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Communication

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LASIC: Light Activated Site-Specific Conjugation of Native IgGs

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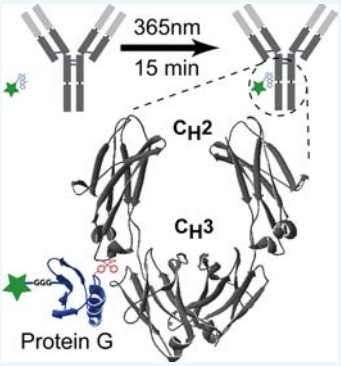
Supporting Information

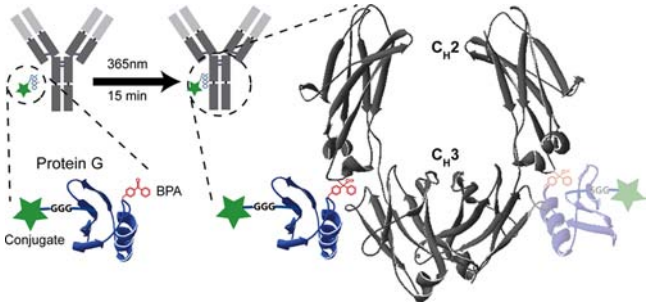
ABSTRACT: Numerous biological applications, from diagnostic assays to immunotherapies, rely on the use of antibody-conjugates. The efficacy of these conjugates can be significantly influenced by the site at which Immunoglobulin G (IgG) is modified. Current methods that provide control over the conjugation site, however, suffer from a number of shortfalls and often require large investments of time and cost. We have developed a novel adapter protein that, when activated by long wavelength UV light, can covalently and site-specifically label the Fc region of nearly any native, full-length IgG, including all human IgG subclasses. Labeling occurs with unprecedented efficiency and speed (>90% after 30 min), with no effect on IgG affinity. The adapter domain can be bacterially expressed and customized to contain a variety of moieties (e.g., biotin, azide, fluorophores), making reliable and efficient conjugation of antibodies widely accessible to researchers at large.



Monoclonal antibodies, because of their broad repertoire of targets and exquisite selectivity, have become an essential component for a wide range of biological applications, from diagnostic assays to immunotherapies. Many of these applications require Immunoglobulin G (IgG) to be modified with a chemical (e.g., biotin, contrast agent, drug, nanoparticle) or biological agent (e.g., enzyme, second antibody).

While these diverse antibody formats are commonplace, their complex structures still pose various developmental and production challenges. A salient hurdle involves how to best attach the functional moiety at specific locations away from the antigen binding Fab domain, so as to preserve binding affinity and obtain homogeneous products. Site-specific modifications have been widely shown to improve the performance and efficacy of antibody-conjugates in almost every known application.[1,2]

Scheme 1. Illustration of IgG Being Photo-Cross-Linked with a Protein G-Based Adapter Protein[a]



[a]The Protein G adapter (blue) contains a customizable conjugate at its C-terminus and the unnatural amino acid benzoylphenylalanine (BPA), whose UV-active benzophenone side chain is shown in red, in the Fc binding domain. When bound to the Fc region of IgG and activated by long wavelength UV light (365 nm), a covalent bond is formed between Protein G and IgG. Either one or two Protein Gs can be conjugated onto each Fc (second one is shown faded).

Several enzymatic and recombinant based approaches have been utilized to enable the site-specific modification of IgG; however, these methods are lengthy and expensive, and often require cloning and cell line development for each construct.[3−6] Despite the exploding interest in site-specifically modified antibody conjugates, these barriers limit their production to specially equipped laboratories and severely constrain the number and types of conjugates that can be made. This not only prevents the use of optimal antibody constructs for common laboratory assays, but also stunts the discovery and exploration of new antibody-based therapeutics, and hampers our understanding into the mechanisms of actions of these new formats.

An ideal approach for developing antibody conjugates would take advantage of the large library of existing antibodies. A means to conjugate existing native antibodies site-specially, rapidly, and inexpensively can become an enabling technology to further antibody conjugate discovery and design. We have developed such as a platform, termed LASIC (Light Activated SIte-specific Conjugation), that enables highly efficient and versatile conjugation of nearly all IgGs, including all human subclasses.

LASIC relies on a small adapter protein that is engineered to contain the photoreactive non-natural amino acid benzoylphenylalanine (BPA) in its IgG binding domain, as well as a customizable reactive moiety at its C-terminus (Scheme 1).

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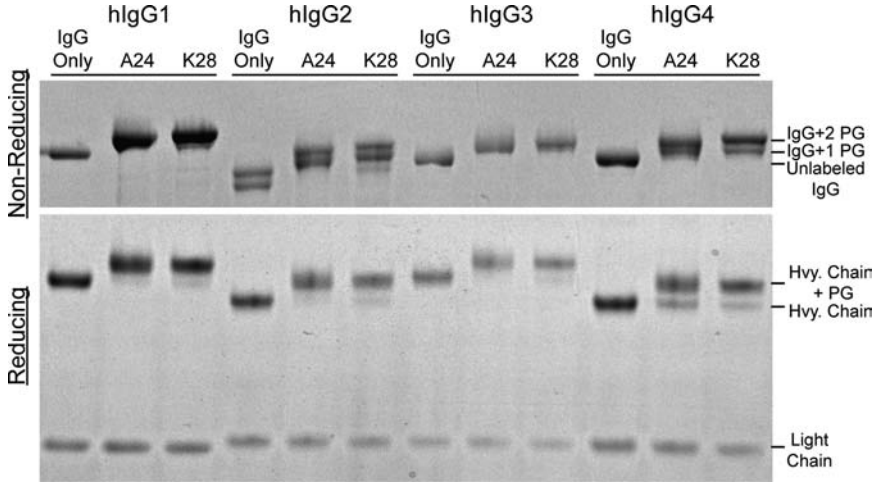

Figure 1. Nonreducing and reducing SDS-PAGE gels of various human IgG subclasses alone or after photo-cross-linking with Protein G-based adapter proteins. The adapter proteins possessed either a A24BPA or K28BPA substitution. Conjugation was done for 1 h and 30 min using 4 equiv of Protein G. Note hIgG2 isotype used here naturally presents as two bands on nonreducing gel.

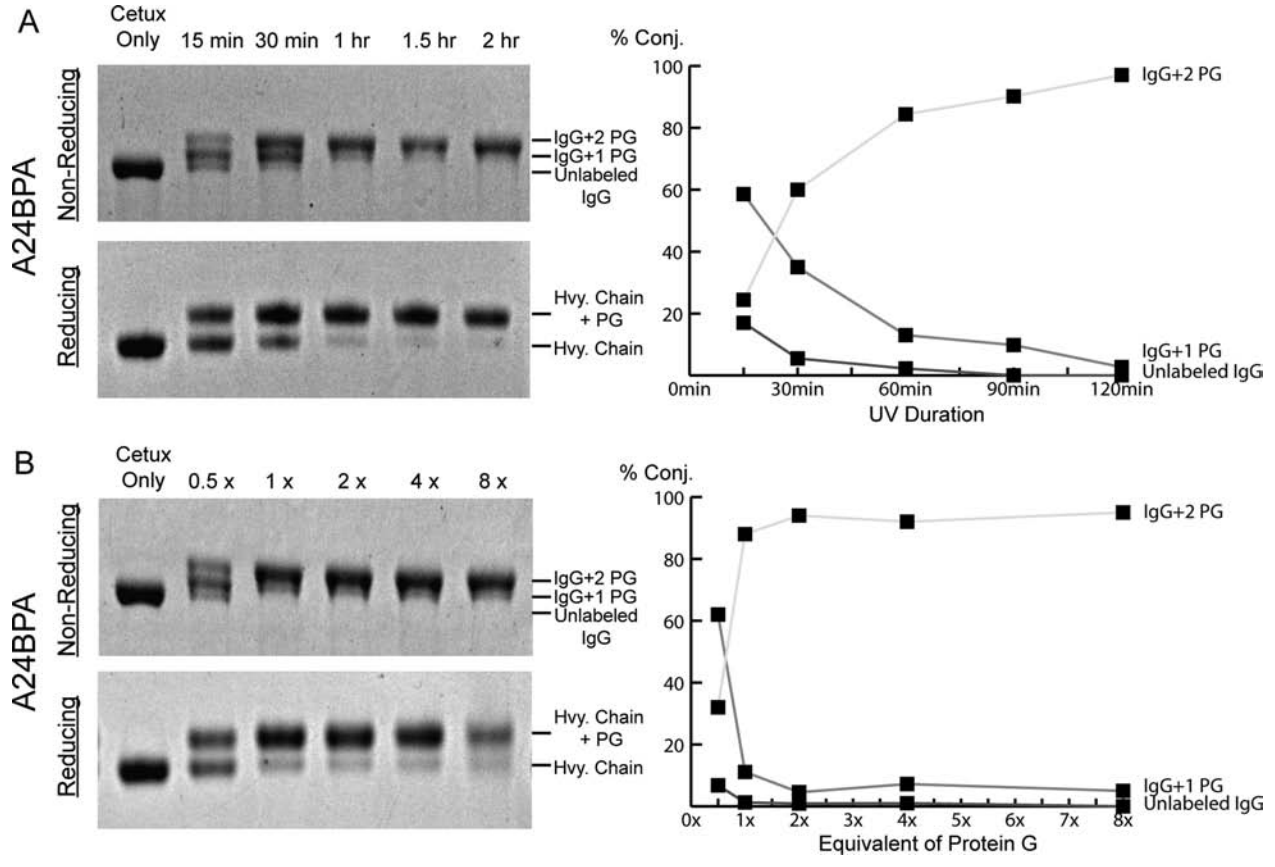

Figure 2. Kinetics and efficiency of IgG-adapter protein cross-linking. (A) Nonreducing and reducing SDS-PAGE of cetuximab (Cetux, human IgG1) alone or after photo-cross-linking with Protein G (PG)-based adapter possessing a A24BPA substitution. UV cross-linking was performed for varying periods of time using 4 equiv of the adapter proteins. Image analysis of nonreducing gels are shown on the right. (B). Nonreducing and reducing SDS-PAGE gels of cetuximab alone or after photo-cross-linking with A24BPA adapter proteins. UV cross-linking was performed for 1 h and 30 min with various molar ratios of adapter protein to IgG heavy chain. Image analysis of nonreducing gels are shown on the right.

While we previously developed an adapter protein based on Protein A,[7,8] it showed moderate to no conjugation toward human IgG subclasses. We therefore reasoned that the more broadly binding Protein G might serve as a better platform for LASIC. Protein G is derived from Streptococcal bacteria and can naturally bind to a broad range of IgGs at the C_H2-C_H3 junction.[9] The noncovalent binding of IgG by both Protein A and Protein G has been used to construct antibody conjugates for a wide variety of applications.[10−12] However, noncovalently attached IgG carry the risk of detaching during long-term storage or in serum, where endogenous IgG may be present in vast excess. To overcome these limitations, Protein G has been covalently linked to IgG using both chemical and photo-activated means, but these methods have been plagued either

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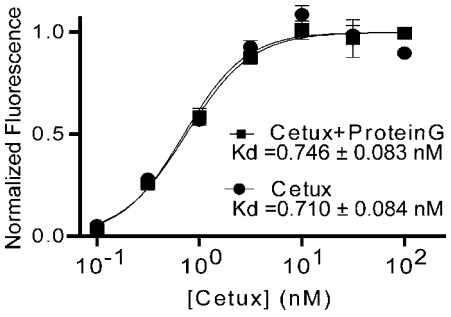

Figure 3. Antibody binding affinity with and without LASIC. Unmodified cetuximab (Human IgG1 anti-human EGFR antibodies) or cetuximab that was subject to LASIC using the A24BPA Protein G adapter protein were applied to EGFR positive KB cells. The extent of cell labeling was quantified by a plate-reader using a fluorescent anti-human secondary antibody.

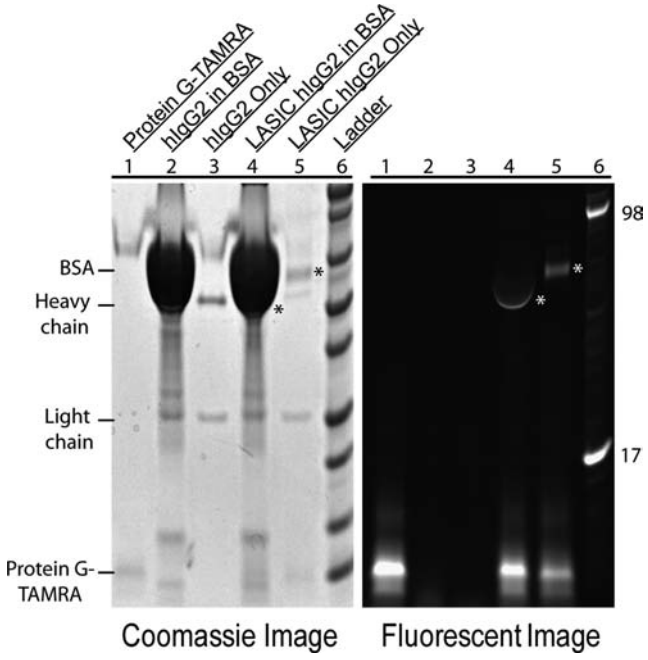

Figure 4. Effect of impure IgG samples on LASIC. 0.25 μg of Human IgG2, either alone (lanes 3, 5) or with 25 μg BSA (lanes 2,4), was conjugated with a TAMRA labeled Protein G adapter protein (lane 1). Samples were run on an SDS-PAGE reducing gel and white light and fluorescence images of the gel were acquired. * indicates LASIC conjugated heavy chain.

by decreased IgG affinity or by complex production and poor efficiency.[13,14]

LASIC adapters, which possess a BPA cross-linker only in the Fc-binding domain, give homogeneous products by forming only one covalent bond with each IgG (Scheme 1), rather than randomly labeling lysines as is the case with chemical cross-linking.[15] In addition, by recombinantly producing LASIC adapters using a well established *E. coli* expression system that can incorporate BPA into proteins via an amber-tRNA suppressor aminoacyl-synthase pair, adapters with BPA in different locations can be efficiently produced and tested for their ability to label many different antibody subclasses.[16]

To minimize the "footprint" of the LASIC adapter and to ensure Fc-specific conjugation, we chose to use a small (6.5 kDa), thermally stable domain of Protein G (HTB1), with a mutation to disable Fab-binding, as the parental molecule.[14,17] We successfully designed and expressed nine Protein G variants, each having an Fc-facing amino acid substituted by BPA: V21, A24, K28, I29, K31, Q32, D40, E42, W42 (SI Figure S1). The yields of expression for all variants were high at around 5 mg/L, consistent with previous reports of BPA incorporation into proteins.[8,18] Next, we screened these variants for their ability to covalently label a range of IgG subclasses from various hosts upon exposure to long wavelength UV light (SI Figure S2A–C). Since each IgG is composed of two identical heavy chains, it can be labeled with up to two Protein G-based adapters, which can be deciphered using nonreducing SDS-PAGE. We found two variants, A24BPA and K28BPA, that allowed nearly 100% of all human IgG subclasses to be labeled with at least one adapter protein on their heavy chains (Figure 1). More than 90% of all human IgG subclasses were labeled with two adaptor proteins (i.e., one adapter protein per heavy chain). In addition, A24BPA is also capable of conjugating most mice (mIgG 2a,2b,2c,3) as well as some rat and rabbit subclasses (rat 2c, rabbit polyclonal) with similar efficiencies (SI Figure S2B,C). The photochemistry of benzophenone has been well described, and in particular, it has been found that BPA preferentially cross-links methionine residues.[19] Indeed, a three-dimensional model of the IgG-Protein G complex shows that A24 and K28 come in very close proximity to Met252 and Met482 on IgG, respectively (SI Figure S3A). In fact, Met252 is found on all IgG that are efficiently labeled with A24BPA, while the same applies for Met428 and K28BPA (SI Figure S3B). These results suggest that Met 252 and Met 482 are the likely site of conjugation by A24BPA and K28BPA adapters, respectively.

LASIC using A24BPA and K28BPA demonstrated unprecedented fast kinetics. After only 15 min of light exposure, more than 80% of IgG were conjugated by one or two A24BPA adapters, while the level reached 95% by 30 min (Figure 2 A). The reaction was nearly stoichiometric with complete conjugation of IgG using only 1 equiv of A24BPA (Figure 2 B). K28BPA reacted quickly as well, reaching 75% and 90% conjugation after 30 min and 1 h, respectively (SI Figure S4). Similar conjugation efficiencies are reproducible for different IgGs of the same subclass. Similar results are also achievable using other readily available UV light sources and in a variety of common buffers (data not shown).

The structural stability of the Protein G HTB1 domain gives LASIC adapters a long shelf life even at room temperature, with no detectable loss of activity even after weeks of storage (SI Figure S5). The use of BPA, which is only activated by nonharmful long wavelength UV light (365 nm) and is only quenched if in close proximity to a target (10 Å) with which it can form a covalent bond, makes the LASIC adapter safe to use, stable under ambient light, and nonreactive toward other proteins that it cannot bind (SI Figure S5).[20] To demonstrate the preservation of antigen binding after LASIC, we first conjugated the human IgG1 anti-human EGFR antibody (cetuximab) with the A24BPA adapter. Next, we applied either unmodified cetuximab or LASIC treated cetuximab to EGFR-positive KB cells followed by detection using a fluorescent anti-human secondary antibody. Analysis of the fluorescent signals by a plate-reader indicated that both the unmodified and LASIC treated cetuximab showed similar binding affinities to the target cell line, demonstrating the gentle nature of photoconjugation (Figure 3). LASIC's exquisite specificity toward IgG allows conjugation to be done even in the presence of other proteins. This was shown by labeling hIgG2, either by

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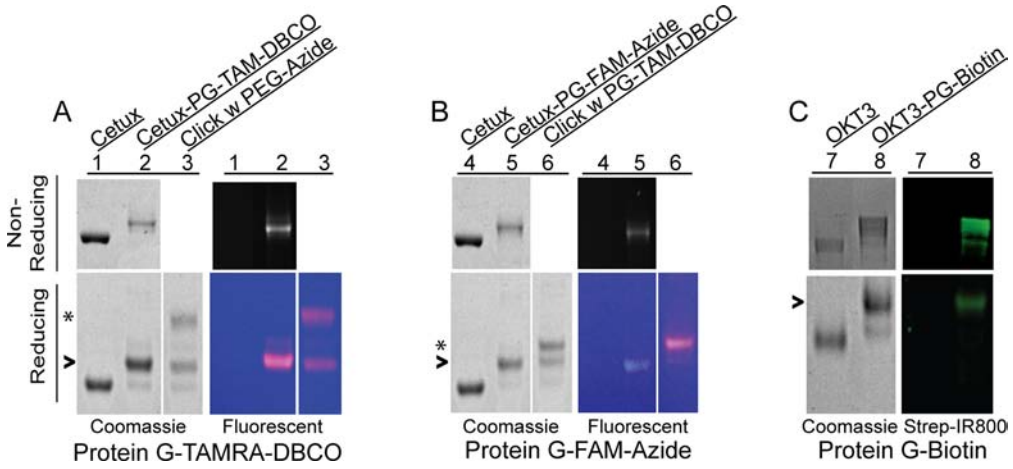

Figure 5. Modification of IgG with various functional moieties using Protein B adapters. Protein G adapters made with peptides containing either (A) TAMRA-DBCO (MW: 1680 Da), (B) FAM-Azide (MW: 1032 Da), or (C) Biotin (MW: 458 Da) were LASIC conjugated onto human IgG1 (Lanes 1 and 2; 4 and 5) or mouse IgG2a (Lanes 7 and 8). Conjugates remained active as demonstrated by click reactions (Lane 3: Click with PEG-Azide; Lane 6: Click with PG-TAMRA-DBCO) or by Western blot with Streptavind-IRdye800. The arrow (>) indicates Protein G-labeled heavy chains; The asterisk (*) indicates click product. Full image available as SI Figure S5.

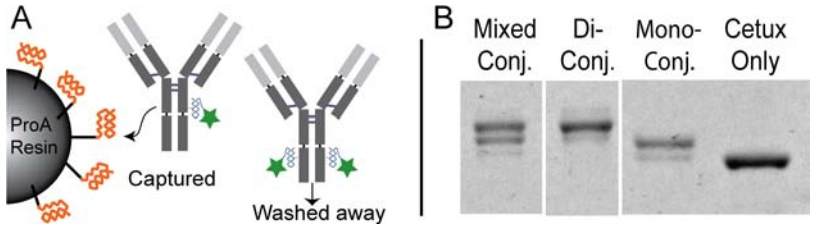

Figure 6. Controlled labeling of IgG with one or two Protein G adapters. (A) Schematic describing the purification of IgG labeled with a single Protein G adapter (monoconjugated product) by capturing it from a mixture of mono- and diconjugated products. Diconjugated IgGs cannot bind to Protein A or G resin. (B) Nonreducing SDS-PAGE gel showing monoconjugated cetuximab having being purified from mixed product using the method described in (A).

itself or in 1% BSA solution, with a TAMRA (5-Carboxytetramethylrhodamine) dye-tagged LASIC adapter, followed by analysis using reducing SDS-PAGE gel (Figure 4). While similarly high levels of IgG2 heavy chains were labeled by the Protein G with or without the background BSA, as determined from the fluorescent image, no apparent labeling of BSA was seen in the gel despite it being present at more than 200 times molar excess.

In order to produce LASIC adapters with a variety of C-terminal modifications we used the sortase expressed protein ligation (STEPL) technology, developed in our lab, to incorporate various moieties during the recombinant protein purification process.[21] To demonstrate the versatility of this approach, we introduced three different Gly-Gly-Gly N-terminated peptides containing either a biotin, a 5-TAMRA dye along with a dibenzocyclooctyl (DBCO), or a 5-FAM (5-Carboxyfluorescein) dye along with an azide. The resulting adapters were then photoconjugated to IgG (Figure 5 and SI Figure S6). As assayed by SDS-PAGE, nearly all of the heavy chains of IgGs were conferred with the functionalities carried by their respective Protein G adapters. There was no decrease in the conjugation efficiency as the moieties are on the C-terminus of the LASIC adapter and hence do not interfere with IgG binding. Since N-terminal triglycine peptides can be quickly and inexpensively synthesized, other reactive groups can be efficiently conjugated onto IgGs just as easily using LASIC.

One feature of using a Protein G-based adapter is that both IgG heavy chains can be modified. While this may be preferred when maximum conjugation is important, in some instances it may be desirable to introduce only a single modification onto IgG. With LASIC it is possible to obtain monoconjugated IgGs by slightly altering the purification protocol (Figure 6). Since the Fc site bound by LASIC adapters overlaps with the natural binding sites of wild-type Protein G and Protein A, monoconjugated product, but not diconjugated products, can be captured from a mixture of the two using commercial Protein A or Protein G resins. The ability to regulate the number of conjugates on the IgG affords an additional level of control, by allowing, for example, one to tailor the drug to antibody ratio when making antibody−drug conjugates (ADCs).[22] Additionally, the ability of monoconjugated product to bind to Protein A and G columns also greatly eases the purification of these conjugates. In addition, monoconjugated IgG leaves one Fc-receptor binding site available for natural effector functions, including antibody dependent cell-mediated cytotoxicity (ADCC) and FcRn-mediated IgG recycling.[23,24]

In summary, we have demonstrated that by using a recombinant Protein G-based adapter, one can efficiently photoconjugate IgGs with a variety of moieties using the LASIC technique. It is worth noting that LASIC may not be universally suitable for all applications. For example, the size and potential immunogenicity of the Protein G domain may render antibody−drug conjugates (ADCs) less effective by interfering with pharmacokinetics, cell internalization, or drug

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release. Additionally, although it is possible to preserve one FcR binding site by monoconjugating the LASIC adapter, it is not known whether this will ensure sufficient FcRn-mediated IgG salvaging or other Fc-dependent activities for in vivo applications. To better delineate these potential concerns, we are working to radiolabel LASIC adapters and plan to study the biodistribution of the resulting conjugate in vivo. Despite these limitations, LASIC is nonetheless promising due to its simplicity and versatility. Given the tremendous potential of site-specific antibody conjugates, there is a need to generate them more efficiently, ideally from full length IgGs so as to take advantage of their existing vast library, validated binding properties, and ready accessibility. Thus, the ability to site-specially conjugate nearly any off-the-shelf IgG is an enabling technology that opens up a variety of applications and may allow the development of antibody conjugates to be "crowd-sourced" by researchers at large.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental methods and supplemental figures. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.5b00275.

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Author Contributions

‡The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. James Z. Hui and Shereen Tamsen contributed equally.

Notes

The authors declare no competing financial interest.

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