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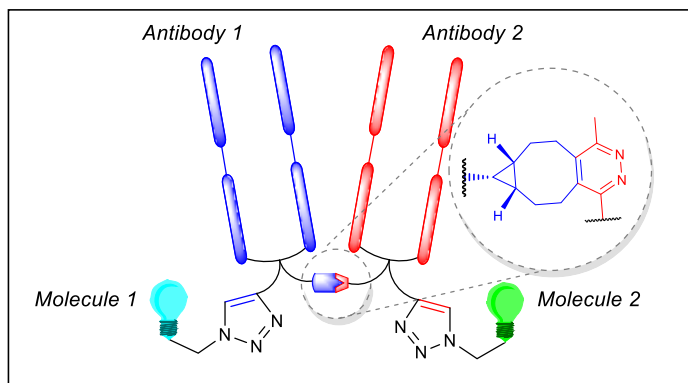
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A plug-and-play approach for the *de novo* generation of dually functionalised bispecifics

Antoine Maruani[†], Peter A. Szijj[†], Calise Bahou[†], João C. F. Nogueira[†], Stephen Caddick[†], James R. Baker^{*†} and Vijay Chudasama^{*†‡}

[†] Department of Chemistry, University College London, 20 Gordon Street, WC1H 0AJ, London, UK.

[‡] Research Institute for Medicines (iMed.Ulisboa), Faculty of Pharmacy, Universidade de Lisboa, 1649-003 Lisbon, Portugal.

* e-mails: v.chudasama@ucl.ac.uk; j.r.baker@ucl.ac.uk

Abstract

Diseases are multifactorial, with redundancies and synergies between various pathways. However, most of the antibody-based therapeutics on the market interact with only one target thus limiting their efficacy. The targeting of multiple epitopes could improve the therapeutic index of treatment and counteract mechanisms of resistance. To this effect, a new class of therapeutics emerged: bispecific antibodies.

Bispecific formation using chemical methods is rare and low yielding and/or requires a large excess of one of the two proteins to avoid homodimerisation and heterogeneity. In order for chemically prepared bispecifics to deliver their full potential, high-yielding, modular and reliable cross-linking technologies are required. Herein, we describe a novel approach not only for the rapid and high-yielding chemical generation of bispecific antibodies from native antibody fragments, but also for the site-specific dual functionalisation of the resulting bioconjugates. Based on orthogonal clickable functional groups, this strategy enables the assembly of functionalised bispecifics with controlled loading in a modular and convergent manner.

Introduction

Monoclonal antibodies (mAbs) have the ability to recognise the epitope of an antigen with high specificity. mAbs that bind to a cancer cell and induce an immune response are extensively utilised in cancer treatments.¹ Nevertheless, although some naked antibodies (*e.g.* Herceptin) have revolutionised certain cancer therapies they often fail to induce sufficient anti-tumour activity on their own— and there is an impetus to develop modified variants that can overcome this limitation.^{1, 2}

Advances in the development of the mAbs format have been made—for example the use of fragments and development of functionalised mAbs. These have led to improvements in their efficacy and have overcome many barriers.³ Despite these advances, the safety and efficacy profile of mAbs biomolecules still requires further work to develop approaches to broaden their therapeutic index.

With advances in mAbs format, has come the possibility of moving away from the conventional monospecific approach and developing antibodies to target multiple targets simultaneously. This is potentially attractive as diseases are multifactorial, with redundancies and synergies between various pathways.⁴ Moreover, targeting multiple epitopes could potentially improve the therapeutic index of treatment (*e.g.* by reducing systemic toxicity) and counteract mechanisms of tumour survival.⁵ To address this opportunity in the mid-1980s, a new class of therapeutics emerged: bispecific antibodies (BsAbs).^{6, 7} The principle behind BsAbs is to target two epitopes through a single entity and

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2 simultaneously affect multiple pathways to increase overall efficacy.
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4 The production of high quality BsAbs is non-trivial and in principle they can be produced either by
5 chemically linking two proteins together (*e.g.* disulfide exchange on native antibody, crosslinking *via*
6 heterobifunctional linker) or by protein, cellular, and genetic engineering (*e.g.* quadroma approach,
7 genetic recombination).^{4, 8, 9} When compared to biological approaches, chemical methods tend to have
8 the advantage of being more general and straightforward as they typically do not require specific
9 engineering for each protein of interest thus enabling the use of commercially available mAbs as
10 precursors which decreases overall costs and time required. Initially, clinical trials with chemically-
11 linked antibodies and antibody fragments were promising, but their costly preparation, low final
12 product yields, time-consuming purification and product heterogeneity ultimately prevented further
13 development.¹⁰⁻¹⁴ Thanks to advances in protein engineering (*e.g.* recombinant DNA technologies), the
14 production of BsAbs *via* biological approaches superseded chemical ones,⁹ however with recent
15 development in site-specific modification of proteins and bioorthogonal reactions, chemical methods
16 for protein–protein preparation are now re-surfacing.¹⁵⁻²² Nevertheless, bispecific formation using such
17 methods from non-engineered scaffolds are rare and none of the current bispecifics in the clinic are
18 made by chemical means. They often have limited conversion or require an excess of one of the two
19 proteins to avoid homodimerisation and heterogeneity, are restricted to IgG2 antibodies and do not
20 enable further site-selective functionalisation.^{18, 23, 24} In order for chemically-prepared bispecifics to
21 deliver their full potential, high-yielding, modular and reliable cross-linking technologies are therefore
22 required.
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27 Bioorthogonal chemistry has revolutionised the field of chemical biology by providing powerful
28 chemical tools for the selective modification of biomolecules. Click chemistry and bioorthogonal
29 chemistry overlap significantly, reflecting the same underlying chemical principles, *i.e.* regioselective
30 conjugation under mild conditions.²⁵ In practice, click reactions have been used extensively in a
31 biological context, particularly for the site-selective introduction of a *single* chemical moiety. However,
32 the deployment of the same approach for the site-selective and bioorthogonal *dual* modification of
33 biomolecules is rare,²⁶ especially in the context of non-engineered scaffolds. Although the introduction
34 of multiple modules enables a plethora of permutations/combinations and can generate a variety of
35 bioconjugates with many potential applications, it remains a challenge.²⁶
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39 Recently, we described a new approach to the synthesis of doubly modified antibodies with non-
40 proteinogenic cargoes.²⁷ Our approach was a chemoselective “dual click” strategy based on
41 dibromopyridazinedione (PD) scaffolds which have then been employed extensively to functionally re-
42 bridge disulfide, *e.g.* in the generation of ADCs,²⁸ antibody conjugates,²⁹⁻³¹ antibody-directed
43 photosensitisers,^{32, 33} and a targeted nanotherapeutic.³⁴ These applications highlighted some key
44 features of this platform, *e.g.* site-selectivity, long-term blood plasma stability, resistance to hydrolysis,
45 compatibility with common mild reducing reagents [tris(2-carboxyethyl)phosphine – TCEP],²⁹ and
46 retention of binding capability of the modified antibody. However, these applications did not take
47 advantage of the double click capability of PDs in the context of forming protein–protein conjugates.
48 This represents a challenge due to the steric hindrance that needs to be overcome when attaching large
49 molecules to each other, and the need to use stoichiometric (or-near stoichiometric) amounts of both
50 reagents. These disfavoured conditions need to be overcome by careful optimisation and experiment
51 design.
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55 Herein, we describe a novel modular approach both for the rapid synthesis of bispecific antibodies from
56 native antibody fragments, and for the site-specific dual functionalisation of the resulting
57 bioconjugates. By incorporating orthogonal clickable functionalities, we are able to rapidly assemble
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2 functionalised bispecifics with controlled loading (0, 1, 2, or “1+1”) in a modular and convergent
3 manner.
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5 **Results**

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7 Three FDA-approved monoclonal antibodies, *i.e.* rituximab, cetuximab and trastuzumab, were selected
8 to evaluate our new approach. We selected Fab fragments for the study as they retain the targeting
9 specificity of full antibodies, usually have better tissue penetration properties, and can be produced
10 more economically.³⁵
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13 Rituximab is a chimeric mAb against CD20 which is found on the surface of B cells. Rituximab is
14 widely used to treat autoimmune diseases and cancer, and fragments derived from rituximab have been
15 engineered for use in a variety of bispecific antibodies in order to help bypass resistance
16 mechanisms.³⁶⁻³⁸
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18 Cetuximab and trastuzumab are both monospecific antibodies that bind to and interfere with signalling
19 of HER family members (HER1 and HER2, respectively). They have been successfully used in the
20 treatment of a variety of cancers (*e.g.* breast cancer, metastatic colorectal cancer, metastatic non-small
21 cell lung cancer). BsAbs directed at multiple members of the HER family are being pursued with the
22 aim of improving therapeutic efficacy.^{39, 40}
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25 *Design of linkers for bispecific formation*

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27 The choice and design of linkers were key to achieve a method that is modular, reliable,
28 straightforward and widely applicable to non-engineered scaffolds. It represented two independent yet
29 related challenges in chemical orthogonality and bioorthogonality as it had to not only enable efficient
30 crosslinking of two antibody fragments, but it should also allow for further dual functionalisation with
31 small molecules of interest.
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34 To design the adequate linkers for protein cross linking, four main parameters needed to be taken into
35 consideration:
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- 37 - All reactions needed to be click compatible to enable modularity;
- 38 - The click reaction to assemble the bispecific by reacting the two proteins needed to be rapid and
39 quantitative even with near stoichiometric quantities;
- 40 - A long and flexible enough spacer would be needed to prevent a potential entropic barrier to the
41 reaction between two proteins;
- 42 - The linkers had to be stable to the various conditions used throughout the entire process (*e.g.*
43 pH, temperature, redox).
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47 To this end, several click approaches were trialled (*i.e.* CuAAC, SPAAC with electron rich and
48 electron poor azide/alkyne combination and Michael addition, data not shown) but all resulted in partial
49 conversion to the desired conjugate. Thus, we decided to focus our efforts on strain-promoted inverse
50 electron demand Diels–Alder cycloaddition (SPIEDAC), a widely used bioorthogonal reaction known
51 for its fast reaction kinetics.⁴¹ Classically, the two partners in SPIEDAC are a strained alkene (*e.g.*
52 trans-cyclooctene [TCO], norbornene) and an electron-poor diene (*e.g.* tetrazine) but stability over an
53 extended period with TCO is often a concern⁴¹ and the reactivity of norbornene towards tetrazine is
54 limited (*i.e.* reaction rates of *ca.* 1 M⁻¹ s⁻¹).^{42, 43} It was therefore decided to mitigate this risk by using a
55 slower yet more stable partner, namely electron-rich strained alkyne bicyclo[6.1.0]nonyne (BCN).
56 BCN is commonly used in strain-promoted azide–alkyne click chemistry⁴⁴ with rates of *ca.* 1 M⁻¹ s⁻¹.
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Contrary to most other strained alkyne (*e.g.* DIBAC, DIBO) which are suboptimal partners for SPIEDACs, BCN is an electron-rich strained alkyne which raises its HOMO and thus reduces the activation energy in inverse demand Diels–Alder reactions. For this reason, BCN has been used in SPIEDAC with reaction rates $>20\,000\text{ M}^{-1}\text{ s}^{-1}$ under aqueous conditions when applied for protein labelling⁴⁵ and therefore represents a promising partner for protein–protein crosslinking.

The substituents on the tetrazine ring are also a key factor in SPIEDACs. Electron-withdrawing substituents such as pyrimidine greatly increase the rate of reaction (by lowering the energy of the LUMO_{tetrazine}) but they may also promote their degradation.⁴⁶ Thus a trade-off between stability and reactivity is often needed depending on the application. Both highly reactive yet less stable tetrazine **1** and highly stable yet less reactive tetrazine **2** (Figure 1) were compared for protein–protein formation *via* SPIEDAC and it was found that for our approach, high stability was preferred over very fast kinetics. Indeed, with highly reactive tetrazine **1** or with a partner with limited reactivity (*e.g.* azides), only partial conversion was observed (data not shown) thus greatly limiting the yield of the reaction.

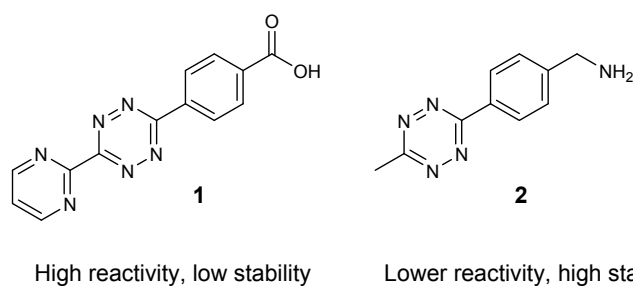
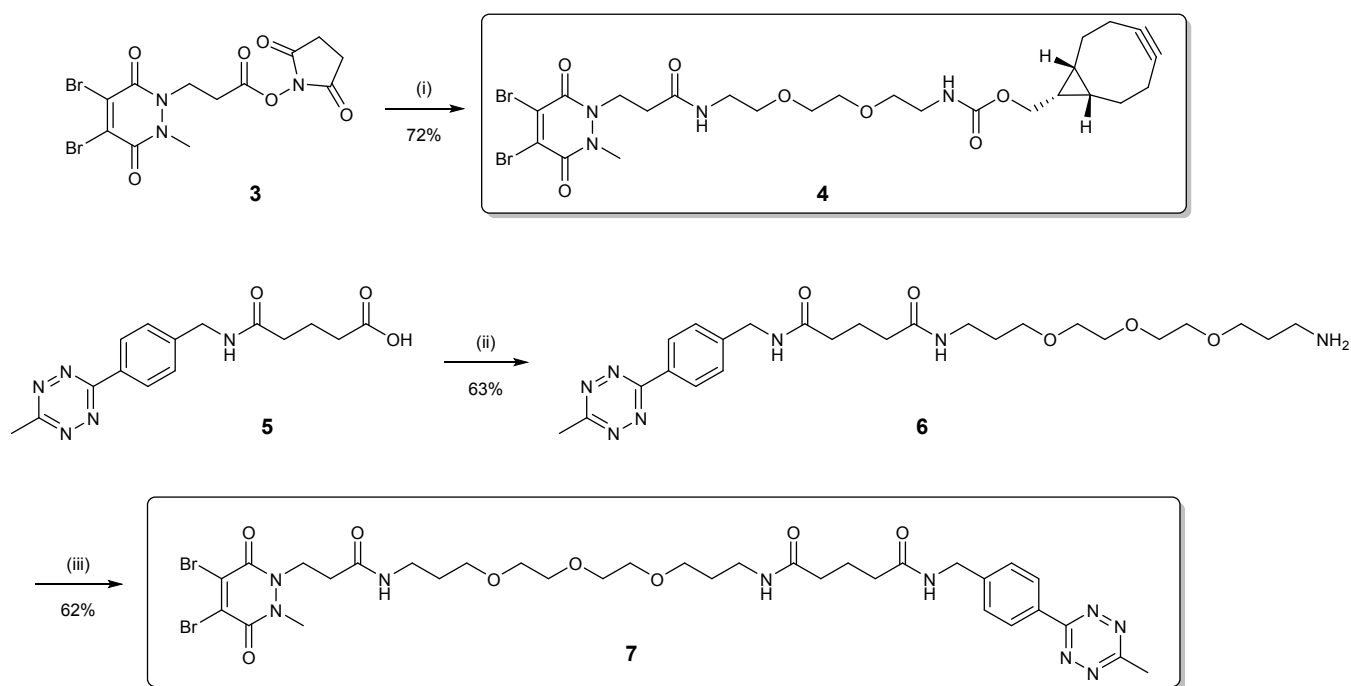


Figure 1: Tetrazines compared for their efficacy for SPIEDAC between proteins.

Two PDs were thus prepared following our recently described protocol,⁴⁷ one bearing a BCN handle and the other bearing tetrazine **2**. To reduce aggregation, minimize steric hindrance, and enhance solubility both linkers incorporated a short PEG spacer.

Briefly, starting from our previously described reactive intermediate **3** for divergent synthesis of PDs,⁴⁷ commercially available BCN-amine was coupled to NHS-PD **3** and gave BCN-PD **4** in 72% yield. The synthesis of Tetrazine-PD **7** started from the preparation of tetrazine **5**⁴⁸ which can be obtained in one step from commercially available material. Carboxylic acid **5** was then coupled to bis(amine) PEG (*i.e.* *O,O'*-bis(3-aminopropyl)diethylene glycol) in 63% yield. The resulting amine **6** was then attached to NHS-PD **3** and gave Tetrazine-PD **7** in 62% yield (Figure 2).



25 Figure 2: *Reagents and conditions*: (i) BCN-amine (*N*-[(1*R*,8*S*,9*S*)-bicyclo[6.1.0]non-4-yn-9-ylmethoxycarbonyl]-1,8-diamino-3,6-dioxaoctane), MeCN, rt, 16 h; (ii) bis(amine) PEG, HATU, NEt₃, CH₂Cl₂, rt, 16 h; (iii) **3**, MeCN, rt, 16 h.

30 Tetrazine-PD **7** and BCN-PD **4** were then appraised for the functional rebridging of a Fab fragment of trastuzumab (Fab_{TRA}). Both conversions to Fab_{TRA}-BCN **8** and Fab_{TRA}-Tetrazine **9** occurred in near quantitative yield (Figure 3) with no thiol-yne addition. Removal of the small excess of the reducing agent used to reduce the disulfide bonds (*i.e.* TCEP) proved to be essential for the formation of Fab_{TRA}-Tetrazine **9** as tetrazines can be reduced by such reagents. Following the isolation of **8** and **9**, conditions to form Fab_{TRA}-Fab_{TRA} homodimer were trialed.

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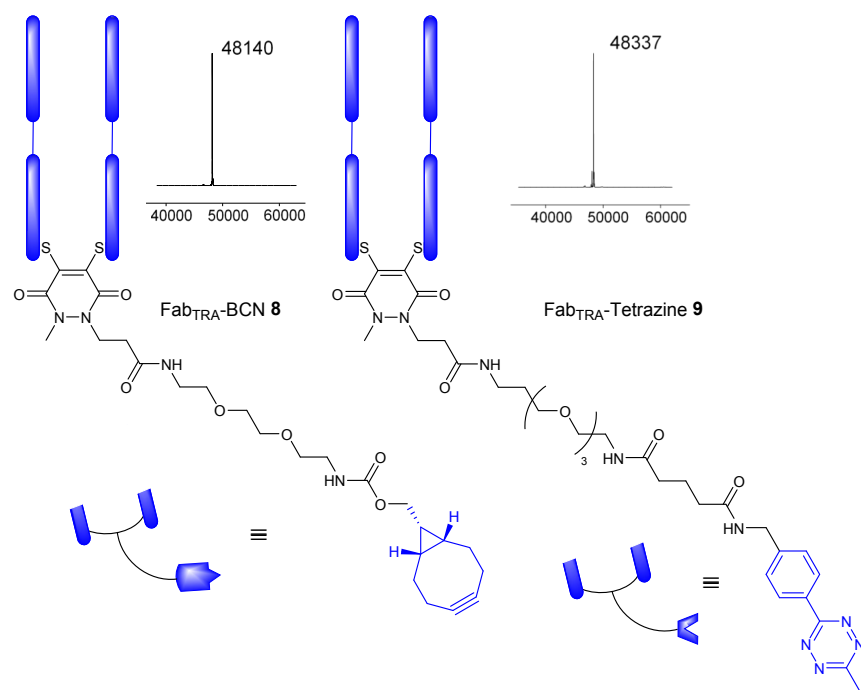


Figure 3: Structures of Fab_{TRA}-BCN **8** and Fab_{TRA}-Tetrazine **9**.

To effect near quantitative conversion whilst using stoichiometric amount of each Fab_{TRA}, three key variables were identified: concentration, pH, and temperature. High concentration would increase the reaction rate but increase potential aggregation whilst low concentration would avoid issues with aggregation but decrease the conversion of the reaction by increasing the residency time (and potential degradation) of BCN and/or tetrazine in buffer. Similarly, high temperature would promote degradation but also increase the reaction rate whilst low temperature would decrease degradation but also reaction rate. Even though pH has no observable impact on SPIEDACs kinetics,⁴⁶ tetrazines are known to degrade faster in basic pH, thus slightly acidic pH was preferred.⁴⁹ Following optimisation of these parameters, it was found that reaction at pH 7 or 8, at low temperature or at concentrations below 60 μM did not reach completion due to competing degradation but when reacting the two partners at 100 μM , 30 $^{\circ}\text{C}$, pH 6.0 near quantitative conversion using near stoichiometric amount (*i.e.* 1.1 equivalents) of each Fab_{TRA} could be obtained after 12 h (Figure 4).

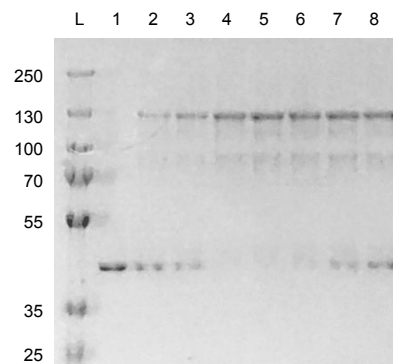


Figure 4: SDS-PAGE analysis of reaction between Fab_{TRA}-BCN **8** and Fab_{TRA}-Tetrazine **9** in acetate buffer pH 6.0 at 30 $^{\circ}\text{C}$ for 12 h. L – ladder; lane 1 – **8** + 0 eq. **9**; lane 2 – **8** + 0.5 eq. **9**; lane 3 – **8** + 0.7 eq. **9**; lane 4 – **8** + 0.9 eq. **9**; lane 5 – **8** + 1.0 eq. **9**; lane 6 – **8** + 1.1 eq. **9**; lane 7 – **8** + 1.3 eq. **9**; lane 8 – **8** + 1.5 eq. **9**.

Using these optimal parameters, Fab_{TRA}-BCN **8** and Fab_{TRA}-Tetrazine **9** were reacted. The reaction reached completion with no remaining Fab_{TRA}-BCN **8** after 12 h. Following purification, Fab_{TRA}-Fab_{TRA} homodimer **10** was isolated in 55–65% yield (Figure 5).

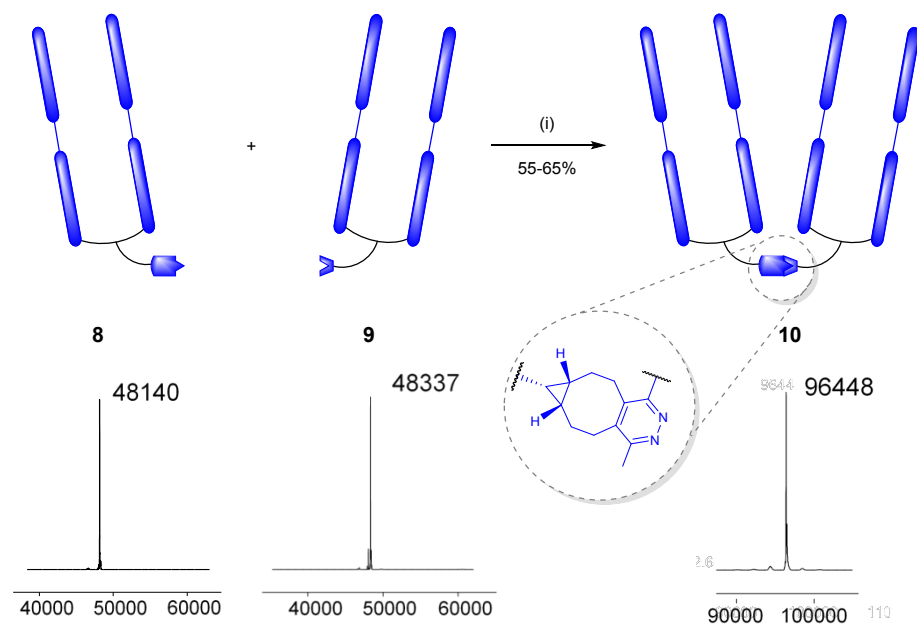


Figure 5: *Reagents and conditions:* (i) 100 μ M, acetate buffer pH 6.0, 30 $^{\circ}$ C, 12 h.

With these optimal conditions in hand, the method was applied to cetuximab Fab (Fab_{CET}) and rituximab Fab (Fab_{RIT}) to generate chemically-prepared bispecifics from non-engineered antibodies. First, Fab_{TRA}-BCN **8**, Fab_{CET}-Tetrazine **11** and Fab_{RIT}-Tetrazine **12** were prepared in near quantitative yield. Then Fab_{TRA}-BCN **8** + Fab_{RIT}-Tetrazine **12**, and Fab_{TRA}-BCN **8** + Fab_{CET}-Tetrazine **11** were reacted for 12 h at 100 μ M, 30 $^{\circ}$ C, pH 6.0.

Gratifyingly, the conditions translated well to other antibodies and BsAbs Fab_{TRA}-Fab_{CET} **13** and Fab_{TRA}-Fab_{RIT} **14** were obtained in good purity in 71% and 78% isolated yield respectively without the need for SEC purification (Figure 6). These promising results demonstrate the versatility of our approach in the generation of bispecifics in near quantitative conversion and high isolated yield from non-engineered antibodies and led to further investigation into the functionalisation of the chemically-formed protein-protein conjugates.

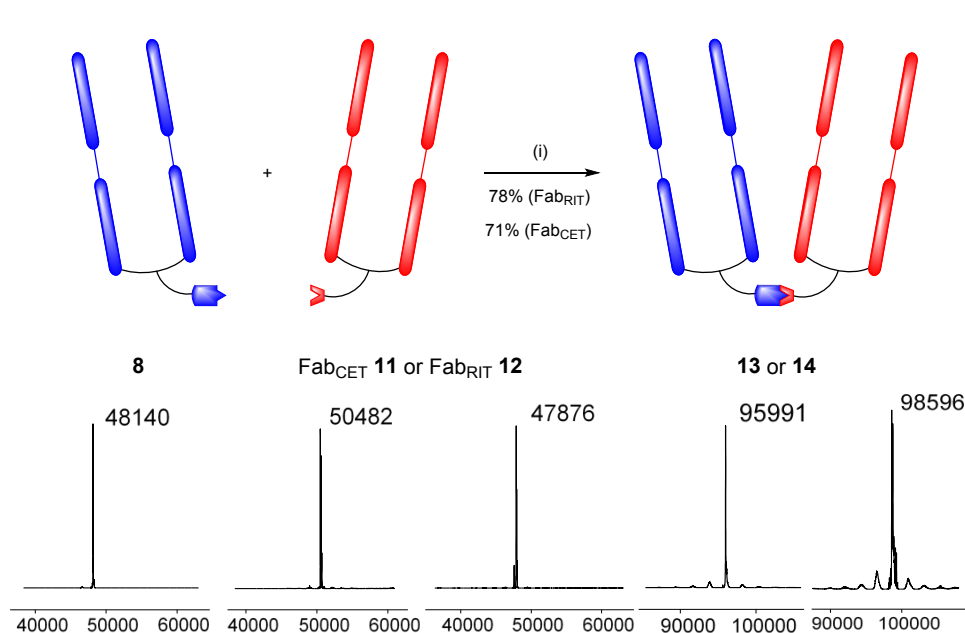


Figure 6: *Reagents and conditions:* (i) [100 μM], acetate buffer pH 6.0, 30 °C, 12 h.

Functionalisation of the bispecific with controlled loading

To further functionalise the bispecific, the methyl group of PDs **4** and **7** needed to be replaced by another reactive functional group. This new group would need to be compatible with the chemical partners and conditions used to assemble the bispecific and yet amenable to facile reaction with a variety of commercially available payloads (*e.g.* fluorophores, drugs, biotin, chelators).

We have previously shown that terminal alkynes and strained alkynes can co-exist on a PD and can be independently functionalised, provided the strained alkyne is reacted first.²⁷ Similar orthogonality has been observed with tetrazines and terminal alkynes. These observations led to the synthesis of two novel PDs bearing unstrained alkyne on one of the nitrogen atoms and BCN or tetrazine **2** on the other. Briefly, the synthesis of **4** and **7** was adapted to add this second reactive species; starting from hydrazine alkyne **16** obtained *via* selective alkylation of hydrazine **15**, a second alkylation led to disubstituted hydrazine **17**, which was converted to PD **18** by condensation with dibromomaleic acid. Following activation with NHS, **19** was reacted with BCN-amine or tetrazine amine **6** to give PD **20** in 58% yield and PD **21** in 43% yield, respectively (Figure 7).

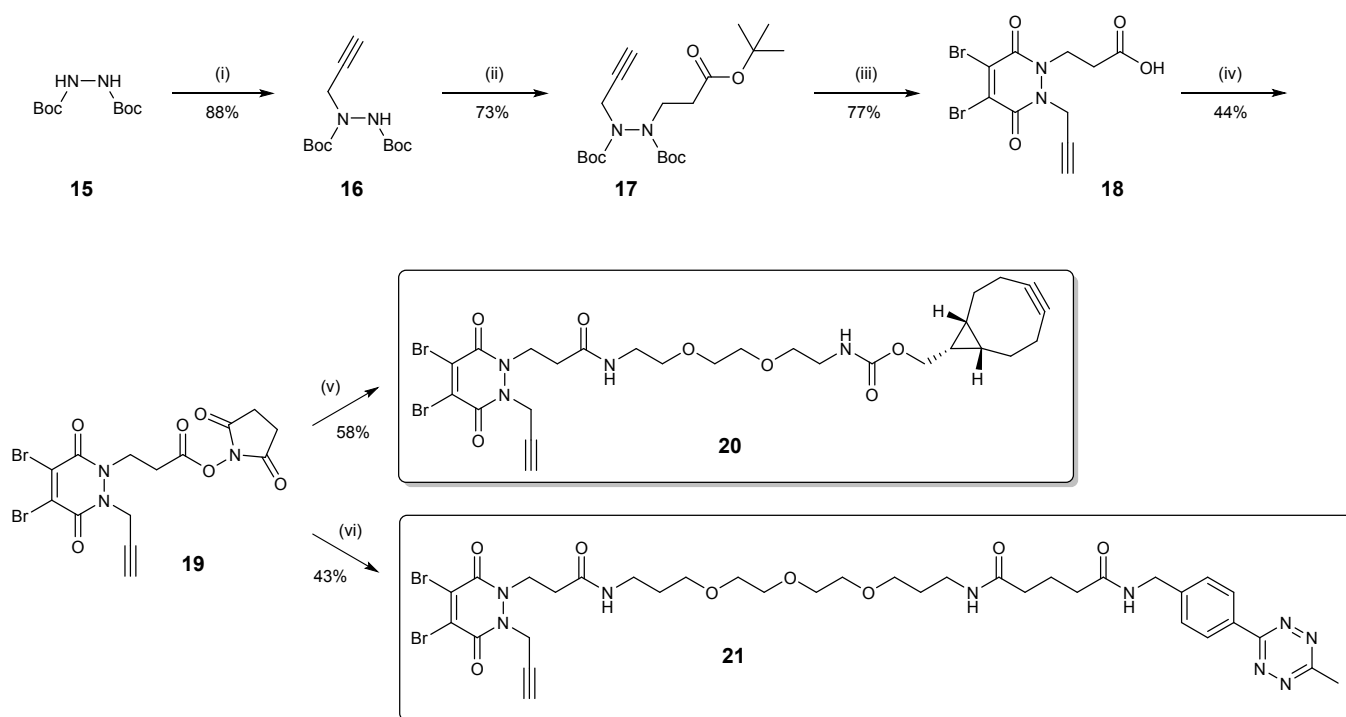


Figure 7: *Reagents and conditions:* (i) Propargyl bromide, TBAB, toluene/5% NaOH_(aq), rt, 16 h; (ii) *t*-butyl acrylate, *t*-BuOH, 10% NaOH_(aq), 60 °C, 24 h; (iii) dibromomaleic acid, AcOH, reflux, 4 h; (iv) DCC, THF, 0 °C for 30 min then NHS, rt, 16 h; (v) BCN amine, MeCN, rt, 16 h; (vi) **6**, MeCN, rt, 16 h.

BCN-Alkyne-PD **20** and Tetrazine-Alkyne-PD **21** were then appraised for the functional rebridging of Fab_{TRA} and Fab_{RIT}. The optimised conditions translated well to these novel PDs and both Fab_{TRA}-BCN-Alkyne **22**, Fab_{TRA}-Tetrazine-Alkyne **23** and Fab_{RIT}-Tetrazine-Alkyne **24** were isolated in near quantitative yield (see SI for details).

To optimise copper(I)-catalysed azide-alkyne cycloaddition (CuAAC) conditions and appraise the stability of the additional alkyne moiety, the click strategy was attempted on a chemically formed Fab_{TRA}-Fab_{TRA} homodimer bearing two additional alkyne moieties. This homodimer was obtained by functionally rebridging Fab_{TRA} with the novel PDs to prepare Fab_{TRA}-BCN-Alkyne **22** and Fab_{TRA}-Tetrazine-Alkyne **23** which were then reacted under optimised conditions to yield Fab_{TRA}-Alkyne-Fab_{TRA}-Alkyne **25** (Figure 8). To enable additional readout of the CuAAC reaction by UV-Vis spectrometry, a commercially available azide-functionalised fluorophore (*i.e.* AlexaFluor488 [AF488] azide) was selected as the first payload. Fab_{TRA}-Alkyne-Fab_{TRA}-Alkyne **25** was subjected to CuAAC conditions, the reaction proceeded with complete conversion with no unreacted Fab_{TRA}-Alkyne-Fab_{TRA}-Alkyne **25** and thus, Fab_{TRA}-AF488-Fab_{TRA}-AF488 **27** was isolated in high purity in 56% yield over two steps. Following analysis by LC-MS and UV-Vis spectrometry, a loading of 2 was confirmed (Figure 8 and SI for details).

To demonstrate the potential for controlled loading, a BsAb bearing a single additional alkyne moiety was generated by reaction of Fab_{TRA}-BCN **8** and Fab_{RIT}-Tetrazine-Alkyne **24** (Figure 8). BsAb Fab_{TRA}-Fab_{RIT}-Alkyne **26** was obtained in 75% yield and was then subjected to CuAAC conditions. Fab_{TRA}-Fab_{RIT}-AF488 bispecific **28** was isolated in high purity in 82% yield. Following analysis by LC-MS, a

loading of **1** was confirmed (Figure 8). Similarly, when reacting Fab_{TRA}-BCN **8** or Fab_{TRA}-BCN-Alkyne **22** with Fab_{CET}-Tetrazine-Alkyne, “clickable” BsAbs Fab_{TRA}-Fab_{CET}-Alkyne and Fab_{TRA}-Alkyne-Fab_{CET}-Alkyne were obtained in high yield and purity (see SI for details).

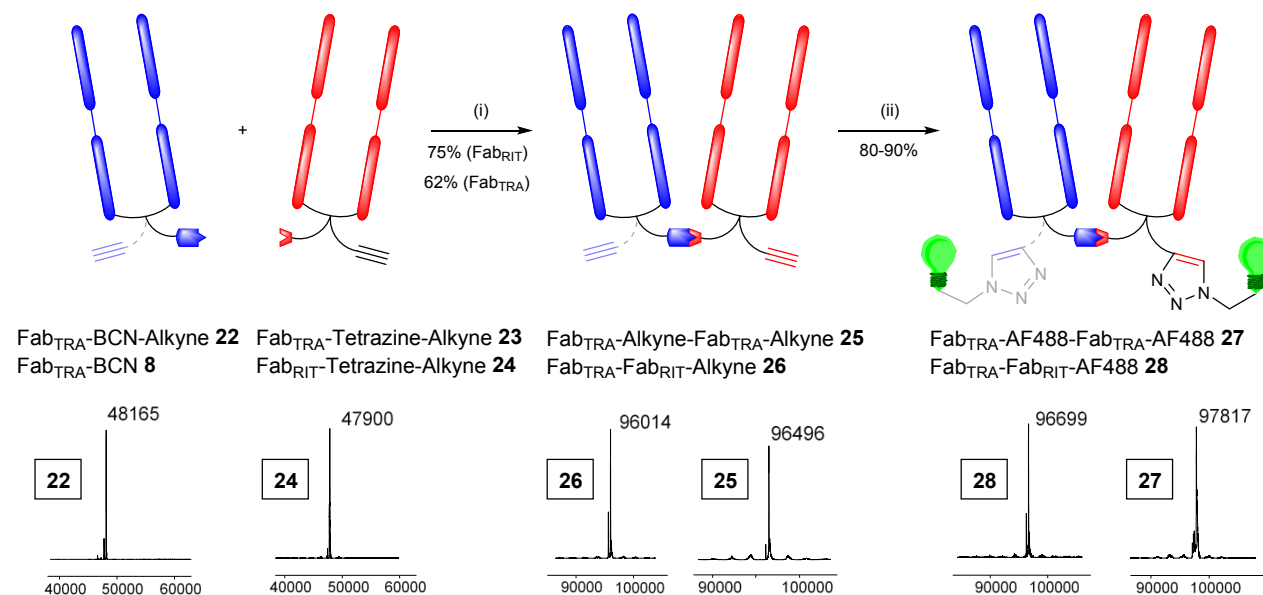


Figure 8: *Reagents and conditions:* (i) acetate buffer pH 6.0, 30 °C, 12 h; (ii) AF488 azide, CuSO₄, THPTA, sodium ascorbate, phosphate buffer pH 7.0, 25 °C, 1 h. The smaller peak observed is due to Fab_{RIT} showing as two peaks by LCMS (see SI for details).

Interestingly, tetrazines have been shown to withstand CuAAC under certain conditions. It was envisaged that this would allow for the functionalisation of the alkyne of Fab_{RIT}-Tetrazine-Alkyne **24** prior to bispecific formation by SPIEDAC thus enabling the addition of a second (different) azide after reacting the tetrazine with BCN. For this strategy, controlling the amounts of copper and sodium ascorbate was critical to alleviate tetrazine reduction. Following optimisation, 1 equivalent of copper(II) sulfate and 10 equivalents of sodium ascorbate were found to quantitatively yield the desired cycloaddition product without detectable tetrazine degradation by LCMS. The unaffected tetrazine from Fab_{RIT}-Tetrazine-AF488 **29** was then reacted with Fab_{TRA}-BCN-Alkyne **22** to form bispecific functionalised with AF488 with a remaining alkyne handle, Fab_{RIT}-AF488-Fab_{TRA}-Alkyne **30** (Figure 9).

To analyse the effectiveness of the “dual click” approach on the chemically prepared mono-functionalised BsAb **30**, a second light absorbing moiety that absorbs at a distinct wavelength to AF488 was needed to enable facile analysis by UV-visible spectrometry of the loading of each fluorophore. To this end, a photostable, water-soluble, cyanine-based fluorophore with a maximum absorbance at 673 nm (sulfo-Cy5.5) was selected and reacted with **30** to yield dually-functionalised bispecific Fab_{RIT}-AF488-Fab_{TRA}-Cy5.5 **31** in 55% yield over three steps. The 1:1 loading of each fluorophore was confirmed by UV-Vis spectrometry and LC-MS (Figure 10) thus demonstrating the modularity of the approach to obtain a loading of 0, 1, 2 or “1+1”. Thus, in essence, what we have been able to achieve is the development of: (i) a robust method in which one can form bispecifics using well-tolerated “click” chemistry; (ii) a protocol that uses Fabs that can be readily generated from native full antibodies (*i.e.* no antibody engineering is required); and (iii) a procedure in which said bispecifics can be functionalised with multiple entities in a modular fashion with exquisite control over their loading(s).

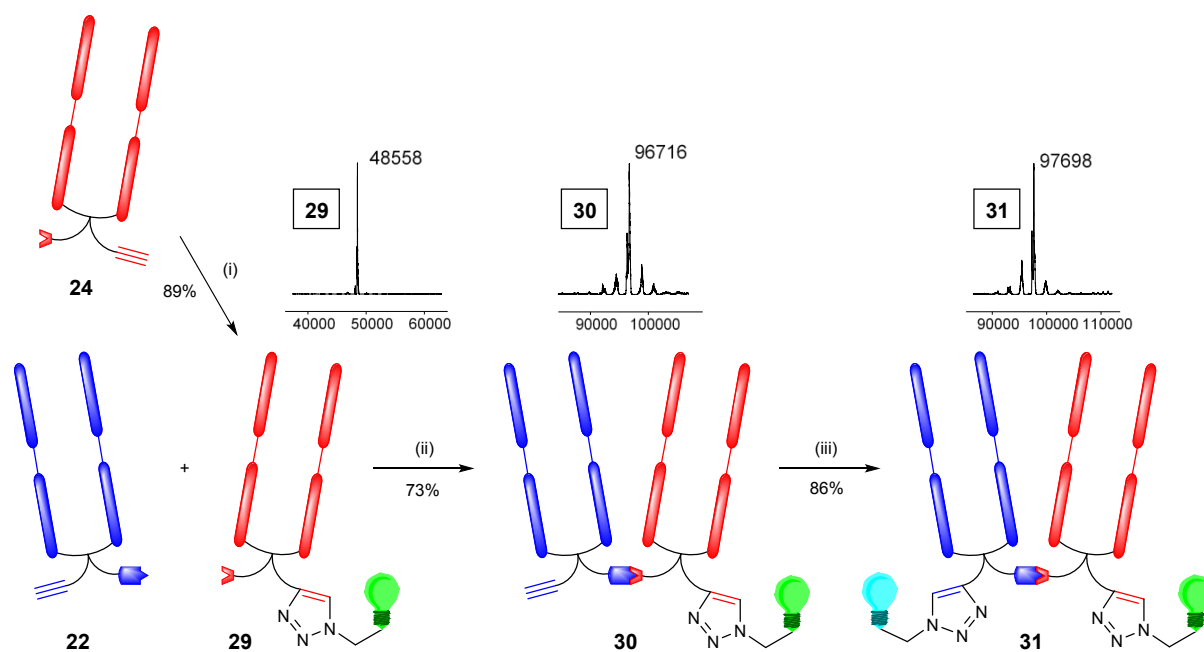


Figure 9: *Reagents and conditions:* (i) AF488 azide, CuSO₄, THPTA, sodium ascorbate, phosphate buffer pH 7.0, 25 °C, 1 h; (ii) acetate buffer pH 6.0, 30 °C, 12 h; (iii) sulfo-Cy5.5 azide, CuSO₄, THPTA, sodium ascorbate, phosphate buffer pH 7.0, rt, 1 h.

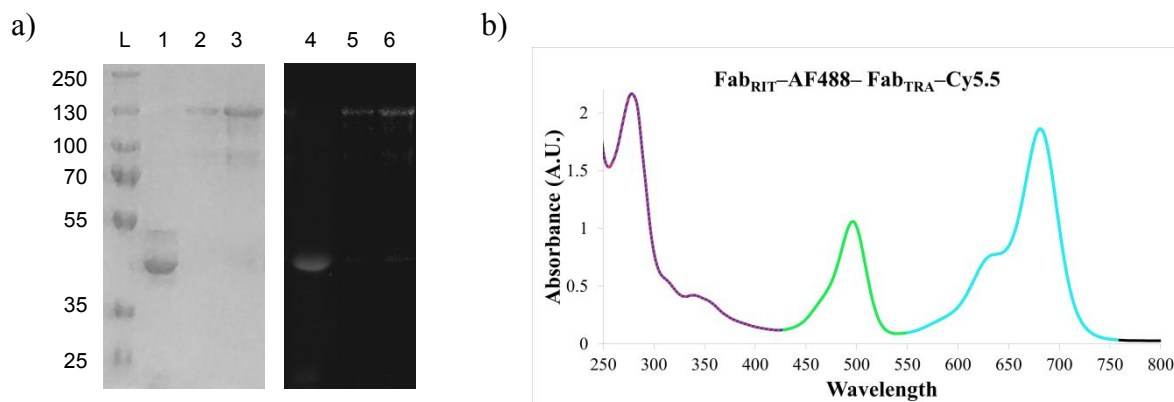


Figure 10: (a) SDS-PAGE analysis of reaction between Fab_{TRA}-BCN-Alkyne **22** and Fab_{RIT}-Tetrazine-AF488 **29**. L – ladder; lane 1 – **29**; lane 2 – **30**; lane 3 – **31**; in-gel fluorescence lane 4 – **29**; lane 5 – **30**; lane 6 – **31**; (b) UV-Vis analysis of Fab_{RIT}-AF488-Fab_{TRA}-Cy5.5 **31**.

Binding affinity

As previously described, conjugates obtained from PD-based antibody modifications are stable over a wide range of conditions (*e.g.* pH, temperature, serum) for protracted periods and do not affect binding nor internalisation of the antibody of interest as the modification is distal from the binding site.^{27, 28, 47} When compared to their parent monospecific Fab by ELISA, bispecific **31** indeed showed no decrease in K_D for each individual Fab arm (Figure 11). Following this, we wanted to confirm that binding of both Fab arms was simultaneously possible in this bispecific format, thus sandwich ELISA protocols in which only a bispecific would result in binding were established (see SI for details). Pleasingly, in all cases all non-bispecific controls showed no binding when compared with Fab_{RIT}-AF488-Fab_{TRA}-Cy5.5

31 and $\text{Fab}_{\text{TRA}}\text{-Fab}_{\text{CET}}$ **13** and for both constructs, binding was detected for both antigens indicating that $\text{Fab}_{\text{TRA}}\text{-Fab}_{\text{CET}}$ **13**/ $\text{Fab}_{\text{RIT}}\text{-AF488-Fab}_{\text{TRA}}\text{-Cy5.5}$ **31** can indeed engage EGFR + HER2 / CD20 + HER2 simultaneously (Figure 11).

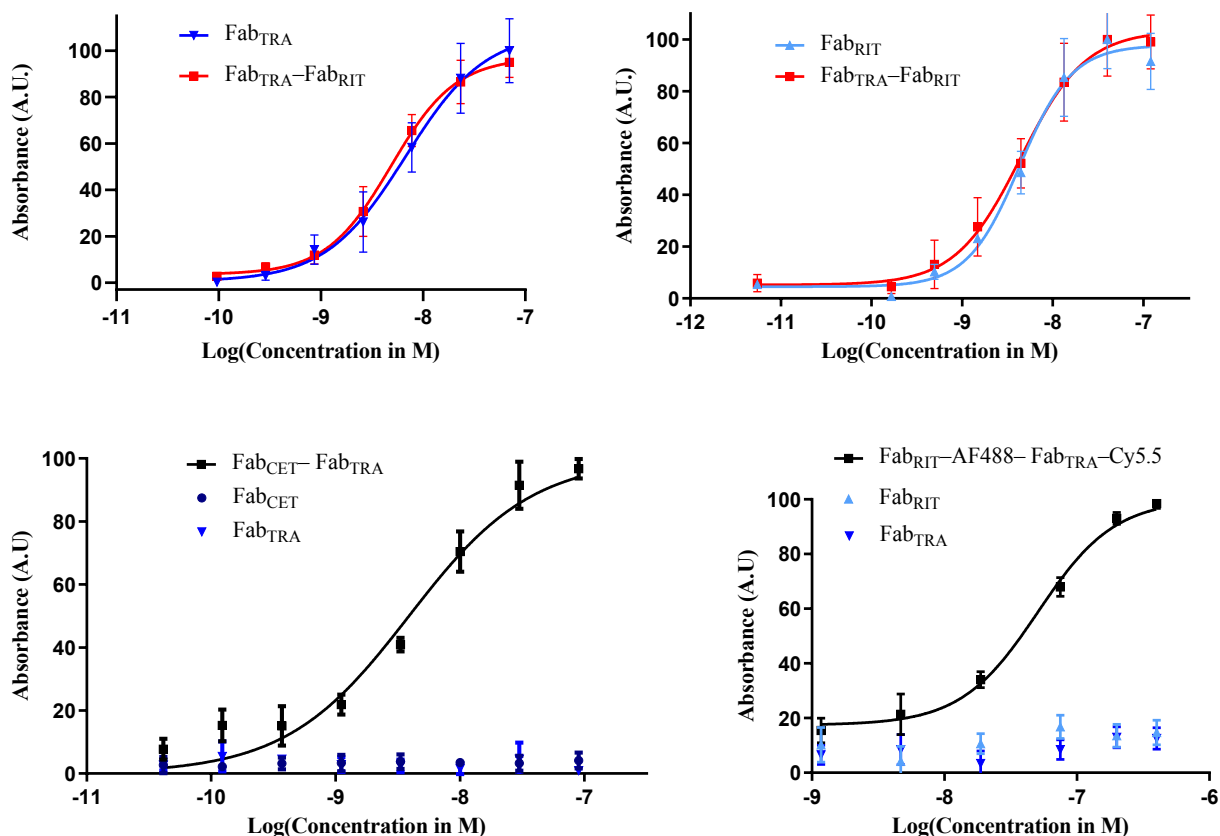


Figure 11: ELISA and sandwich ELISA analyses of $\text{Fab}_{\text{RIT}}\text{-AF488-Fab}_{\text{TRA}}\text{-Cy5.5}$ **31** and $\text{Fab}_{\text{TRA}}\text{-Fab}_{\text{CET}}$ **13**.

Discussion

In conclusion, we have developed a novel modular approach to assemble native antibody fragments to generate bispecifics in high yield and purity. Our approach enables further controlled and site-selective dual modifications of the chemically-constructed BsAbs which retains binding for both its targets. At its core, this strategy relies on the fine tuning of chemically orthogonal and bioorthogonal reactions to sequentially assemble all the building blocks in a reliable and modular fashion. This allows facile combination of antibodies and could be used to rapidly generate a library of BsAbs without the need for genetic engineering. We envisaged that this versatility can pave the way not only to novel bispecifics-based therapeutics, especially in laboratories where antibody engineering techniques are not easily accessible, but also in fields outside of antibody-based therapeutics in which protein-protein conjugates need to be prepared and functionalised.

Conflicts of interest

There are no immediate conflicts to declare, but we make clear that J.R.B., S.C. and V.C. are Directors of the spin-out ThioLogics.

Author contribution

V.C., A.M., S.C. and J.R.B. conceived and designed the project; A.M., V.C. and J.R.B. conceived and designed the chemistry and chemical biology experiments; A.M., C.J.B. and P.S. performed the chemistry experiments; A.M., J.C.F.N. and P.S. performed the chemical biology experiments; A.M., V.C. and C.B. analysed the data; AM, S.C. and V.C. co-wrote the paper.

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Supporting information

Supporting information containing general experimental details including characterisation and evaluation of bioconjugates is available.

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