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Communication

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Design and Synthesis of Megamolecule Mimics of a Therapeutic Antibody

Justin A. Modica, Tsatsral Iderzorig, and Milan Mrksich*

Northwestern University, Departments of Chemistry and Biomedical Engineering, 2145 Sheridan Road, Evanston, IL 60208

ABSTRACT: This communication describes the design, synthesis and biological activity of a megamolecule mimic of an anti-HER2 antibody. The antibody mimic was prepared by linking two Fabs from the therapeutic antibody trastuzumab, which are fused through the heavy chain variable domain to either cutinase or SnapTag, with a linker terminated in an irreversible inhibitor for each enzyme. This mimic binds HER2 with comparable avidity to trastuzumab, has similar activity in a cell-based assay, and can arrest tumor growth in a mouse xenograft BT474 tumor model. A panel of 16 bivalent anti-HER2 antibodies were prepared wherein each varied in the orientation of the fusion domain on the Fabs. The analogs displayed a range of cytotoxic activity and surprisingly, the most active mimic binds to cells with a ten-fold lower avidity than the least active variant suggesting that structure plays a large role in their efficacy. This work suggests that the megamolecule approach can be used to prepare antibody mimics having a broad structural diversity.

Monoclonal antibodies (mAbs) are an important class of therapeutics and have emerged as the decade's new blockbuster drugs. Yet, nearly all approved therapeutic mAbs are based on structures that are genetically encoded, and the majority are based on the natural Y-shaped immunoglobulin (IgG) scaffold. Protein engineering approaches have increased the number of mAb variants but are still quite limited in accessing a broader structural space.[1-2] For example, the IgG scaffold enforces a constant distance and orientation of the two Fab domains that are organized for recognition of a pair of identical epitopes on the cell surface, and efforts to vary this distance, expand valency, or create multi-specific variants of the core structure are still challenging.[3] In this paper, we demonstrate the use of 'megamolecule' assembly to prepare a family of antibody mimics that present two Fabs for the HER2 receptor and show that these analogs vary in their activity, and that the most active analog halts tumor growth *in vivo*. Further, this work reveals that complex relationships exist between antibody structure and function and suggests that antibody engineering efforts may benefit from approaches that can vary structural parameters over a wider space.

We recently introduced the 'megamolecule' strategy to prepare structures that are perfectly defined—in the sense that every atom and bond is specified—and have dimensions greater than 10 nm and molecular weights exceeding 100,000 Da.[4-7] These molecules are prepared from fusion proteins that include an enzyme domain that reacts selectively and covalently with an irreversible inhibitor. In this way, reactions of the proteins with linkers terminated in these inhibitors are used to assemble the structures. Our work has used three enzyme-inhibitor systems—the reaction of cutinase with phosphonate esters,[8] of HaloTag with alkylchlorides,[9] and of SnapTag with benzylguanine or benzylchloropyrimidine derivatives[10]—and several other pairs are now in development.

We prepared antibody mimics by first making genetic constructs encoding the Fab domain from the antibody trastuzumab (Herceptin) fused to either cutinase or SnapTag at

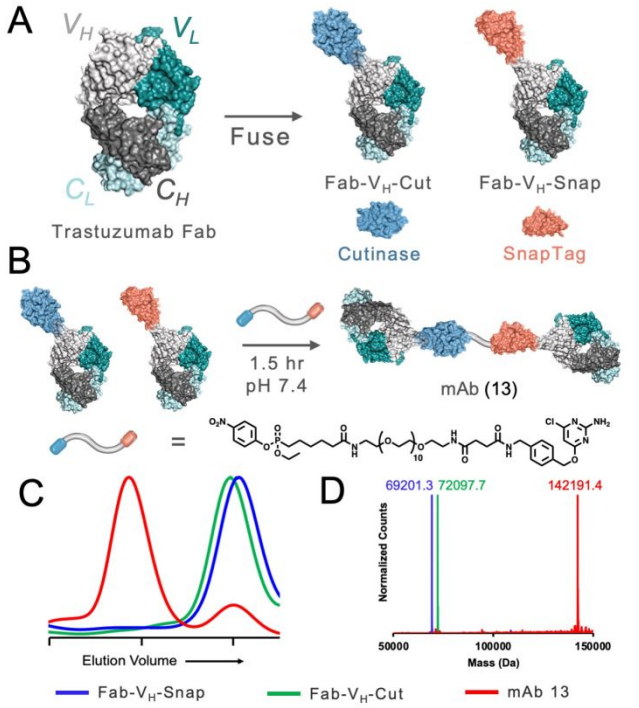

Figure 1. The preparation and characterization of an anti-HER2 antibody mimic. (A) Antibody building blocks were generated by fusing cutinase or SnapTag to the heavy chain variable domain of the Fab of the antibody trastuzumab. The Fab (PDB ID – 1cly), and the fusion protein structures pictured here are not true representations of the actual structures but are presented in this manner for clarity. Detailed models of these fusion proteins and mAb **13** can found in the Supporting Information. (B) The antibody mimic is assembled by joining the two fusion proteins with a PEG-based linker. (C) A size-exclusion chromatograph of the two Fab fusions and of the crude linking reaction. (D) Deconvoluted ESI-MS of the two purified fusion proteins and bivalent antibody product.

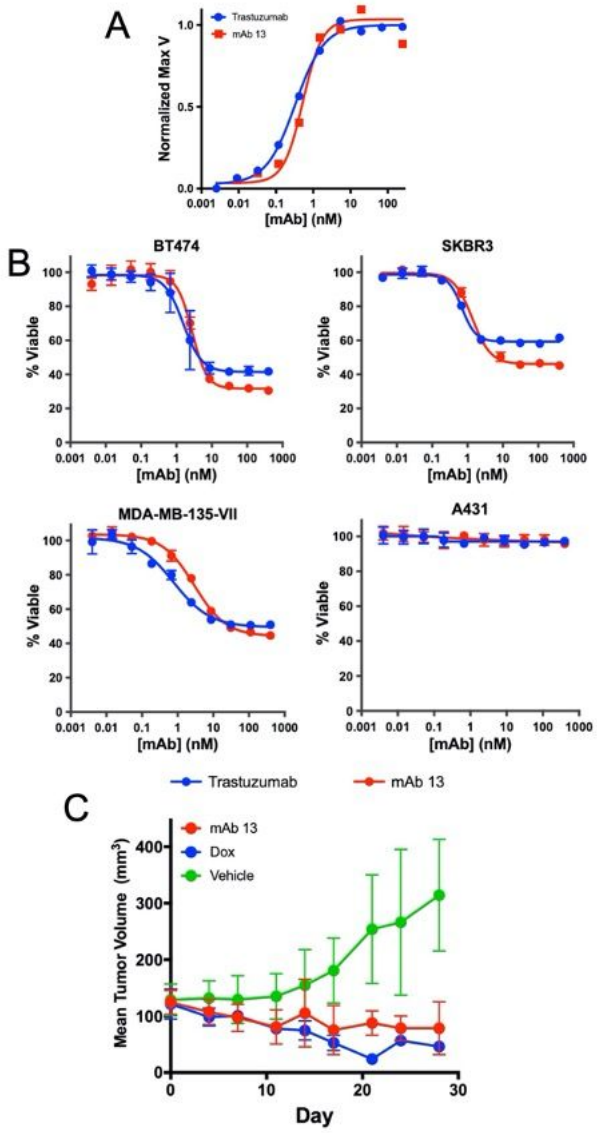

Figure 2. Measured avidity, *in vitro*, and *in vivo* activity of trastuzumab mimic **13**. (A) Direct ELISA of trastuzumab and **13** against HER2 extracellular domain. (B) In vitro cytotoxicity data for **13** and trastuzumab in three HER2 positive and one HER2 negative cell lines. (C) A plot of mean tumor volume vs time for the mouse BT474 xenograft model.

the N-terminus of the heavy chain variable domain (V_H) as shown in Figure 1A. We chose the trastuzumab Fab because it allows a direct comparison of our mimics with the approved therapeutic in a well-studied biological system. We then expressed each fusion in SHuffle Express B *E. coli*,[11] with typical yields of ~15 mg / L after purification. For the linker, we used a dodecaethylene glycol spacer functionalized with a p-nitrophenyl phosphonate ester on one side and a benzylchloropyrimidine on the other (Figure 1B).[6] Combining this linker and the two Fab fusion proteins in equal amounts at 10 μM gave the desired bivalent antibody, **13** (numbering explained later in the text), in 85% yield after 90 min of reaction and purification by size exclusion chromatography (SEC). Electrospray ionization mass spectrometry (ESI-MS) of the product gave an experimental mass of 142191.4 Da, in good agreement with the calculated value of 142181.6 Da.

Trastuzumab is a therapeutic mAb used in the treatment of cancers that overexpress the epidermal growth factor receptor HER2.[12] The primary mechanism for trastuzumab-mediated growth arrest of HER2 positive tumors is the inhibition of signaling through the disruption of receptor dimerization. The Fab of trastuzumab accomplishes this by binding to domain IV of the HER2 extracellular domain thereby blocking the dimer interface.[13] To verify that our mimic had comparable avidity for HER2 as the parent antibody, we used a direct ELISA assay to measure binding of **13** to the HER2 extracellular domain. The apparent dissociation constant for **13** was 0.5 nM, similar to that for trastuzumab (0.3 nM) (Figure 2A).

We then used a cytotoxicity assay to compare the activity of **13** with trastuzumab in the HER2 positive cell lines BT474, SKBR3 and MDA-MB-135-VII. We treated cells cultured in 96-well plates with trastuzumab and **13** in concentrations ranging from 400 nM to 4 pM for 96 hours and then measured their viability after this period using the Alamar Blue reagent. The results of these experiments are shown in Figure 2B. The determined EC_{50} values were similar in all cases: 2.8, 1.4, and 2.9 nM for **13**, and 1.6, 0.7, and 0.8 nM for trastuzumab in the three cell lines, respectively. Both molecules showed no cytotoxicity in a HER2 negative carcinoma cell line A431, indicating insignificant off-target effects associated with mimic **13** in this assay. Control experiments using just the Fab, and the V_H-cutinase and V_H-SnapTag fusions gave EC_{50} values that were similar to each other and were approximately 10-fold higher than those for the bivalent molecule **13** (Supporting Information, Figure S1). This result shows that the fusion partners did not interfere in the engagement of HER2 or enhance the blocking effect of the Fab domains.

As an additional comparison, we monitored the stability of trastuzumab and **13** by SDS-PAGE in serum-containing cell culture media at 37°C and in PBS at 55, 65, and 75°C and found that the two had comparable stability under the conditions of the cytotoxicity experiments, yet different stability at 65°C with **13** decomposing within 1 hr and trastuzumab slowly decomposing over 10 hours (Figures S4 and S5).

Having established that our mimic displays comparable activity to trastuzumab in *in vitro* assays, we next characterized the efficacy of **13** in a mouse xenograft BT474 tumor model. After establishing the tumor, SCID-beige mice (n = 5 per treatment group) were treated once daily for 28 days with **13** (2.5 mg / kg) or with doxorubicin (2.5 mg / kg). As megamolecule **13** lacks the Fc domain and thus the ability to bind the neonatal Fc receptor (FcRn),[14-15] its half-life is short ($t_{1/2}$ ~2.6 hr, Figure S2) and for this reason we implemented a daily dosing schedule. For a positive control, we chose doxorubicin as it is a highly potent chemotherapeutic agent, has a serum half-life closer to our mimic than an IgG and is widely used as a comparison in efficacy studies of novel chemotherapeutics.[16-17] At the conclusion of the experiment, the groups treated with **13** and doxorubicin showed significant arrest of tumor growth compared to that of the negative control group ($p = 0.034$) (Figure 2C). Additionally, no statistically significant difference in total body or organ weight was observed between the treatment group and control suggesting that the molecule was well-tolerated at this dose (Figure S3).

We selected antibody **13** from a panel of 16 trastuzumab mimics we prepared. Each of these molecules presents two copies of the Fab on a scaffold formed by joining a cutinase and SnapTag domain with the linker, as explained earlier. The structures differ in the spacing and relative orientations of the Fab domains because each fusion can be assembled by tethering the enzyme to either the heavy or light chain of the Fab, and either the N- or C- terminus of each chain. Hence the antibody mimics were prepared by joining one of the four Fab-cutinase

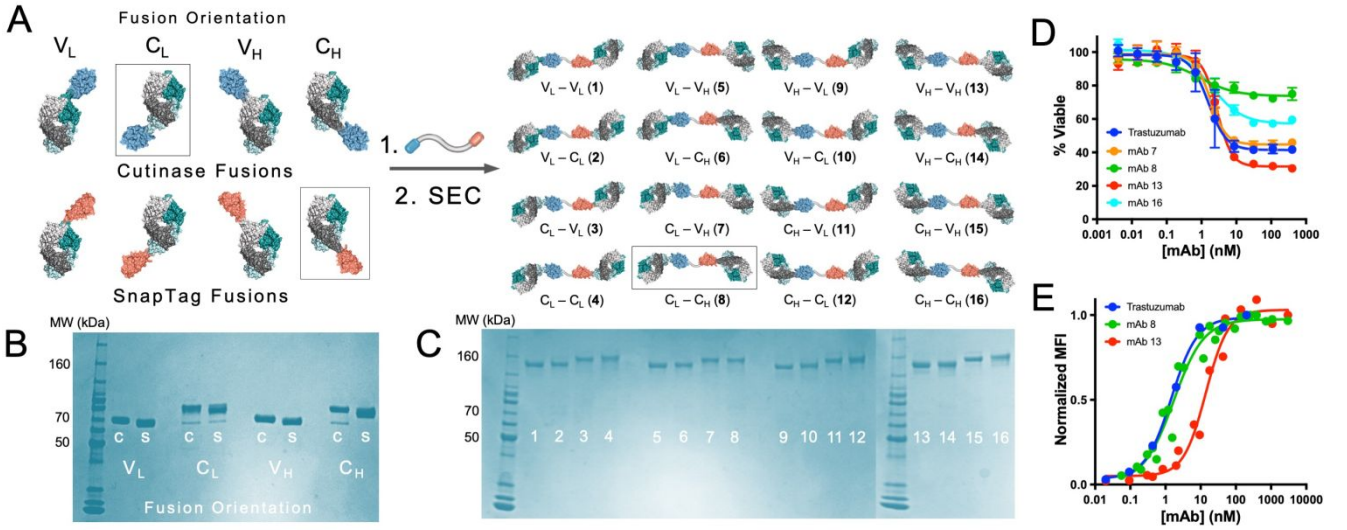

Figure 3. Preparation and characterization of 16 trastuzumab mimics. (A) Cutinase or SnapTag can be fused to each of the four domains of the trastuzumab Fab to yield 8 Fab fusion proteins. These fusions are joined in separate reactions using the megamolecule linker to yield 16 possible heterobivalent antibodies. Labels indicate the connectivity of the products. Boxes outline the two fusions used to construct mAb **8.** (B) Non-reducing SDS-PAGE gel of the Fab fusion building blocks. Band labels denote which enzyme is fused to the Fab: c = cutinase, s = SnapTag. (C) Non-reducing SDS-PAGE gel of the 16 antibodies showing masses near the expected molecular weights. (D) BT474 cytotoxicity data from four of the 16 mimics. (E) HER2 binding curves of the mimics and trastuzumab on BT474 cells obtained using flow cytometry.

fusions with one of the four Fab-SnapTag fusions to give 16 different structures (Figure 3A). Notably, we found that all sixteen constructs were produced in similar yield, and we believe that the similarity of the reaction kinetics owe to the initial binding of irreversible inhibitor to the enzyme, which is expected to be the same in all cases when steric effects are not significant. We determined the ability of each mimic to arrest growth in BT474 cells, and we found that they spanned a broad range of cytotoxic activity in this assay, with mimic **8** as the least active and **13** as the most active (and this was the reason we used **13** in the studies described earlier) (Figure 3D). Interestingly, in mimic **13**, the fusions were placed at the V_H domains, and we observed that the other mimics that were tethered through the variable domains generally showed better activity than those connected by way of the constant domains. As Fabs are attached to the IgG structure in their native context via the C_H domain, and the complimentary determining regions (CDRs) are located on the V domains, it is somewhat surprising that fusing the enzyme at these locations would not interfere in binding and yield the most active variants. With **13** this is especially striking as it is known that V_H CDR3 confers a large part of the specificity of trastuzumab for HER2.[18]

To address whether differences in activity between the least and most cytotoxic analogs **8** and **13**, were due to a structural organization of the Fab domains or the avidity of the molecules for the cell surface, we used flow cytometry to measure the binding of the megamolecules to HER2 on BT474 cells. These data are shown in Figure 3E. Surprisingly, we found that the least active mimic **8** showed an approximate 10-fold greater avidity for the cell surface than did mimic **13**, where the former had an avidity similar to trastuzumab (K_D = ~1.5 nM). Hence, even though mimic **13** has lower avidity for the cell surface, it is more effective in the cytotoxicity assay. From these data, we surmise that this analog may be more effective at holding the HER2 receptors apart and preventing signaling, but we have not addressed the role that differences in receptor internalization,[19] proteolytic shedding,[20] or some other process may play in their observed activity. Indeed, it is plausible that some analogs lead to receptor-antibody complexes that are weak in effect or that may even promote activation.

This work illustrates how the megamolecule approach can be adapted to prepare antibody mimics. Moreover, we demonstrate that these simple trastuzumab analogs are effective and well-tolerated in *in vivo* models. The use of reactions between an enzyme and covalent inhibitor provide a means for the modular assembly of a broad range of antibody scaffolds, and offers a number of unique benefits compared to recently developed methods such as those involving click chemistry-mediated crosslinking of antibody fragments incorporating non-canonical amino acids,[21] the SpyCatcher-SpyTag system,[22] split-inteins,[23] and others.[24] These benefits include the large number of available enzyme-inhibitor pairs that can be employed in the preparation of antibodies with expanded valency and specificity, the ability to control geometry using alternate linker designs, the potential of creating well-defined drug / imaging agent conjugates and access to a large number of designs in parallel using combinatorial methods. We believe that this approach offers a new opportunity to develop antibody mimics for a broad range of therapeutic applications.

ASSOCIATED CONTENT

Supporting Information

Detailed experimental procedures, protein sequences; yield, and molecular weight calculations; and extended figures including raw flow cytometry data can be found in the supporting information.

The Supporting Information is available free of charge on the ACS Publications website.

AUTHOR INFORMATION

Corresponding Author

* milan.mrksich@northwestern.edu

ORCID

Milan Mrksich: 0000-0002-4964-796X
Justin Modica: 0000-0002-3218-7311

Conflicts of Interest

M.M. and J.A.M are co-inventors on US patent application #2019016155 that incorporates methods outlined in this manuscript and have equity in ModuMab Therapeutics, Inc.

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REFERENCES

1. Strohl, W. R., Current progress in innovative engineered antibodies. *Protein Cell* **2018,** *9* (1), 86-120.
2. Richards, D. A., Exploring alternative antibody scaffolds: Antibody fragments and antibody mimics for targeted drug delivery. *Drug Discov Today Technol* **2018,** *30*, 35-46.
3. Sifniotis, V.; Cruz, E.; Eroglu, B.; Kayser, V., Current Advancements in Addressing Key Challenges of Therapeutic Antibody Design, Manufacture, and Formulation. *Antibodies (Basel)* **2019,** *8* (2).
4. Modica, J. A.; Skarpathiotis, S.; Mrksich, M., Modular Assembly of Protein Building Blocks To Create Precisely Defined Megamolecules. *Chembiochem* **2012,** *13* (16), 2331-2334.
5. Modica, J. A.; Lin, Y.; Mrksich, M., Synthesis of Cyclic Megamolecules. *Journal of the American Chemical Society* **2018,** *140* (20), 6391-6399.
6. Taylor, E. L.; Metcalf, K. J.; Carlotti, B.; Lai, C. T.; Modica, J. A.; Schatz, G. C.; Mrksich, M.; Goodson, T., 3rd, Long-Range Energy Transfer in Protein Megamolecules. *J Am Chem Soc* **2018,** *140* (46), 15731-15743.
7. Kimmel, B. R.; Modica, J. A.; Parker, K.; Dravid, V.; Mrksich, M., Solid-Phase Synthesis of Megamolecules. *J Am Chem Soc* **2020,** *142* (10), 4534-4538.
8. Hodneland, C. D.; Lee, Y. S.; Min, D. H.; Mrksich, M., Selective immobilization of proteins to self-assembled monolayers presenting active site-directed capture ligands. *Proc Natl Acad Sci U S A* **2002,** *99* (8), 5048-52.
9. Los, G. V.; Encell, L. P.; McDougall, M. G.; Hartzell, D. D.; Karassina, N.; Zimprich, C.; Wood, M. G.; Learish, R.; Ohana, R. F.; Urh, M.; Simpson, D.; Mendez, J.; Zimmerman, K.; Otto, P.; Vidugiris, G.; Zhu, J.; Darzins, A.; Klaubert, D. H.; Bulleit, R. F.; Wood, K. V., HaloTag: a novel protein labeling technology for cell imaging and protein analysis. *Acs Chem Biol* **2008,** *3* (6), 373-82.
10. Keppler, A.; Kindermann, M.; Gendreizig, S.; Pick, H.; Vogel, H.; Johnsson, K., Labeling of fusion proteins of O6-alkylguanine-DNA alkyltransferase with small molecules in vivo and in vitro. *Methods* **2004,** *32* (4), 437-44.
11. Lobstein, J.; Emrich, C. A.; Jeans, C.; Faulkner, M.; Riggs, P.; Berkmen, M., SHuffle, a novel Escherichia coli protein expression strain capable of correctly folding disulfide bonded proteins in its cytoplasm. *Microb Cell Fact* **2012,** *11*, 56.
12. Hudis, C. A., Drug therapy: Trastuzumab - Mechanism of action and use in clinical practice. *New Engl J Med* **2007,** *357* (1), 39-51.
13. Cho, H. S.; Mason, K.; Ramyar, K. X.; Stanley, A. M.; Gabelli, S. B.; Denney, D. W.; Leahy, D. J., Structure of the extracellular region of HER2 alone and in complex with the Herceptin Fab. *Nature* **2003,** *421* (6924), 756-760.
14. Kuo, T. T.; Aveson, V. G., Neonatal Fc receptor and IgG-based therapeutics. *Mabs-Austin* **2011,** *3* (5), 422-430.
15. Unverdorben, F.; Richter, F.; Hutt, M.; Seifert, O.; Malinge, P.; Fischer, N.; Kontermann, R. E., Pharmacokinetic properties of IgG and various Fc fusion proteins in mice. *Mabs-Austin* **2016,** *8* (1), 120-128.
16. LundgrenEriksson, L.; Carlsson, A.; Eksborg, S.; Ryd, W.; Vesanen, R.; Hultborn, R., Pharmacokinetics of doxorubicin and epirubicin in mice during chlorpromazine-induced hypothermia. *Cancer Chemoth Pharm* **1997,** *40* (5), 419-424.
17. Tacar, O.; Sriamornsak, P.; Dass, C. R., Doxorubicin: an update on anticancer molecular action, toxicity and novel drug delivery systems. *J Pharm Pharmacol* **2013,** *65* (2), 157-170.
18. Gerstner, R. B.; Carter, P.; Lowman, H. B., Sequence plasticity in the antigen-binding site of a therapeutic anti-HER2 antibody. *J Mol Biol* **2002,** *321* (5), 851-862.
19. Fehling-Kaschek, M.; Peckys, D. B.; Kaschek, D.; Timmer, J.; de Jonge, N., Mathematical modeling of drug-induced receptor internalization in the HER2-positive SKBR3 breast cancer cell-line. *Sci Rep* **2019,** *9*, 12709-12724.
20. Molina, M. A.; Codony-Servat, J.; Albanell, J.; Rojo, F.; Arribas, J.; Baselga, J., Trastuzumab (Herceptin), a humanized anti-HER2 receptor monoclonal antibody, inhibits basal and activated HER2 ectodomain cleavage in breast cancer cells. *Cancer Res* **2001,** *61* (12), 4744-4749.
21. Kim, C. H.; Axup, J. Y.; Dubrovska, A.; Kazane, S. A.; Hutchins, B. A.; Wold, E. D.; Smider, V. V.; Schultz, P. G., Synthesis of Bispecific Antibodies using Genetically Encoded Unnatural Amino Acids. *Journal of the American Chemical Society* **2012,** *134* (24), 9918-9921.
22. Yumura, K.; Akiba, H.; Nagatoishi, S.; Kusano-Arai, O.; Iwanari, H.; Hamakubo, T.; Tsumoto, K., Use of SpyTag/SpyCatcher to construct bispecific antibodies that target two epitopes of a single antigen. *J Biochem* **2017,** *162* (3), 203-210.
23. Han, L.; Chen, J. S.; Ding, K.; Zong, H. F.; Xie, Y. Q.; Jiang, H.; Zhang, B. H.; Lu, H. L.; Yin, W. H.; Gilly, J.; Zhu, J. W., Efficient generation of bispecific IgG antibodies by split intein mediated protein trans-splicing system. *Sci Rep* **2017,** *7,* 8360-8370.
24. Witte, M. D.; Theile, C. S.; Wu, T. F.; Guimaraes, C. P.; Blom, A. E. M.; Ploegh, H. L., Production of unnaturally linked chimeric proteins using a combination of sortase-catalyzed transpeptidation and click chemistry. *Nat Protoc* **2013,** *8* (9), 1808-1819.

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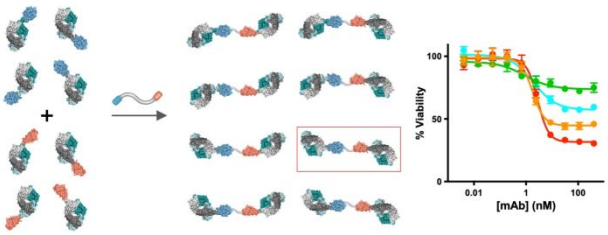
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