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Chemical Production of Cytotoxic Bispecific Antibodies Using the Ugi Multicomponent Reaction

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Bispecific antibodies (bsAbs) have recently emerged as a promising platform for the treatment of several conditions, most importantly cancer. Based on the combination of two different antigen-binding motifs in a single macromolecule; bsAbs can either display the combined characteristics of their parent antibodies, or new therapeutic features, inaccessible by the sole combination of two distinct antibodies. While bsAbs are traditionally produced by molecular biology techniques, the chemical development of bsAbs holds great promises and strategies have just begun to surface. In this context, we took

advantage of a chemical strategy based on the use of the Ugi reaction for the site-selective conjugation of whole antibodies and coupled the resulting conjugates in a bioorthogonal manner with Fab fragments, derived from various antibodies. We thus managed to produce five different bsAbs with 2:1 valency, with yields ranging from 20% to 48%, and showed that the affinity of the parent antibody was preserved in all bsAbs. We further demonstrated the interest of our strategy by producing two other bsAbs behaving as cytotoxic T cell engagers with IC₅₀ values in the picomolar range in vitro.

Introduction

Immunotherapy is a medical treatment that stimulates the immune system of patients to fight their disease. While being only a few decades old, the field of immunotherapy has already had a profound impact in several diseases, most particularly in cancer. Central to the immunotherapy arsenal are monoclonal antibodies (mAbs), designed to selectively target proteins that are overexpressed in certain cancers. With around forty of them marketed for oncology indications already, mAbs have shown spectacular results since the first approval of rituximab in

1997.^[1] This opened new perspectives and paved the way toward the development of new formats, among which antibody-drug conjugates (ADCs) and bispecific antibodies (bsAbs) are probably the most famous examples.^[2–4] As their name suggests, bsAbs describe a large family of biomolecules designed to recognize two different epitopes or antigens that can be used as surrogates to the combination of two independent therapeutic mAbs. Coined combinatorial bsAbs, these can lead to the simultaneous inhibition of two cell surface receptors, two ligands, or to the cross-linking of two receptors,^[5,6] and can be used in the treatment of multifactorial disorders such as cancer, inflammatory disorders and diabetes.^[7–9] However, bsAbs can also display new properties, not accessible by the simple combination of their two individual parent mAbs. Called obligate bsAbs, their importance is best epitomized by bispecific T-cell engagers (BiTE). Historically composed of two single chain variable fragments (scFv) connected by a short glycine-rich peptide linker (Figure 1), BiTEs display the dual ability of binding to both a receptor on an undesirable cell in the host and to the T-cell receptor complex, triggering a cytotoxic immune response.^[10,11] Currently, there are ten bsAbs approved by either the EMA or the FDA – or both – for cancer therapy, the vast majority of which follow an obligate mechanism, by binding both the T lymphocyte antigen CD3 and a cancer antigen. Structurally speaking, all these bsAbs put on the form of a heterodimer of half-mAb with 1:1 valency – i.e., one binding site for each targeting molecule (Figure 1) – and are classically produced by molecular biology tools. First developed in the early 1980s by the quadroma technique,^[12] progresses in genetic engineering helped to improve the mediocre yields caused by statistically disfavoured pairing, epitomized by knobs-into-holes technology,^[13–15] charge interaction-based pairing techniques,^[16] or the CrossMab approach developed by Roche.^[17]

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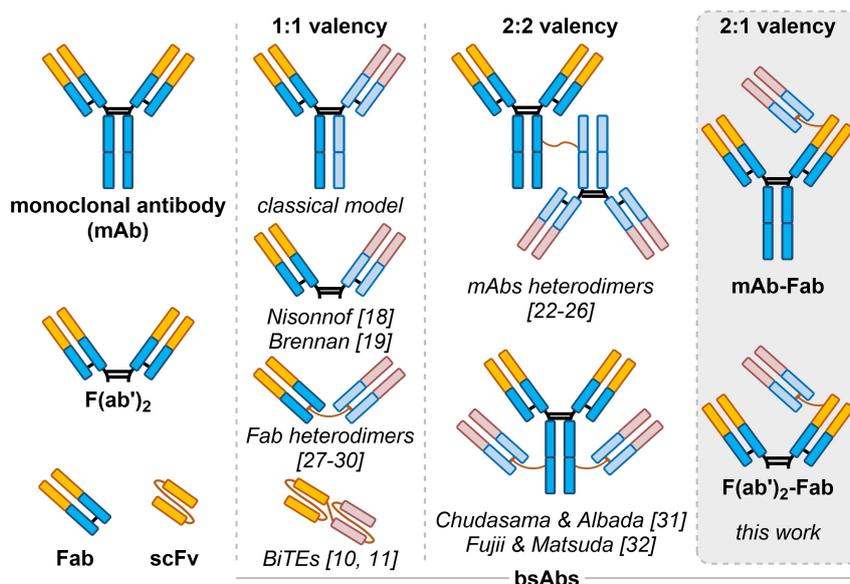


Figure 1. Graphical overview of monoclonal antibodies (mAb), including two of their fragments – the fragment antigen-binding (Fab) and $F(ab')_2$ – and a derived fusion protein format – the single-chain variable fragment (scFv) –, as well as the structures of some previously reported bispecific antibodies (bsAbs), classified according to their valency. Bibliography references for each structure are given in brackets.

In parallel, chemists saw the opportunity of applying their skills to the improvement of bsAb production. Early work from Nisonnof and Brennan, in 1960 and 1985, respectively, focused on murine bispecific $F(ab')_2$ fragments, produced by smooth reduction-reoxidation of disulfide bonds of the hinge region,^[18,19] a strategy later applied to whole mAbs in the 2010s.^[20,21] Access to bsAbs with 2:2 valency via heterodimerisation of mAbs was also extensively investigated.^[22–26] Using various sorts of chemoselective homo- or hetero-cross-linkers, this approach led however to high structural heterogeneity. These selectivity issues were finally addressed with the emergence of site-selective bioconjugation techniques and bioorthogonal chemistry, allowing fast generation of molecularly defined bsAbs libraries with great flexibility and few side products.^[27–30] While most of these studies were aimed at the production of Fab dimers with 1:1 valency, a very recent work from the Chudasama and Albada groups published during the finalisation of our work reported the production of two bsAbs with 2:2 valency, composed of a whole mAb site-selectively adorned with two identical Fab fragments (Figure 1).^[31] Using both their expertise in the field, these were produced by biogenic tyrosine-based click chemistry followed by an inverse electron-demand Diels–Alder (IEDDA) reaction with rebridged Fabs, ensuring the generation of molecularly defined bispecific species. While partial trimerisation led to unseparable mixtures of bsAbs with 2:1 and 2:2 valencies, their strategy still led to a potent HER2xCD3 bispecific T-cell engager with IC_{50} values in the picomolar range. Concomitantly, researchers at Ajinomoto described the successful production of an EGFRxCD3 T-cell engager bsAb built on the same 2:2 valency format.^[32] Using the second generation of their proprietary technology AJICAP, rebridged Fab fragments were site-selectively conjugated to the

mAb's Lys248 residue in the Fc domain – notably, this did not alter the neonatal Fc receptor-binding affinity.

In this expansive field of chemistry-driven conjugation approaches,^[33] our team recently reported the site-selective modification of proteins using the Ugi reaction.^[34,35] Reacting the anti-HER2 antibody trastuzumab with an azide-containing aldehyde and ethyl isocynoacetate, we have notably shown that this multicomponent reaction could selectively label *N*-terminal E1 residues without affecting the antibody's affinity. Taking advantage of the azide group thus incorporated onto the mAb, we assumed that its reaction with site-selectively bicyclo[6.1.0]non-4-yne (BCN)-functionalized Fab fragments would allow access to homogeneous bsAbs through strain-promoted azide-alkyne cycloaddition (SPAAC) reaction.

Results & Discussion

We thus set out to first produce a collection of Fab from various mAb precursors. Through the development of bespoke digestion and purification procedures, we managed to isolate five different Fab fragments – i.e., anti-PD-L1 avelumab and durvalumab, anti CD20 rituximab, anti-CD3 muromonab as well as anti-HER2 trastuzumab – with acceptable to good yields (54 to 85 % yield) and excellent purity (see SI for more details). Each of these Fab were next conjugated in a site-selective manner, using the dibromomaleimide (DBM) rebridging reagent functionalised with a BCN group **DBM-BCN** (Figure 2). This reagent was constructed using a BCN acid group instead of the more conventional BCN alcohol, the former allowing amide bond formation, which were shown to give superior stability over carbamate bonds over time.^[36] Complete conversion and excellent chemoselectivity and purity – average degree of

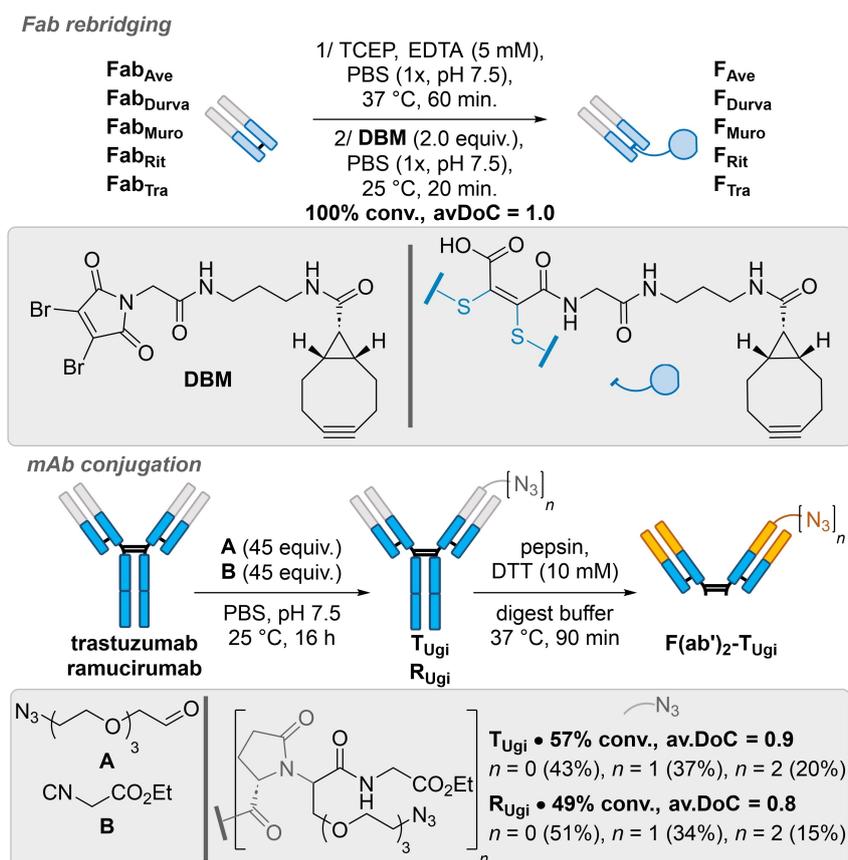


Figure 2. Site-selective conjugation of whole mAb and Fab fragments, poised for bsAb production by bioorthogonal SPAAC reaction.

conjugation (avDoC) = 1.0 – were systematically obtained, delivering our second SPAAC partners. Importantly, all observed molecular weights (MW_{obs}) values were found to be 18 Da higher than the calculated (MW_{th}) values, suggesting the hydrolysis of the maleimide ring in all rebridged adducts. This is consistent with previous observations made on such C-2 linkers, displaying hydrolysis half-lives of less than 20 minutes and thus offering better stability of the thiovinyl motif by making it unreactive towards serum thiols.^[37,38]

Having now in hands complementary azide-labelled mAbs and BCN-labelled Fab, we turned to the evaluation of bsAb production through SPAAC reaction. After extensive optimisation using E1-conjugated trastuzumab T_{Ugi} and the rebridged Fab of rituximab F_{Rit} , we found out that complete conversion of T_{Ugi} was obtained after 48 h at 25 °C in PBS 1X buffer (pH 7.5), using 100 μM and 200 μM solutions of the two coupling partners, respectively (Figure 3A). Purification and analysis by size exclusion chromatography coupled to native mass spectrometry (SEC-nMS) allowed the separation and identification of three main peaks (Figure 3B). Peak 3, eluting at 11.08 min., was found to correspond to unreacted rebridged F_{Rit} that had been added in excess. The second peak, eluting at 7.78 min., contained unlabelled trastuzumab, as expected from the 60% conversion of the Ugi conjugation reaction. Excitingly, peak 1, with an elution time of 7.10 min and a shoulder peak at around 6.30 min, was found to contain a major compound, whose

molecular weight matched that of the bsAb T- F_{Rit} with a 2:1 valency.

Purification and isolation of this bsAb was then performed by preparative SEC. The separation was found to be extremely challenging, as also observed by our predecessors,^[31] with an overlapping between the peaks corresponding to the bsAbs and that of trastuzumab. Varying the eluent did not lead to better resolution, while increasing the loading concentration of the crude was found to be beneficial only to a certain extent. We suspect that this could be due to a stacking of the Fab portion onto the whole mAb, resulting in a clear separation of the different species under denaturing conditions but in a poor increase of the hydrodynamic radius of the bsAb and hence mediocre separation under native conditions. Two consecutive runs of purification were thus necessary to fully separate the bsAbs from both its parent mAb and pure unreacted F_{Rit} – which could be recycled for further bsAb production – delivering 48% of T- F_{Rit} (yield based on the amount of azide-bearing trastuzumab).

Analysis by SEC-nMS confirmed the isolation of the desired product, with MW_{obs} closely matching MW_{th} alongside a minor side product (less than 20% according to relative quantification of UV signals) whose MW_{obs} was found to be in good agreement with that of a T- F_{Rit} bsAb with 2:2 valency (i.e., T-2 F_{Rit}). The formation of this bsAb is probably the result of a second SPAAC reaction between T- $\text{F}_{\text{Rit}}\text{-N}_3$ – bearing a second unreacted Ugi

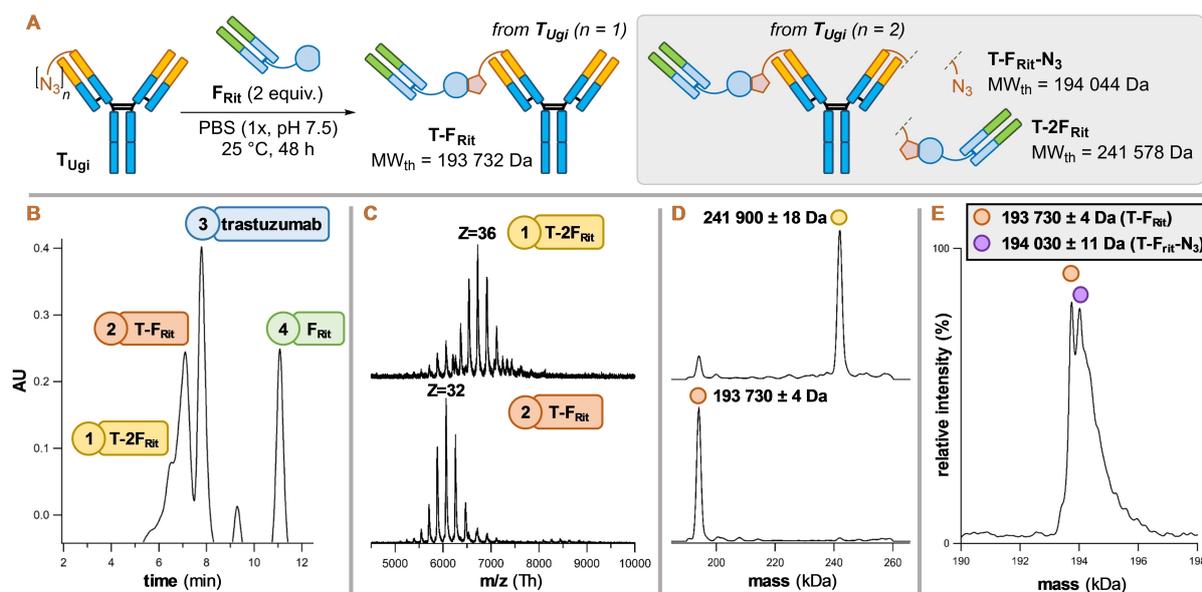


Figure 3. BsAb production, analysis and purification. A) Synthetic scheme describing the formation of our model bsAb T-F_{Rit} from site-selectively Ugi-conjugated trastuzumab T_{Ugi} and the rebridged Fab fragment of rituximab F_{Rit} – side species are indicated in the grey box; B) SEC chromatogram of the crude reaction mixture, with peaks individually assigned; C) Native mass spectra of purified T-F_{Rit} showing the presence of the T-2F_{Rit} side species, with 2:2 valency; D) deconvoluted values of the two main bsAb species T-F_{Rit} (orange) and T-2F_{Rit} (yellow); E) enlarged deconvoluted mass spectrum of T-F_{Rit} showing the presence of the T-F_{Rit}-N₃ side species (purple), bearing a second unreacted Ugi conjugation site.

conjugation site, consistent with the analytical data shown in Figure 3E – and another equivalent of F_{Rit}. In an attempt to push this bsAb formation further, we tried to increase either the concentration in F_{Rit} or the reaction time. While precipitation was observed in the first case, leaving the reaction for up to 120 h led to no detectable change, confirming the favoured formation of 2:1 T-F_{Rit} and the absence of higher molecular weight species. While less common than their 2:2-valency counterparts, bispecifics with 2:1 formats have been already documented, especially for the formation of T-cell bsAbs. In this latter case, they usually outperform their 1:1-valency analogues while minimising non-specific T-cell activation in the extratumoral space and tightening the tumor/T-cell synapse area.^[39–42]

Pleasingly, the affinity of our bsAb toward HER2 receptor was maintained: a K_D value of 0.35 nM was measured on live HER2-positive SKBR3 cell line using LigandTracer technology, a value comparable to that of native trastuzumab ($K_D = 0.65$ nM), measured under the same conditions. Quite counter-intuitively, this demonstrates that even the attachment of large biomolecular fragments on the N-terminal position of trastuzumab does not impair its antigen recognition.

Building on these positive results, we applied our procedure to the production of other bsAbs by varying the rebridged Fab fragment – i.e., F_{Tra}, F_{Durva}, F_{Ave} and F_{Muro} – but also by using another site selectively Ugi-functionalized mAb, ramucirumab R_{Ugi} (Figure 4). We also showed that the pepsin-digested F(ab')₂ fragment of trastuzumab F(ab')₂-T_{Ugi} could be employed, delivering a bsAb deprived of a Fc portion after reaction with F_{Muro}. We were delighted to notice that our method proved to be well applicable to all of these other proteins and fragments, allowing access to seven different 2:1 bsAbs, albeit with lower

yields than those observed for the formation of T-F_{Rit}. As previously observed, the presence of bsAb species with an unmodified Ugi conjugation site was systematically detected (purple circles in Figure 4), even though bsAbs with 2:2 valencies could only be observed in T-F_{Tra} experiments (see SI). Pleasingly, all bsAbs whose valency of 2 originated from trastuzumab showed a maintained affinity toward HER2 receptors, with subnanomolar K_D values. The only loss in affinity was observed with the 2:1 bsAb R-F_{Tra}, built by the combination between Ugi-modified ramucirumab R_{Ugi} and the rebridged Fab fragment of trastuzumab F_{Tra}, that showed a 4-fold decrease in affinity compared to native trastuzumab ($K_D = 0.65$ nM) and a 3-fold decrease compared to its Fab fragment (F_{Tra}, $K_D = 0.97$ nM). While this could reinforce our previous hypothesis of electrostatic interactions between the whole mAb and the Fab fragment in the bsAb construct, it shows that inverting Fab and whole mAb can still lead to affine constructs, showcasing the modularity of our approach even further.

Having validated our strategy and demonstrated that the site-selective Ugi reaction is a valuable conjugation method to access 2:1 bsAbs, we wanted to engage into in-depth pharmacological studies of certain of our dimers, namely T-F_{Muro} and F(ab')₂-T-F_{Muro}. Indeed, beside a HER2 recognition motif, these two bsAbs also feature a CD3-targeting Fab fragment capable of binding and activating T-cells, thus making them potential bispecific T-cell engagers.

Preliminary receptor occupancy studies by flow cytometry were conducted with human breast cancer HER2⁺-CD3⁻ HCC1954 cells and human T lymphocyte CD3⁺-HER2⁻ Jurkat cells. For CD3-binding evaluation, both bsAbs were first incubated with Jurkat cells at 4°C to prevent any receptor

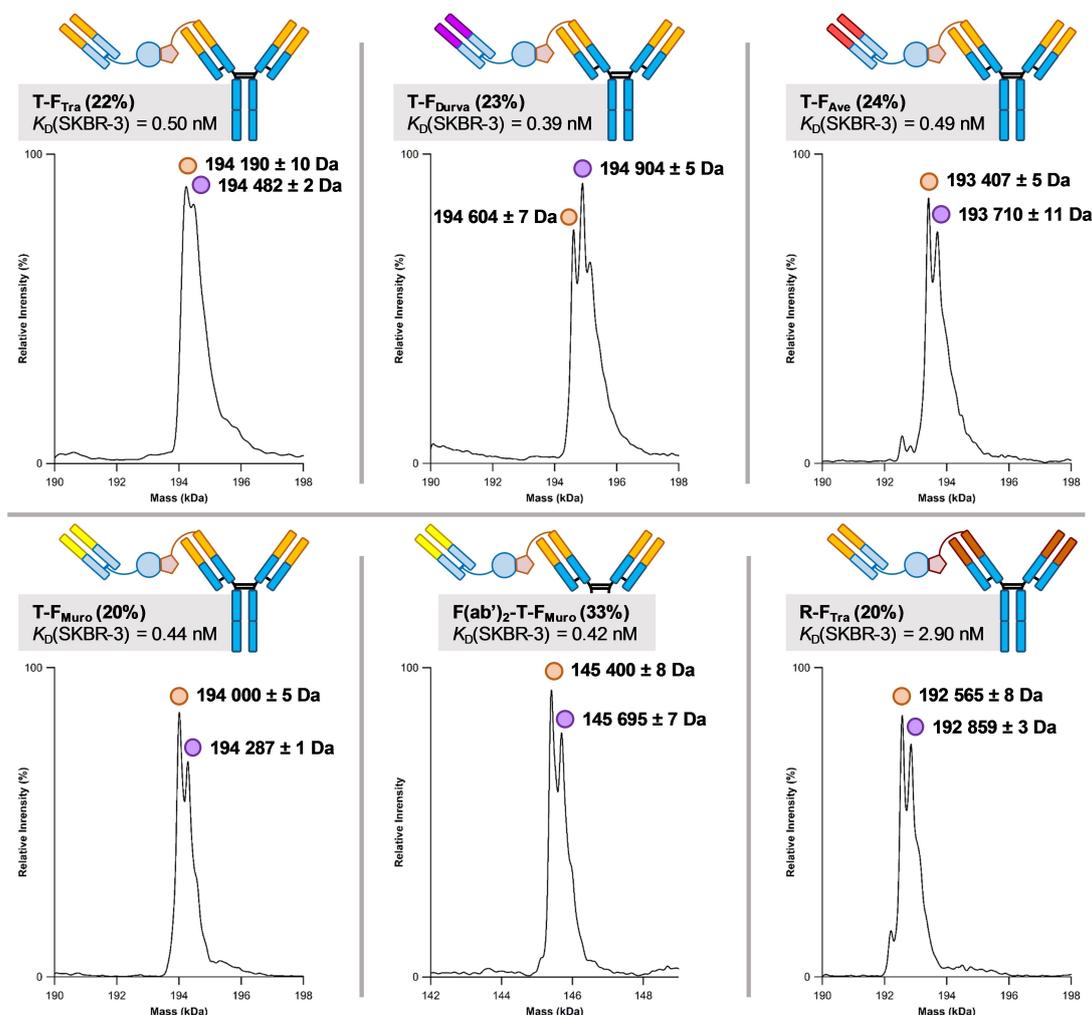


Figure 4. Deconvoluted native mass spectra of all produced bsAbs. Yields and binding affinity (K_D) values are indicated for each bsAb. The purple circle indicates the bsAb-N₃ side species, following the same colour code as the one used in Figure 3.

internalization. Following incubation, samples were washed to remove any unbound bsAb and stained with allophycocyanin (APC)-conjugated anti-CD3 secondary antibody. The results of the receptor occupancy studies showed clear binding of both T-F_{Muro} and F(ab')₂-T-F_{Muro} bispecific constructs to Jurkat cells, indicated by a significant reduction in fluorescence of the bsAb-incubated samples in comparison with the untreated, APC-stained controls, thus validating CD3-receptor occupancy by the bsAbs. Similar study conducted on HCC1954 cells also demonstrated HER2-receptor occupancy, confirming the results of our affinity studies (see SI). Following confirmation of the ability of both T-F_{Muro} and F(ab')₂-T-F_{Muro} to bind to their target antigens, their capacity to induce T cell-mediated cytotoxicity was then evaluated in a dose-response study (Figure 5). Briefly, HCC1954 cells were cultured with primary T cells isolated from buffy coats of three different healthy donors at an effector-to-target (E:T) ratio of 10:1 and treated with increasing concentrations of T-F_{Muro} or F(ab')₂-T-F_{Muro} (i.e., 9.9 pM – 2.53 nM and 13.7 pM – 3.5 nM, respectively, with the top concentrations equating 1 $\mu\text{g mL}^{-1}$ of bsAbs). After 72 h of incubation, tumor cell viability was measured using CellTiter-Glo® assay. Gratify-

ingly, both bsAbs showed to induce notable levels of cytotoxicity in a dose-dependent manner in HCC1954 cancer cells when co-cultured with T cells, with T-F_{Muro} and F(ab')₂-T-F_{Muro} giving average IC₅₀ values of 77 pM and 83 pM, respectively (Figure 5A). This is in line with values observed previously for similar constructs.^[31] In addition, we also showed that the observed potency of the two bsAbs was not caused by the mAb or the Fab taken individually, nor by a potential combined effect, but rather by their covalent combination (Figure 5B). Finally, the ability of the bsAbs to induce interferon gamma (IFN- γ) release from the T cells was assessed. This analysis showed that in the presence of the bsAb and coculture, IFN- γ was released, consistent with the intercellular engagement of the bsAb to drive T cell activation and induce subsequent target cell cytotoxicity (Figure 5C). Finally it is worthy to note that as the the Fc portion of mAbs can trigger immunogenicity against the bsAb, whilst also increasing the occurrence of adverse effects through their complement-dependent cytotoxicity and antibody-dependent cell-mediated cytotoxicity mechanisms,^[43,44] the facile generation of Fc-deprived F(ab')₂-

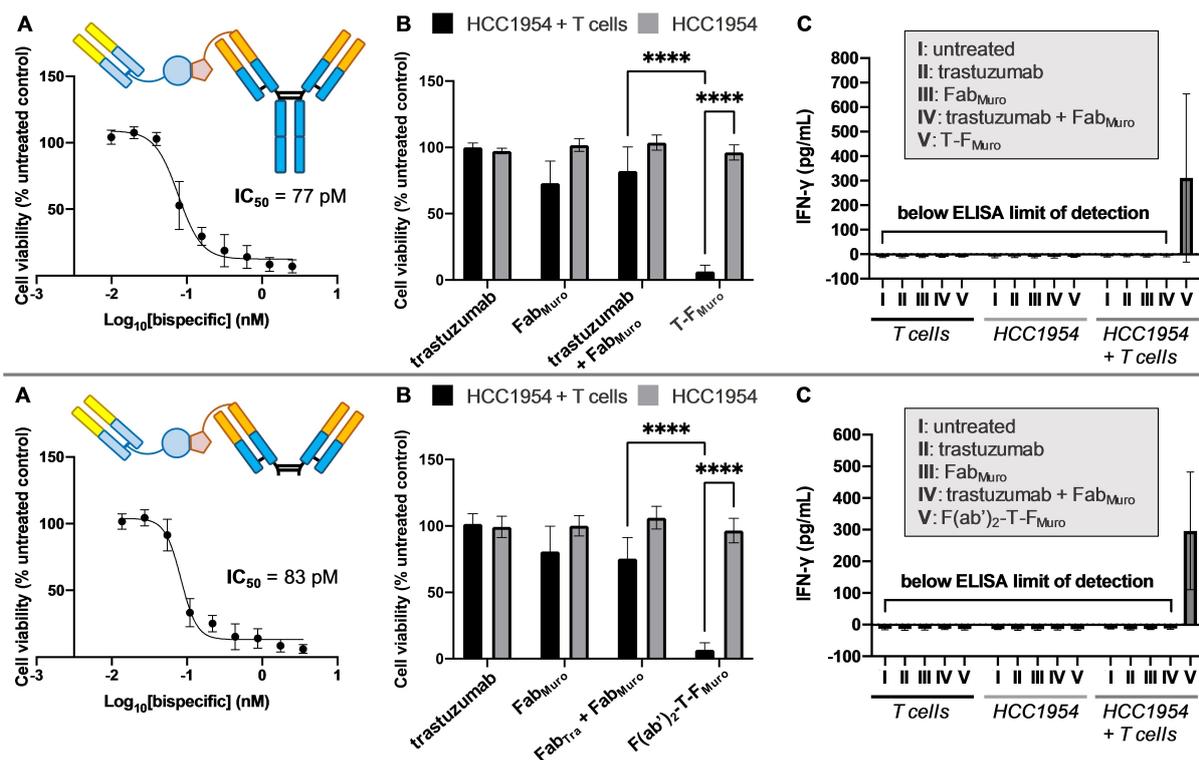


Figure 5. T-cell-dependent cytotoxicity of T-F_{Muro} and F(ab')₂-T-F_{Muro} bsAbs. A) Dose-response study in T cell – HCC1954 cell coculture (E:T 10:1; 72 h); T cells were isolated from three different healthy donors. B) Single-dose 72-h treatment in T cell – HCC1954 cell cocultures (E:T 10:1) and HCC1954 cells only; for T-F_{Muro} concentrations of 2.5 nM were used for all tested compounds; for F(ab')₂-T-F_{Muro} concentrations of 3.5 nM were used. C) IFN-γ production following 72 h treatment of T cell – HCC1954 cell cocultures (E:T 10:1), HCC1954 cells only or T cells only; for T-F_{Muro} concentrations of 2.5 nM were used for all tested compounds; for F(ab')₂-T-F_{Muro} concentrations of 3.5 nM were used.

Fab formats such as F(ab')₂-T-F_{Muro} represents a key advantage of our approach.

Conclusions

In conclusion, we have developed a modular method for the selective formation of 2:1 bsAbs, based on a whole mAb-Fab architecture. Using off-the-shelf mAbs, we site-selectively conjugated them with our previously developed Ugi reaction before coupling the resulting conjugates with rebridged Fab fragments. Seven different bsAbs with 2:1 valency were produced, with yields ranging from 20 to 48% and a maintained affinity toward HER2 receptors on live cells when whole trastuzumab was employed. Two of our bsAbs, namely T-F_{Muro} and its partially digested analogue F(ab')₂-T-F_{Muro}, were shown to be capable of eliciting high levels of T cell-mediated cytotoxicity in vitro, with IC₅₀ values in the picomolar range. This tends to demonstrate that a 2:1 valency is already sufficient to yield bioactive bsAbs and that the conjugation site on the whole mAb partner can be varied without altering the potency of the constructs.

Associated Content

The Supporting Information contains experimental procedures, chemical synthesis procedures, all analytical data – including SEC-nMS profiles, affinity studies, and copies of ¹H and ¹³C NMR spectra – and in vitro assays.

Author Contributions

V. V.: Conceptualisation; Data curation; Formal analysis; Investigation; Methodology; Validation; Writing – original draft. I. K.: Data curation; Formal analysis; Investigation; Methodology; Validation. S. E.: Data curation; Formal analysis; Investigation; Methodology; Validation; Visualization. B. J.: Data curation; Formal analysis; Investigation; Methodology; Validation; Visualization. R. B.: Data curation; Formal analysis; Investigation; Methodology; Validation; Visualization. H. C.: Data curation; Visualization; Writing – review & editing. A. D.: Resources; Writing – review & editing. O. H.-A.: Project administration; Resources; Supervision. C. J. S.: Funding acquisition; Methodology; Project administration; Resources; Supervision; Writing – review & editing. S. C.: Funding acquisition; Methodology; Project administration; Resources; Supervision; Writing – review & editing. G. C.: Conceptualisation; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Proj-

ect administration; Supervision; Validation; Visualization; Resources; Writing – review & editing.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

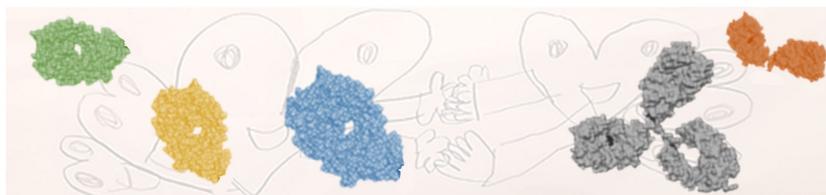
The data that support the findings of this study are available in the supplementary material of this article.

Keywords: bioconjugation · antibodies · chemical biology · cancer

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RESEARCH ARTICLE



This work reports the chemical synthesis of bispecific antibodies using two site-selective conjugation reactions. Seven constructs with 2:1

valency were produced, including two cytotoxic T-cell engagers with IC_{50} values in the picomolar range in vitro.

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Chemical Production of Cytotoxic Bispecific Antibodies Using the Ugi Multicomponent Reaction

