_2$ **9**. The resulting Fc_{CD20}-$(Tz)_2$ **10** is then reacted with Fab_{HER2}-BCN **11** to generate Fc_{CD20}-(Fab_{HER2})-Tz **12** after SEC purification. Fc_{CD20}-(Fab_{HER2})-Tz **12** is reacted further with Fab_{CD3}-BCN **15** to generate bispecific Fc_{CD20}-(Fab_{HER2})-Fab_{CD3} SynAb **13** after SEC purification. **B |** The bis-PD$(Tz)_2$ **9** used for bispecific SynAb synthesis. **C |** Br_2PD-BCN **4** used for bispecific SynAb synthesis. **D |** UV trace of SEC purification of Fc_{CD20}-(Fab_{HER2})-Tz **12**. **E |** SDS-PAGE analysis of Fc_{CD20}-(Fab_{HER2})-Tz **12** intermediate formation and SEC purification. Lane 1: Ladder. Lane 2: Anti-HER2 mAb (trastuzumab). Lane 3: Fc_{CD20}-$(Tz)_2$ **10**. Lane 4: Fab_{HER2}-BCN **11**. Lane 5: Crude Fc_{CD20}-(Fab_{HER2})-Tz **12**. Lane

6-7 & 13: Fc_{CD20}-$(Fab_{HER2})_2$ **2** by-product/larger species. Lane 8-9: Purified Fc_{CD20}-(Fab_{HER2})-Tz **12**. Lane 10-12: Left-over Fab and Fc species. **F |** LC-MS analysis of Fc_{CD20} **5**. Observed mass: 49868 Da. **G |** LC-MS analysis of Fc_{CD20}-$(Tz)_2$ **10**. Expected mass: 51246 Da. Observed mass: 51250 Da. **H |** LC-MS analysis of Fab_{HER2}-BCN **11**. Expected mass: 48141 Da. Observed mass: 48141 Da. **I |** LC-MS analysis of Fab_{CD3}-BCN **15**. Expected mass: 47948 Da. Observed mass: 47950 Da. **J |** LC-MS analysis of Fc_{CD20}-(Fab_{HER2})-Tz **12**. Expected mass: 99359 Da. Observed mass: 99367 Da. **K |** UV trace of SEC purification of bispecific Fc_{CD20}-(Fab_{HER2})-Fab_{CD3} SynAb **13**. **L |** SDS-PAGE analysis of SEC purification of Fc_{CD20}-(Fab_{HER2})-Fab_{CD3} SynAb **13**. Lane 1: Ladder. Lane 2: Anti-HER2 mAb (trastuzumab). Lane 3: Fc_{CD20}-$(Tz)_2$ **10**. Lane 4: Fab_{HER2}-BCN **11**. Lane 5: Fc_{CD20}-(Fab_{HER2})-Tz **12** intermediate. Lane 6-8: Large species. Lane 9-10: Fc_{CD20}-(Fab_{HER2})-Fab_{CD3} SynAb **13**. Lane 11-12: Left-over Fc_{CD20}-(Fab_{HER2})-Tz **12**. **M |** LC-MS analysis of bispecific Fc_{CD20}-(Fab_{HER2})-Fab_{CD3} SynAb **13**. Expected mass: 147284 Da and 147398 Da. Observed mass: 147292 Da and 147396 Da.

SEC purification allowed for the isolation of Fc_{CD20}-(Fab_{HER2})-Tz **12** in reasonable purity, although with small amounts of unwanted Fc_{CD20}-$(Fab_{HER2})_2$ SynAb **2** remaining according to a highly concentrated SDS-PAGE (Figure 3/E). Carrying out the purification on a larger SEC column should improve the separation of these species. Even so, the SynAb **2** did not appear in the LC-MS spectrum and the Fc_{CD20}-(Fab_{HER2})-Tz **12** intermediate was quite pure (Figure 3/J, see ESI for complete spectrum). Purified Fc_{CD20}-(Fab_{HER2})-Tz **12** was then mixed with an excess of freshly prepared Fab_{CD3}-BCN **15** (1.3 eq.) in water at 37 °C for 16 h. Satisfyingly, SEC purification of the crude mixture allowed for isolation of the desired bispecific Fc_{CD20}-(Fab_{HER2})-Fab_{CD3} SynAb **13** with excellent purity as confirmed by SDS-PAGE and LC-MS analysis (Figure 3/K-M). The additional peak visible in the LC-MS spectrum is due to Fab_{CD3} being digested to two species with a single amino acid difference by papain (see ESI for details). To the best of our knowledge, this result constitutes the first example of an IgG-like bsAb produced *via* purely chemical methods from the corresponding Fab and Fc fragments. The overall process including protein digestion, protein reduction and re-bridging, both protein–protein click reactions and purifications was carried out in only 5 days. Importantly, due to the highly modular nature of this method, it could easily be adapted to generate many other Fc_Z-(Fab_X)-Fab_Y IgG-like bispecific SynAbs with various Fab/Fc combinations. Only the initial parent antibodies would need to be changed, but all other steps (reduction, re-bridging, protein–protein click) would still function, likely requiring only minor or no optimisation. The speed, modularity, homogeneity, and efficiency of this chemical method to produce IgG-like bsAbs makes it a valuable complement or alternative to bioengineering, with characteristics particularly suited for high-throughput screening and quick identification of hits.

3. Biological evaluation of SynAb **2** and bispecific SynAb **13**

To further validate our chemical method for production of IgG-like proteins, we evaluated the biological functions of the Fc_{CD20}-$(Fab_{HER2})_2$ SynAb **2** and bispecific Fc_{CD20}-(Fab_{HER2})-Fab_{CD3} SynAb **13** in binding and cell viability assays *in vitro*. Binding of both constructs to HCC1954 ($HER2^+CD3^-$) cancer cells and Jurkat ($CD3^+HER2^-$) cells was investigated by flow cytometry (three independent experiments in both cases, Figure 4/A). SynAb **2** or bispecific SynAb **13** compounds were incubated with HCC1954 ($HER2^+CD3^-$) cells and then stained with PE-labelled anti-IgG Fc. PE fluorescence was analysed *via* flow cytometry. The assay revealed no increase in fluorescence for cells incubated with PE-labelled anti-IgG Fc alone in comparison to untreated cells, while a clear increase in fluorescence was observed in case the cells were pre-incubated with either SynAb. This experiment indicated that both antibody constructs exhibited binding to $HER2^+$ cells, as expected. Not unexpectedly, the monospecific Fc_{CD20}-$(Fab_{HER2})_2$ SynAb **2** exhibited increased binding to $HER2^+$ cells compared to the bispecific SynAb **13**. This observation could be explained by the monospecific construct being bivalent for HER2-binding as opposed to the bispecific construct which is monovalent. Thus, the avidity effect would dictate stronger binding of the bivalent but monospecific SynAb **2.** These results confirm that the HER2 binding capacity of the constructs is retained after the digestion, reduction, re-bridging and assembly steps used during SynAb production. While the function of the Fc moiety was not directly tested, the results show that it retains its epitope for the PE-labelled anti-IgG Fc, the secondary antibody used for fluorescent detection of the constructs.

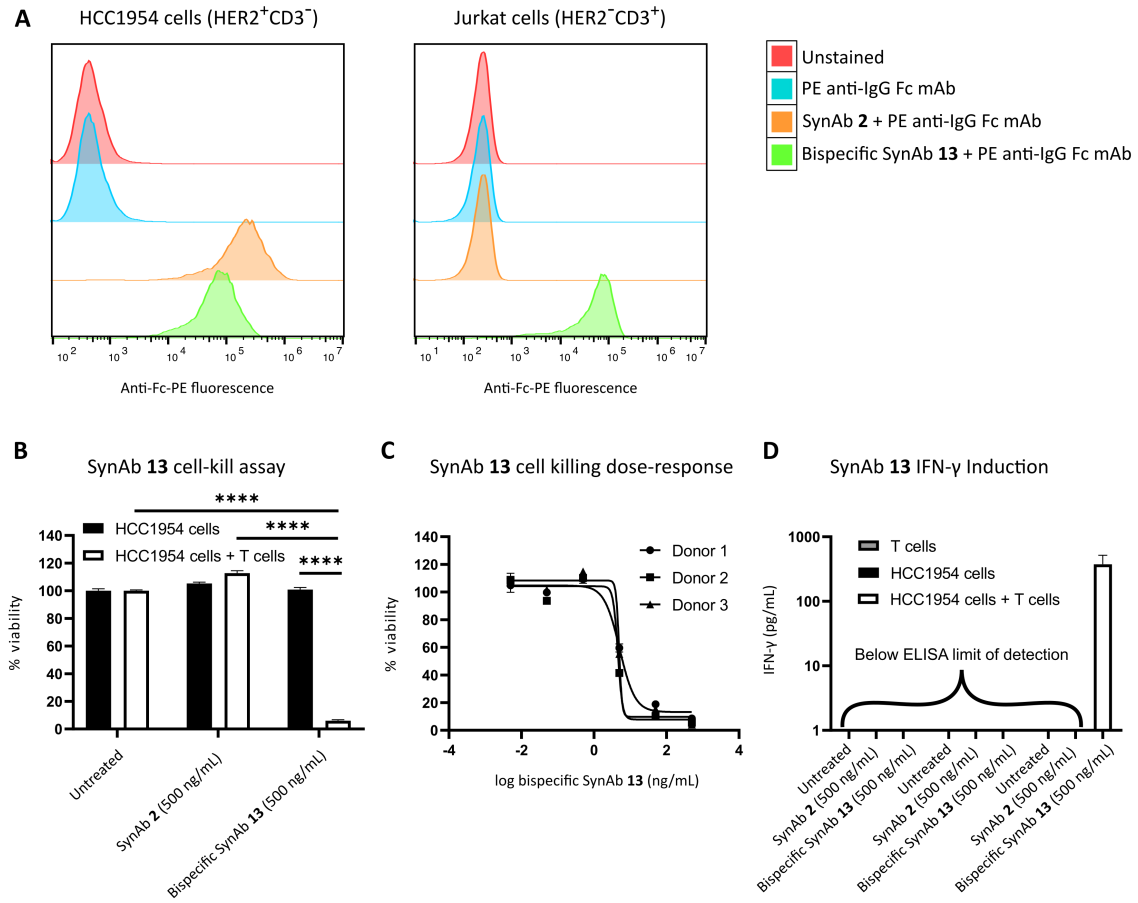


Figure 4 **| Biological testing of SynAbs 2 and 13. A |** Binding of SynAb **2** and bispecific SynAb **13** to HCC1954 (HER2$^+$CD3$^-$) and Jurkat (HER2$^-$CD3$^+$) cells. Flow cytometry histograms representative of three independent experiments. Cells were incubated with SynAb **2** or bispecific SynAb **13** followed by incubation with PE-labelled anti-IgG Fc antibody. PE fluorescence was measured by flow cytometry. **B |** Cytotoxicity assay of SynAb **2** and bispecific SynAb **13**. HCC1954 (HER2$^+$CD3$^-$) cells alone or HCC1954/T cell co-cultures (E:T ratio of 10:1) were either not treated or incubated with 500 ng/mL (3.4 nM) SynAb **2** or 500 ng/mL (3.4 nM) bispecific SynAb **13**. HCC1954 viability was assessed by CellTiter-Glo at 48 h following treatment. Data from three biologically independent experiments (three different blood donors) with three replicates each. **C |** Cytotoxicity dose-response curve of bispecific SynAb **13**. HCC1954/T cell co-cultures (E:T ratio of 10:1) were incubated with varying concentrations of bispecific SynAb **13** (serial dilutions ranging from 0.005 to 500 ng/mL, 0.034 pM to 3.4 nM). HCC1954 viability was assessed by CellTiter-Glo at 48 h following treatment. Data from three biologically independent experiments (three different blood donors) with three replicates each. **D |** Induction of IFN-γ production by bispecific SynAb **13**. T cells alone, HCC1954 (HER2$^+$CD3$^-$) cells alone or HCC1954/T cell co-cultures (E:T ratio of 10:1) were either not treated or treated with 500 ng/mL (3.4 nM) SynAb **2** or 500 ng/mL (3.4 nM) bispecific SynAb **13**. Culture supernatant IFN-γ was quantified by ELISA at 48 h following treatment. Data from three biologically independent experiments (three different blood donors) with three replicates each. **|** Data represented as means + SEM. For statistical analysis, two-way ANOVA was used followed by post-hoc Tukey's honestly significant difference multiple comparisons test with multiplicity-adjusted P values with $\alpha = 0.05$. ****P < 0.0001. Curves in **C** fitted with non-linear regression model "Sigmoidal, 4PL, X is log(concentration)".

Binding to Jurkat ($CD3^+HER2^-$) cells was carried out under similar conditions, by incubating SynAb **2** or bispecific SynAb **13**, followed by staining with PE-labelled anti-IgG Fc (Figure 4/A). As expected, only the bispecific Fc_{CD20}-(Fab_{HER2})-Fab_{CD3} SynAb **13** induced an increase in fluorescence when compared to untreated cells, confirming the capacity of the bispecific SynAb **13** to bind to $CD3^+$ cells, driven by the presence of the Fab_{CD3} moiety present in the construct. Again, these results suggest that the affinity of the Fab_{CD3} for the CD3 receptor was not abrogated by previous digestion, reduction, re-bridging and assembly steps. Monospecific Fc_{CD20}-(Fab_{HER2})$_2$ SynAb **2** staining, on the contrary, and as expected, lead to no increase in PE fluorescence compared to untreated cells, due to the lack of any CD3 binding module.

After validation of the target-binding ability of the SynAb constructs, the capacity of the bispecific SynAb **13** to recruit T cells to HCC1954 ($HER2^+$) cells and induce T cell-mediated cell death was evaluated on three blood donor samples. T cell / HCC1954 co-cultures (E:T ratio of 10:1) or HCC1954 monocultures were treated with 500 ng / mL of bispecific SynAb **13** or SynAb **2** (as a control), or not treated, and HCC1954 viability was assessed after 48h. As expected, cell viability did not decrease for cells treated with Fc-(Fab_{HER2})$_2$ SynAb **2** with or without T cells, and for Fc-(Fab_{HER2})-Fab_{CD3} bispecific SynAb **13** without T cells, when compared to untreated cells. On the contrary, to our delight, HCC1954 cell viability was reduced when both bispecific SynAb **13** and T cells were present (Figure 4/B). This reduction in viability was also confirmed to be dose-dependent (Figure 4/C). This suggests that the construct exhibited T cell engagement activity through simultaneous binding of CD3 and HER2 in trans (i.e., on different cells). To further confirm that target cell-killing was due to increased T cell activation, expression of IFN-γ (a T cell activation marker) was evaluated. For this purpose, T cell / HCC1954 cocultures (E:T ratio of 10:1), HCC1954 monocultures or T cell monocultures were treated with 500 ng / mL bispecific SynAb **13** or SynAb **2.** Culture supernatant IFN-γ was quantified by ELISA at 48 hours following treatment (Figure 4/D). As expected, only the condition where bispecific SynAb **13** was incubated with HCC1954 cells co-cultured with T cells induced expression of IFN-γ confirming that the construct drove T cell activation in the presence of target cells. It is also important to note that no IFN-γ production was observed when the construct was incubated with T cells alone, suggesting that immune activation would be primarily localized to the tumour environment, decreasing the potential for side effects, such as cytokine release syndrome (CRS), and increasing the therapeutic window.[51] Based on the three blood donor samples, the HCC1954 cell viability IC_{50} for bispecific SynAb **13** was determined to be 4.9 ± 0.2 ng / mL. This value is similar to IC_{50} values reported in literature for engineered (HER2 × CD3) BiTEs — with or without an Fc — and evaluated *in vitro* with similar E:T ratio on high HER2 expressing cell lines.[36,37,52,53] Overall, these biological results demonstrate potent T cell engager activity for the chemically produced bispecific SynAb **13**.

We have described a novel, modular, and rapid, purely chemical approach for the assembly of IgG-like constructs, dubbed SynAbs, from parent Fc and Fab modalities. This first-in-class plug-and-play strategy allows for the generation of both mono- and bispecific constructs, *via* pyridazinedione-mediated disulfide re-bridging followed by Cu-free click chemistry for protein–protein ligation without the need for any protein engineering. It may represent a major development for the chemical biology community as it provides a branch point in enabling fundamental novel opportunities in the area of preparing and appraising synthetic antibodies at will. As a biologically relevant example, an IgG-like bispecific T cell engager SynAb, Fc_{CD20}-(Fab_{HER2})-Fab_{CD3} **13**, was generated in only 5 days starting from commercial parent anti-CD20, anti-HER2 and anti-CD3 mAbs. Biological evaluation of the bispecific SynAb **13** confirmed preservation of the binding capacities of the Fab_{HER2} and Fab_{CD3} modalities for their respective HER2 and CD3 receptors, while the Fc moiety was recognized by an anti-IgG Fc antibody. Importantly, the construct was able to redirect T cells to HCC1954 ($HER2^+$) cancer cells to induce T cell activation and T cell-dependent cancer cell death (IC_{50} = 4.9 ± 0.2 ng / mL). The method can be applied to any mAb with a single solvent-accessible disulfide in the Fab region, to investigate a wide range of Fab pairs as targeted arms for an IgG-like bsAb. This high versatility, combined with the speed, selectivity, homogeneity, and efficiency of the method makes it a valuable tool for the high-throughput production of a wide range of homogenous IgG-like bsAbs, with the potential to speed up the screening and identification of hits. The linkers between the components could also be readily altered/tuned (e.g., length, rigidity, etc.), and cleavage could be introduced between the component proteins. Additionally, the constructs would need not be limited to construction solely from antibody fragments — the Fc could be used as a platform to attach additional functionality through click-chemistry such as immunomodulatory enzymes (e.g., sialidase),[54] cytokines (e.g., IL2),[55] or immunomodulatory molecules (e.g., CTLA-4)[56] to unlock novel mechanisms of action. Furthermore, IgG-like bsAb-payload conjugates — Fc_Z-(Fab_X-payload)-Fab_Y-payload — can also be envisioned if a Br_2PD having two orthogonal click handles (e.g., Br_2PD-(BCN)-DBCO) is used to re-bridge the Fab moieties. This would give access to a post-assembly functionalisation step *via* click reaction (e.g., with an azide-linked small payload) to introduce drugs and/or fluorophores on the IgG-like bispecific construct. As a further potential improvement to the method, the synthesis of an asymmetric, bifunctional bis-Br_2PD linker for the re-bridging of the Fc is under investigation in our group, in order to allow for the carrying out of the three-protein assembly in one orthogonally-controlled step. Moving away from sequential assembly this way would help avoid the intermediate SEC purification step. We believe that the numerous assets of the described chemical method would make it a valuable complementary tool to bioengineering, especially for the initial, high-throughput, low scale screening stages of IgG-like bsAb

discovery. While scalability remains to be explored, its validation could highlight this chemical method as an attractive alternative to bioengineering for the production of IgG-like bsAbs in general, due to the rapidity, modularity and homogeneity it affords, as well as the potential for an in-built platform for further bsAb functionalization. In the meantime, the SynAb strategy constitutes a quick, efficient, and versatile way to access homogenous IgG-like bsAbs for researchers across disciplines having a restricted access to bioengineering technology. Hopefully, this breakthrough approach will be fertile ground for the future production and evaluation of numerous IgG-like bsAb, IgG-like bsAb–payload conjugates and Fc-bearing antibody–protein conjugates and assist in the discovery of new therapies.

Acknowledgements

We gratefully acknowledge the Wellcome Trust for funding P.S., the EU for funding L.N.C.R. (project 859458) and F.T. (project 838703), the EPSRC for funding I.A.T (EP/T517793/1), and the Ramsay Memorial Fellowships Trust for funding A.M. We also acknowledge Dr Kersti Karu of the UCL Chemistry Mass Spectrometry (MS) Facility, Dr Abil Aliev of the UCL NMR service, as well as Dr Nikos Pinotsis of the ISMB Biophysics Centre. This research was funded in part by the Wellcome Trust [Grant number: 175282, 214941/Z/18/Z]. For the purpose of Open Access, the authors have applied a CC BY public copyright licence to any Author Accepted Manuscript version arising from this submission.

Authors' contributions

F.T., P.S. and L.N.C.R. prepared the antibody fragments. F.T., P.S., L.N.C.R. and I.A.T. synthesized the small molecules. P.S. generated the monospecific SynAbs. F.T. generated the bispecific SynAb. P.S. carried out the SEC purifications. F.T. and P.S. analysed the protein constructs by LC-MS and SDS-PAGE. M.G. performed the biology experiments. J.B. carried out the statistical analysis. F.T., P.S., A.M., J.R.B., C.J.S. and V.C. conceived and designed the project and/or experiments. F.T., P.S. and V.C. co-wrote the manuscript. All authors read and approved the final manuscript. As F.T. and P.S. contributed equally to this work, they are permitted to list their names as first on the author list on any C.V., grant or fellowship application, etc. Their names were merely listed in this order arbitrarily, determined by a game of Keyforge.

Competing interests

V.C. and J.R.B. are directors of the spin-out ThioLogics and are inventors of the patent filed by UCL Business PLC (European Patent Office EP-2464654-B1) titled 'Thiol protecting group' published on October 08, 2014 and other patents derived thereof, as well as directly related patents in the other jurisdictions that fall within the patent family. However, there are no competing financial interests to declare.

Data Availability Statement

The detailed procedures required to duplicate this work are available in the electronic supplementary information (ESI) document along with full LC-MS and NMR spectra where appropriate. Any additional data or unique materials (through a materials transfer agreement) are available from the corresponding authors on reasonable request.

(1) Kontermann, R. E. Dual Targeting Strategies with Bispecific Antibodies. *MAbs* **2012**, *4* (2), 182–197.

(2) Thoreau, F.; Chudasama, V. Enabling the next Steps in Cancer Immunotherapy: From Antibody-Based Bispecifics to Multispecifics, with an Evolving Role for Bioconjugation Chemistry. *RSC Chem. Biol.* **2022**, *3* (2), 140–169.

(3) Sellmann, C.; Doerner, A.; Knuehl, C.; Rasche, N.; Sood, V.; Krah, S.; Rhiel, L.; Messemer, A.; Wesolowski, J.; Schuette, M.; et al. Balancing Selectivity and Efficacy of Bispecific Epidermal Growth Factor Receptor (EGFR) x c-MET Antibodies and Antibody-Drug Conjugates. *J. Biol. Chem.* **2016**, *291* (48), 25106–25119.

(4) Ahn, E. R.; Vogel, C. L. Dual HER2-Targeted Approaches in HER2-Positive Breast Cancer. *Breast Cancer Res. Treat.* **2012**, *131* (2), 371–383.

(5) List, T.; Casi, G.; Neri, D. A Chemically Defined Trifunctional Antibody-Cytokine-Drug Conjugate with Potent Antitumor Activity. *Mol. Cancer Ther.* **2014**, *13* (11), 2641–2652.

(6) Yu, Y. J.; Atwal, J. K.; Zhang, Y.; Tong, R. K.; Wildsmith, K. R.; Tan, C.; Bien-Ly, N.; Hersom, M.; Maloney, J. A.; Meilandt, W. J.; et al. Therapeutic Bispecific Antibodies Cross the Blood-Brain Barrier in Nonhuman Primates. *Sci. Transl. Med.* **2014**, *6* (261), 1–11.

(7) Kontermann, R. E.; Brinkmann, U. Bispecific Antibodies. *Drug Discov. Today* **2015**, *20* (7), 838–847.

(8) Spiess, C.; Zhai, Q.; Carter, P. J. Alternative Molecular Formats and Therapeutic Applications for Bispecific Antibodies. *Mol. Immunol.* **2015**, *67* (2), 95–106.

(9) Brinkmann, U.; Kontermann, R. E. The Making of Bispecific Antibodies. *MAbs* **2017**, *9* (2), 182–212.

(10) Nisonoff, A.; Rivers, M. M. Recombination Antibody of a Mixture of Univalent Fragments of Different Specificity. *Arch. Biochem. Biophys.* **1961**, *93*, 460–462.

(11) Segal, D. M.; Bast, B. J. E. G. Production of Bispecific Antibodies. *Curr. Protoc. Immunol.* **1995**, *14* (1), 1–16.

(12) Ridgway, J. B. B.; Presta, L. G.; Carter, P. “Knobs-into-Holes” Engineering of Antibody C(H)3 Domains for Heavy Chain Heterodimerization. *Protein Eng.* **1996**, *9* (7), 617–621.

(13) Klein, C.; Schaefer, W.; Regula, J. T. The Use of CrossMAb Technology for the Generation of Bi- and Multispecific Antibodies. *MAbs* **2016**, *8* (6), 1010–1020.

(14) Dengl, S.; Mayer, K.; Bormann, F.; Duerr, H.; Hoffmann, E.; Nussbaum, B.; Tischler, M.; Wagner, M.; Kuglstatter, A.; Leibrock, L.; et al. Format Chain Exchange (FORCE) for High-Throughput Generation of Bispecific Antibodies in Combinatorial Binder-Format Matrices. *Nat. Commun.* **2020**, *11* (1), 4974.

(15) Szijj, P.; Chudasama, V. The Renaissance of Chemically Generated Bispecific Antibodies. *Nat. Rev. Chem.* **2021**, *5* (2), 78–92.

(16) Walsh, S. J.; Bargh, J. D.; Dannheim, F. M.; Hanby, A. R.; Seki, H.; Counsell, A. J.; Ou, X.; Fowler, E.; Ashman, N.; Takada, Y.; et al. Site-Selective Modification Strategies in Antibody-Drug Conjugates. *Chem. Soc. Rev.* **2020**.

(17) Hoyt, E. A.; Cal, P. M. S. D.; Oliveira, B. L.; Bernardes, G. J. L. Contemporary Approaches to Site-Selective Protein Modification. *Nat. Rev. Chem.* **2019**, *3* (3), 147–171.

(18) Lang, K.; Chin, J. W. Bioorthogonal Reactions for Labeling Proteins. *ACS Chem. Biol.* **2014**, *9* (1), 16–20.

(19) Dommerholt, J.; Rutjes, F. P. J. T.; van Delft, F. L. Strain-Promoted 1,3-Dipolar Cycloaddition of Cycloalkynes and Organic Azides. *Top. Curr. Chem.* **2016**, *374* (2), 16 (2016).

(20) Ning, X.; Temming, R. P.; Dommerholt, J.; Guo, J.; Ania, D. B.; Debets, M. F.; Wolfert, M. A.; Boons, G. J.; Van Delft, F. L. Protein Modification by Strain-Promoted Alkyne-Nitrone Cycloaddition. *Angew. Chem. Int. Ed.* **2010**, *49* (17), 3065–3068.

(21) Knall, A. C.; Slugovc, C. Inverse Electron Demand Diels-Alder (IEDDA)-Initiated Conjugation: A (High) Potential Click Chemistry Scheme. *Chem. Soc. Rev.* **2013**, *42* (12), 5131–5142.

(22) Oliveira, B. L.; Guo, Z.; Bernardes, G. J. L. Inverse Electron Demand Diels-Alder Reactions in Chemical Biology. *Chem. Soc. Rev.* **2017**, *46* (16), 4895–4950.

(23) Liu, H.; Audisio, D.; Plougastel, L.; Decuypere, E.; Buisson, D. A.; Koniev, O.; Kolodych, S.; Wagner, A.; Elhabiri, M.; Krzyczmonik, A.; et al. Ultrafast Click Chemistry with Fluorosydnones. *Angew. Chem. Int. Ed.* **2016**, *55* (39), 12073–12077.

(24) Chudasama, V.; Maruani, A.; Caddick, S. Recent Advances in the Construction of Antibody-Drug Conjugates. *Nat. Chem.* **2016**, *8* (2), 114–119.

(25) Maruani, A.; Szijj, P. A.; Bahou, C.; Nogueira, J. C. F.; Caddick, S.; Baker, J. R.; Chudasama, V. A Plug-and-Play Approach for the de Novo Generation of Dually Functionalized Bispecifics. *Bioconjugate*

Chem. **2020**, *31* (3), 520–529.

(26) Lucchi, R.; Bentanachs, J.; Oller-Salvia, B. The Masking Game: Design of Activatable Antibodies and Mimetics for Selective Therapeutics and Cell Control. *ACS Cent. Sci.* **2021**, *7* (5), 724–738.

(27) Szijj, P. A.; Kostadinova, K. A.; Spears, R. J.; Chudasama, V. Tyrosine Bioconjugation-an Emergent Alternative. *Org. Biomol. Chem.* **2020**, *18* (44), 9018–9028.

(28) Chaubet, G.; Thoreau, F.; Wagner, A. Recent, Non-Classical, Approaches to Antibody Lysine Modification. *Drug Discov. Today Technol.* **2018**, *30*, 21–26.

(29) Forte, N.; Livanos, M.; Miranda, E.; Morais, M.; Yang, X.; Rajkumar, V. S.; Chester, K. A.; Chudasama, V.; Baker, J. R. Tuning the Hydrolytic Stability of Next Generation Maleimide Cross-Linkers Enables Access to Albumin-Antibody Fragment Conjugates and Tri-ScFvs. *Bioconjugate Chem.* **2018**, *29* (2), 486–492.

(30) Hull, E. A.; Livanos, M.; Miranda, E.; Smith, M. E. B.; Chester, K. A.; Baker, J. R. Homogeneous Bispecifics by Disulfide Bridging. *Bioconjugate Chem.* **2014**, *25* (8), 1395–1401.

(31) Lee, M. T. W.; Maruani, A.; Chudasama, V. The Use of 3,6-Pyridazinediones in Organic Synthesis and Chemical Biology. *J. Chem. Res.* **2016**, *40* (1), 1–9.

(32) Bahou, C.; Richards, D. A.; Maruani, A.; Love, E. A.; Javaid, F.; Caddick, S.; Baker, J. R.; Chudasama, V. Highly Homogeneous Antibody Modification through Optimisation of the Synthesis and Conjugation of Functionalised Dibromopyridazinediones. *Org. Biomol. Chem.* **2018**, *16* (8), 1359–1366.

(33) Lee, M. T. W.; Maruani, A.; Richards, D. A.; Baker, J. R.; Caddick, S.; Chudasama, V. Enabling the Controlled Assembly of Antibody Conjugates with a Loading of Two Modules without Antibody Engineering. *Chem. Sci.* **2017**, *8* (3), 2056–2060.

(34) Bahou, C.; Chudasama, V. The Use of Bromopyridazinedione Derivatives in Chemical Biology. *Org. Biomol. Chem.* **2022**, *20* (30), 5879–5890.

(35) Maruani, A.; Smith, M. E. B.; Miranda, E.; Chester, K. A.; Chudasama, V.; Caddick, S. A Plug-and-Play Approach to Antibody-Based Therapeutics via a Chemoselective Dual Click Strategy. *Nat. Commun.* **2015**, *6*, 6645.

(36) Wuellner, U.; Klupsch, K.; Buller, F.; Attinger-Toller, I.; Santimari, R.; Zbinden, I.; Henne, P.; Grabulovski, D.; Bertschinger, J.; Brack, S. Bispecific CD3/HER2 Targeting FynomAb Induces

Redirected t Cell-Mediated Cytolysis with High Potency and Enhanced Tumor Selectivity. *Antibodies* **2015**, *4* (4), 426–440.

(37) Wang, L.; He, Y.; Zhang, G.; Ma, J.; Liu, C.; He, W.; Wang, W.; Han, H.; Boruah, B. M.; Gao, B. Retargeting T Cells for HER2-Positive Tumor Killing by a Bispecific Fv-Fc Antibody. *PLoS One* **2013**, *8* (9), e75589.

(38) Robinson, E.; Nunes, J. P. M.; Vassileva, V.; Maruani, A.; Nogueira, J. C. F.; Smith, M. E. B.; Pedley, R. B.; Caddick, S.; Baker, J. R.; Chudasama, V. Pyridazinediones Deliver Potent, Stable, Targeted and Efficacious Antibody-Drug Conjugates (ADCs) with a Controlled Loading of 4 Drugs per Antibody. *RSC Adv.* **2017**, *7* (15), 9073–9077.

(39) Blackman, M. L.; Royzen, M.; Fox, J. M. Tetrazine Ligation: Fast Bioconjugation Based on Inverse-Electron-Demand Diels-Alder Reactivity. *J. Am. Chem. Soc.* **2008**, *130* (41), 13518–13519.

(40) Baalmann, M.; Neises, L.; Bitsch, S.; Schneider, H.; Deweid, L.; Werther, P.; Ilkenhans, N.; Wolfring, M.; Ziegler, M. J.; Wilhelm, J.; et al. A Bioorthogonal Click Chemistry Toolbox for Targeted Synthesis of Branched and Well-Defined Protein–Protein Conjugates. *Angew. Chem. Int. Ed.* **2020**, *59* (31), 12885–12893.

(41) Bahou, C.; Love, E. A.; Leonard, S.; Spears, R. J.; Maruani, A.; Armour, K.; Baker, J. R.; Chudasama, V. Disulfide Modified IgG1: An Investigation of Biophysical Profile and Clinically Relevant Fc Interactions. *Bioconjugate Chem.* **2019**, *30* (4), 1048–1054.

(42) Kellner, C.; Otte, A.; Cappuzzello, E.; Klausz, K.; Peipp, M. Modulating Cytotoxic Effector Functions by Fc Engineering to Improve Cancer Therapy. *Transfus. Med. Hemotherapy* **2017**, *44* (5), 327–336.

(43) Ward, E. S.; Ober, R. J. Targeting FcRn to Generate Antibody-Based Therapeutics. *Trends Pharmacol. Sci.* **2018**, *39* (10), 892–904.

(44) Zalevsky, J.; Chamberlain, A. K.; Horton, H. M.; Karki, S.; Leung, I. W. L.; Sproule, T. J.; Lazar, G. A.; Roopenian, D. C.; Desjarlais, J. R. Enhanced Antibody Half-Life Improves in Vivo Activity. *Nat. Biotechnol.* **2010**, *28* (2), 157–159.

(45) Suurs, F. V.; Lorenczewski, G.; Bailis, J. M.; Stienen, S.; Friedrich, M.; Lee, F.; van der Vegt, B.; de Vries, E. G. E.; de Groot, D. J. A.; Lub-de Hooge, M. N. Mesothelin/CD3 Half-Life–Extended Bispecific T-Cell Engager Molecule Shows Specific Tumor Uptake and Distributes to Mesothelin and CD3-Expressing Tissues. *J. Nucl. Med.* **2021**, *62* (12), 1797–1804.

(46) Lorenczewski, G.; Friedrich, M.; Kischel, R.; Dahlhoff, C.; Anlahr, J.; Balazs, M.; Rock, D.; Boyle, M.

C.; Goldstein, R.; Coxon, A.; et al. Generation of a Half-Life Extended Anti-CD19 BiTE® Antibody Construct Compatible with Once-Weekly Dosing for Treatment of CD19-Positive Malignancies. *Blood* **2017**, *130* (Supplement 1), 2815–2815.

(47) Zekri, L.; Vogt, F.; Osburg, L.; Müller, S.; Kauer, J.; Manz, T.; Pflügler, M.; Maurer, A.; Heitmann, J. S.; Hagelstein, I.; et al. An IgG-based Bispecific Antibody for Improved Dual Targeting in PSMA-positive Cancer. *EMBO Mol. Med.* **2021**, *13* (2), e11902.

(48) Riesenberg, R.; Buchner, A.; Pohla, H.; Lindhofer, H. Lysis of Prostate Carcinoma Cells by Trifunctional Bispecific Antibodies (AEpCAM × ACD3). *J. Histochem. Cytochem.* **2001**, *49* (7), 911–917.

(49) Jeger, S.; Zimmermann, K.; Blanc, A.; Grünberg, J.; Honer, M.; Hunziker, P.; Struthers, H.; Schibli, R. Site-Specific and Stoichiometric Modification of Antibodies by Bacterial Transglutaminase. *Angew. Chem. Int. Ed.* **2010**, *49* (51), 9995–9997.

(50) Shi, W.; Li, W.; Zhang, J.; Li, T.; Song, Y.; Zeng, Y.; Dong, Q.; Lin, Z.; Gong, L.; Fan, S.; et al. One-Step Synthesis of Site-Specific Antibody–Drug Conjugates by Reprograming IgG Glycoengineering with LacNAc-Based Substrates. *Acta Pharm. Sin. B* **2022**, *12* (5), 2417–2428.

(51) Strohl, W. R.; Naso, M. Bispecific T-Cell Redirection versus Chimeric Antigen Receptor (Car)-t Cells as Approaches to Kill Cancer Cells. *Antibodies* **2019**, *8* (3), 41.

(52) Yu, S.; Zhang, J.; Yan, Y.; Yao, X.; Fang, L.; Xiong, H.; Liu, Y.; Chu, Q.; Zhou, P.; Wu, K. A Novel Asymmetrical Anti-HER2/CD3 Bispecific Antibody Exhibits Potent Cytotoxicity for HER2-Positive Tumor Cells. *J. Exp. Clin. Cancer Res.* **2019**, *38* (1), 355.

(53) Jäger, M.; Schoberth, A.; Ruf, P.; Hess, J.; Lindhofer, H. The Trifunctional Antibody Ertumaxomab Destroys Tumor Cells That Express Low Levels of Human Epidermal Growth Factor Receptor. *Cancer Res.* **2009**, *69* (10), 4270–4276.

(54) Gray, M. A.; Stanczak, M. A.; Mantuano, N. R.; Xiao, H.; Pijnenborg, J. F. A.; Malaker, S. A.; Miller, C. L.; Weidenbacher, P. A.; Tanzo, J. T.; Ahn, G.; et al. Targeted Glycan Degradation Potentiates the Anticancer Immune Response in Vivo. *Nat. Chem. Biol.* **2020**, *16* (12), 1376–1384.

(55) Hutmacher, C.; Neri, D. Antibody-Cytokine Fusion Proteins: Biopharmaceuticals with Immunomodulatory Properties for Cancer Therapy. *Adv. Drug Deliv. Rev.* **2019**, *141*, 67–91.

(56) Kremer, J. M.; Westhovens, R.; Leon, M.; Di Giorgio, E.; Alten, R.; Steinfeld, S.; Russell, A.; Dougados, M.; Emery, P.; Nuamah, I. F.; et al. Treatment of Rheumatoid Arthritis by Selective Inhibition of T-

Cell Activation with Fusion Protein CTLA4Ig. *N. Engl. J. Med.* **2003**, *349* (20), 1907–1915.