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Site-Specific Bioconjugation and Multi-Bioorthogonal Labeling via Rapid Formation of Boron-Nitrogen Heterocycle

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Abstract

Precise control of covalent bond formation in the presence of multiple functional groups is pertinent in the development of many next-generation bioconjugates and materials. Strategies derived from bioorthogonal chemistries are contributing greatly in that regard; however, the gain of chemoselectivity is often compromised by the slow rates of many of these existing chemistries. Recent work on a variation of the classical aldehyde/ketone condensation based on *ortho*-carbonylphenylboronic acids has uncovered markedly accelerated rates compared to the simple carbonyl counterparts. The products of these reactions are distinct, often in the form of boron-nitrogen heterocycles. In particular, we have shown that 2-formylphenylboronic acid (2fPBA), when coupled with an α -amino-hydrazide, produces a unique zwitterionic and stable 2,3,1-benzodiazaborine derivative. In this work, we apply this chemistry to generate chemically defined and functional bioconjugates, herein illustrated with immunoconjugates. We show that an antibody and a fluorophore (as payload) equipped with the relevant reactive handles undergo rapid conjugation at near-stoichiometric ratios, displaying a reaction half-life of only ~5 min with 2 equivalents of the linker-payload. Importantly, the reaction can be extended to multi-component labeling by partnering with the popular strain-promoted azide-alkyne cycloaddition and tetrazine-*trans*-cyclooctene (Tz-TCO) ligation. The mutual orthogonality to both of these chemistries allows simultaneous triple bioorthogonal conjugations, a rare feat thus far that will widen the scope of various multi-labeling applications. Further collaboration with the Tz-TCO reaction enables rapid one-pot synthesis of a site-specific dual-payload antibody conjugate. Altogether, we envision that the 2fPBA- α -amino-hydrazide ligation will facilitate efficient assembly of diverse bioconjugates and materials, enabling access to more complex modalities via partnership with other orthogonal chemistries.

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List of abbreviations; supplementary methods, including synthesis; supplementary figures; and NMR and mass spectra.

SLB and KM are inventors on a patent pertaining to this chemistry. The other authors declare no competing financial interest.

Graphical Abstract

INTRODUCTION

A number of bioorthogonal chemistries have been developed over the past two decades, providing tools to probe biological processes in living systems. These reactions share the defining feature of orthogonal reactivity to native biological functional groups, enabling their use in a biological setting.¹ In addition to labeling biomolecules in the context of cells and organisms, bioorthogonal chemistries have played an important role in the construction of diverse biomolecule conjugates (e.g. with nanoparticles, therapeutic agents, polymers) and biomaterials.^{2, 3} Amidst the growing repertoire of bioorthogonal chemistries, the quest for new reactions continues, with focus on those that possess advanced capabilities, such as improved speed, unique physical properties, and mutual orthogonality to existing bioorthogonal chemistries.⁴ In this work, we present one bioorthogonal reaction that fulfills some of these demands and we exploit these notable attributes in bioconjugate synthesis, with a narrative catering to emerging interests in the field of antibody-drug conjugates (ADCs) as one potential application.

With four ADCs currently approved by the U.S. Food and Drug Administration and many more under evaluation in the clinical trials, these target-specific biotherapeutics will likely become more prevalent in medical treatment options. Conjugation strategies that enable facile and economical production of homogeneous and stable ADCs are thus highly desired. A variety of bioorthogonal chemistries are being explored to address issues of heterogeneity and premature deconjugation of ADCs that are made using conventional chemistries that target native lysines and cysteines.² One demonstrated class is the aldehyde/ketone condensation. The carbonyl functionality can be introduced to antibodies at specific sites by various approaches.⁵ ADCs with a defined drug-antibody ratio (DAR) of 2 or 4 have been successfully generated via oxime,⁶ hydrazinoiso-Pictet-Spengler,⁷ and Knoevenagel ligations.^{8, 9} None-theless, the advantage of conjugate homogeneity using these chemistries is compromised by less-than-ideal conjugation conditions, namely acidic pH for oxime ligation, slow conjugation kinetics, and the necessity of a large excess of the reactive payload.

Newer bioorthogonal reactions are under investigation for efficient assembly of site-specific ADCs.^{10, 11} While reaction speed and efficiency are often not heavily weighted considerations for the production of bioconjugates, they are desirable especially for ADCs

given the relatively high costs of antibodies and drug-linkers.¹² Furthermore, the limited solubility of frequently hydrophobic drugs can render their use at large excess unfeasible, while increasing the composition of organic solvents to permit drug solubility can result in antibody aggregation.¹² Therefore, the search for new strategies continues that will enable the generation of site-specific ADCs without compromising the biocompatibility, kinetics, and efficiency of the reaction.

We and others have demonstrated that boronic acid-based chemistry can be applied toward the preparation of bioconjugates (for a recent review, see ref. 13).¹³ In one subset, aryl carbonyls containing a boronic acid at the *ortho* position exhibit accelerated condensation kinetics relative to simple aldehydes/ketones.¹⁴ We have previously shown that 2-formylphenylboronic acid (2fPBA, **1**) undergoes a rapid reaction with a phenylhydrazine ($k = \sim 10^3 \text{ M}^{-1}\text{s}^{-1}$) at low stoichiometric concentration in aqueous solution at neutral pH.¹⁵ The initial short-lived hydrazone product undergoes a unimolecular dehydration reaction that produces the final product, a boron-nitrogen heterocycle that is a 2,3,1-benzodiazaborine (DAB) derivative. We subsequently examined the use of hydrazides as nucleophiles, as they are stable, easy to prepare and have previously been introduced to a number of biomolecules.^{16–18} In particular, we have found that inclusion of an amine on the α -carbon of the hydrazide (an α -amino-hydrazide, **2**) yields a tricyclic structure in which the nitrogen of the α -amino group coordinates with boron to secure a tetracoordinate boron arrangement (**3b**; Figure 1a).¹⁹ The extra B-N interaction imparts added stability to the DAB, suggesting its suitability for generating stable bioconjugates. Other examples of introducing such intramolecular interaction to strengthen the stability of boronic acid-containing conjugates have also previously been demonstrated.^{20, 21}

An additional feature to note about the DAB product of 2fPBA and α -amino-hydrazide is its zwitterionic character, which may limit its contribution to the overall hydrophobicity of the construct in which it participates. This is relevant in ADC linker design, as hydrophobicity is associated with increased aggregation and accelerated plasma clearance of the ADCs, resulting in less favorable pharmacokinetics and therapeutic efficacy.²² While most studies have examined the effects of hydrophobicity due to the payload or the spacer of the drug linker,^{22–25} it has also been observed that the hydrophobicity of the conjugating moiety, unique to the coupling chemistry, can affect the aggregation propensity and the hydrophobicity of the ADC.²⁶ Therefore, the polar, zwitterionic nature of the DAB scaffold provides a unique opportunity to minimize the hydrophobicity of the bioconjugate without sacrificing speed or efficiency of conjugation.

As the field of ADCs continues to evolve, more complex formats of ADCs are under development. For example, high loaded ADCs generated using branched linkers to couple multiple drug molecules to a specific site on the antibody have demonstrated enhanced potency.^{27, 28} Other recent work has shown that the dual delivery of two different cytotoxic payload classes may offer opportunities for improved efficacy.^{29, 30} Likewise, bispecific ADCs are gaining interests, as they have shown improved internalization and lysosomal trafficking as compared to mono-targeted ADCs.³¹ ADCs equipped with new functions are also being built by combined attachment of a drug with another type of payload, such as an imaging probe for theranostic utility,³² or a serum-stabilizing moiety for improved lifetime

in vivo.³³ Orthogonal bioconjugation approaches have been used to achieve site-specific assembly of some of these multifunctional ADCs. In particular, aldehyde/ketone condensation is compatible with a number of bioorthogonal reactions³⁴ and it has been used in tandem with strain-promoted azide-alkyne cycloaddition to create bispecific antibodies.³⁵ The mechanistic basis of the 2fPBA- α -amino-hydrazide ligation, which overlaps with the parent alde-hyde condensation reaction, thus hints at its potential to inherit the orthogonality to an array of existing bioorthogonal reactions, opening up the possibilities of multiplex conjugations.

Altogether, given the favorable prospects in speed, stability, hydrophilicity, and orthogonality to other chemistries, we anticipate that the coupling reaction of 2fPBA and α -amino-hydrazide can enable efficient and versatile assembly of assorted bioconjugates. Herein, we assess these potential unique features of the reaction and its applicability toward the construction of site-specific immunoconjugates.

RESULTS AND DISCUSSION

Bioorthogonality of reaction.

We first set out to examine the kinetics and efficiency of the 2fPBA- α -amino-hydrazide reaction. Rate constants were determined according to absorption-based kinetics performed between 2fPBA (**1**) and glycine hydrazide (**2a**) under pseudo-first order conditions in aqueous buffer at pH 7.4. The data were fit to a kinetic model of two sequential first order reactions corresponding to the formation of the hydrazone (**3a**) and the subsequent ring-closing to form the boron-nitrogen heterocycle (**3b**) (Figure 1a). A second order rate constant for hydrazone formation was calculated to be $\sim 700 \text{ M}^{-1}\text{s}^{-1}$ (Figure S1). This represents a significant improvement to the slow condensation kinetics observed for the classical aldehyde-hydrazine/hydrazide reaction, which has rate constants on the order of 10^{-3} – $10^1 \text{ M}^{-1}\text{s}^{-1}$ depending on pH and substitutions on the reactants.^{36, 37} The rate constant of the second ring-closing step that results in the formation of the DAB heterocycle was 0.009 s^{-1} , which is in line with our previous report on coupling 2fPBA with a hydrazine.¹⁵ The reaction was also performed at 1:1 molar ratio of each reactant. Complete conversion to the product was observed within 20 minutes, demonstrating the fast kinetics and the efficiency of this chemistry (Figure 1b).

One drawback of some of the most commonly used bioorthogonal reactions, including strain-promoted azide-alkyne cycloaddition (SPAAC) and tetrazine-*trans*-cyclooctene (Tz-TCO) ligation, is the hydrophobicity of the reagents and the resultant conjugates.³⁸ In contrast, the reactive functional groups and the product of the 2fPBA- α -amino-hydrazide reaction are highly water soluble. For example, in experiments involving the model compounds, we routinely prepare concentrated stocks of the reactants (at least 100 mM for reactants **1** and **2a**) and the product **3b** (at least 5 mM) in completely aqueous solution. The calculated cLogP of **3b** (~ 0.2) is also much smaller than those of the common triazole and dihydropyrazine moieties (~ 3 – 5) that constitute the core of the other ligation reactions (Figure S2). The hydrophilicity and water solubility of the reactants and of the resulting core strongly suggest that this chemistry will be useful for preparing bioconjugates in which hydrophobicity is considered a detriment.

Selective bioconjugation by introducing 2fPBA and α -amino-hydrazide as reactive handles was previously demonstrated in a simple model protein labeling reaction.¹⁹ Here, the bioorthogonality of the reaction was challenged with a more complex environment in the form of cell lysate. A model protein, tubulin, was modified to contain the α -amino-hydrazide handle. Lysate prepared from A549 cells was spiked with α -amino-hydrazide-functionalized tubulin or unmodified tubulin and subsequently treated with a 2fPBA-functionalized BODIPY-FL fluorophore. As demonstrated by SDS-PAGE (Figure 1c), labeling was observed only in the cell lysate sample spiked with α -amino-hydrazide-functionalized tubulin, with a single fluorescent band positioned at the molecular weight of tubulin (~55 kDa). Meanwhile, no labeling was observed for the non-spiked lysate and the lysate spiked with unmodified tubulin. Overall, the kinetics, relative hydrophilicity, and the chemoselectivity of bioconjugation support the biocompatibility and the bioorthogonality of this chemistry.

Site-specific antibody conjugation.

Given the bioorthogonal attributes of the 2fPBA- α -amino-hydrazide reaction and the demand for novel conjugation strategies for ADCs, we further explored the application of this reaction for the generation of site-specific antibody bioconjugates using a clinically relevant antibody, trastuzumab, as our model antibody. Trastuzumab targets human epidermal growth factor receptor 2 (HER2), which is commonly overexpressed in breast cancer. Two engineered trastuzumab antibodies were used to append a 2fPBA functional group at a single site on the light chain or the heavy chain (Scheme 1). Trastuzumab engineered with a C-terminal glutamine tag (LLQG) on the light chain (Tras LC) was functionalized with 2fPBA via a transglutaminase (TG)-mediated ligation. This method, which has previously been reported for the preparation of ADCs, makes use of the enzyme's ability to accept various alkyl amine-functionalized substrates, forming an isopeptide bond with the side chain of glutamine.^{39, 40} Tras LC was thus incubated with an amine-functionalized 2fPBA crosslinker (synthesized according to Scheme S2) in the presence of TG. Successful incorporation of the 2fPBA functionality on the light chain (Tras LC-2fPBA) was confirmed by ESI-MS (Figure S3).

To functionalize the heavy chain with 2fPBA, trastuzumab carrying a K334C mutation on the heavy chain (Tras HC) was used. With the endogenous cysteines all participating in disulfide bonds, the engineered 334C site is available for site-specific modification with a thiol-reactive crosslinker. A maleimide-2fPBA bifunctional crosslinker was thus synthesized (Scheme S1). Tras HC was first deglycosylated with peptide-N-glycosidase F (PNGaseF) in order to simplify subsequent MS analysis, which can be complicated by the presence of different glycoforms of the heavy chain. Overnight incubation of the deglycosylated antibody with the maleimide crosslinker led to successful incorporation of 2fPBA on the heavy chain (Tras HC-2fPBA) as confirmed by ESI-MS (Figure S3).

With the antibodies now equipped with the 2fPBA handle, an α -amino-hydrazide-functionalized fluorophore, Texas Red- α -amino-hydrazide (Tx Red-am-zide), was synthesized to serve as the payload (Scheme S3). Next, Tras LC-2fPBA, Tras HC-2fPBA, or each of its unmodified counterpart was allowed to react with Tx Red-am-zide and then

subjected to SDS-PAGE under reducing condition (Figure 2). Only the 2fPBA-functionalized antibodies, specific to the subunits carrying the 2fPBA functional group, were fluorescently labeled. This result was corroborated by ESI-MS analysis, which showed that the reduced conjugates exhibited proper mass shift on the 2fPBA-modified chain but no mass shift on the unmodified chain, demonstrating the site-specificity of the conjugation (Figure S3).

Kinetics of antibody-fluorophore conjugation.

Kinetics of small molecule reactions are not necessarily recapitulated in reactions with proteins. Therefore, it was important to assess the kinetics with the relevant reactants. The kinetics of conjugation of Tras LC-2fPBA and Tras HC-2fPBA with Tx Red-am-zide was thus examined. At different time points, an aliquot of the reaction mixture was removed, reduced by TCEP, and subjected to MS analysis. The addition of TCEP also quenches the reaction (Figure S4–6).

For the light chain-modified Tras LC-2fPBA, conjugation in the presence of two equivalents of Tx Red-am-zide per 2fPBA reached completion in ~4 h, with a reaction half-life of only ~5 min (Figure 3a; Figure S7). Conjugation using ten equivalents of the payload rapidly achieved near-complete loading in just 5 min and reached completion within 30 min. Although the antibody coupling reaction is relatively slower than the small molecule model reaction, it is still considered fast compared to many bioorthogonal reactions applied for ADCs (Table 1). It is important to note that the rate and the extent of bioorthogonal reactions is dependent on reaction conditions and the nature of the biomolecule and conjugating ligands.⁴¹ The examples shown in Table 1 are specific to reported conjugations between antibodies and drug-linkers.

For the heavy chain-modified Tras HC-2fPBA, its kinetics of conjugation with Tx Red-am-zide is similar to that of Tras LC-2fPBA in that the reaction rapidly reached 50% completion within 5 min, though the time to complete was slower, taking ~4–6 h for both two- and ten-fold excess of the payload (Figure 3b; Figure S8). It is noted that the total loading on Tras HC-2fPBA was slightly less than the expected two payload molecules per antibody. The reason for the sub-stoichiometric ratio in this product is not known, although we speculate that the location of the reactive cysteines, where the 2fPBA modification was made, may play a role. The two 334C residues of Tras HC are located nearby one another in a cavity between the two CH2 domains of the heavy chains.⁴² It is possible that the attachment of the first large polycyclic Texas Red fluorophore kinetically hinders the addition of the second payload, thus limiting the overall DAR. Differences in conjugation efficiency between sites has been reported previously with other bioconjugation approaches.^{7, 43} Indeed, when the 2fPBA functional group was introduced to another site of the heavy chain, complete loading of Tx Red-am-zide was observed (vide infra).

Hydrophobicity of antibody conjugates.

Minimizing the hydrophobicity of ADCs and related bioconjugates has associated positive outcomes, such as reduced aggregation and improved pharmacokinetics.²² The individual components of a bioconjugate can each contribute to its overall hydrophobicity. We thus

examine the hydrophobicity of antibody conjugates as contributed by the conjugating moiety that is the product of the coupling chemistry. Given the increasingly common usage of SPAAC and Tz-TCO reactions in bioconjugation, comparison was made with these chemistries. Antibody “conjugates” carrying the ligation scaffold, namely the DAB, triazole, and dihydropyrazine moieties, without an attached payload were prepared. Difference observed in the apparent hydrophobicity would therefore stem solely from the ligation scaffold.

Using amine-functionalized heterobifunctional crosslinkers as TG substrates, Tras LC was functionalized with 2fPBA, azide (Az), or TCO (Scheme S5). The subsequent Tras LC-2fPBA, Tras LC-Az, and Tras LC-TCO were subjected to conjugation with a minimalist model compound containing an α -amino-hydrazide, an aza-dibenzocyclooctyne, and a tetrazine, respectively (Scheme S5). The hydrophobicity of each antibody conjugate was then evaluated by hydrophobic interaction chromatography (HIC). The result showed that the conjugates representative of SPAAC and Tz-TCO chemistries were more hydrophobic than the DAB conjugate (Figure S12). This is consistent with the trend predicted by the estimated cLogP of the small molecules (Figure S2). The relative hydrophilicity of the DAB scaffold may be attributed in part by its zwitterionic character. To test this hypothesis, the antibody conjugate of Tras LC-2fPBA and α -amino-hydrazide was further compared with a different DAB conjugate formed between Tras LC-2fPBA and hydrazine. The latter produces a neutral DAB derivative¹⁵ and was determined to be more hydrophobic based on HIC (Figure S13). The result suggests that the zwitterionic nature of the DAB that is unique to the coupling of 2fPBA and α -amino-hydrazide does play a role in minimizing the hydrophobicity of this conjugate.

Serum stability.

As prominently exemplified by ADCs generated using endogenous thiol-maleimide chemistry, stability of ADCs in buffer does not necessarily translate into stability in plasma or serum.^{45–47} The succinimide thioether linkage has the potential to undergo cleavage in serum due to retro-Michael reaction. As we assess possible future application of the 2fPBA- α -amino-hydrazide chemistry for constructing antibody conjugates that will be used *in vivo*, we thus examined the serum stability of the antibody-fluorophore conjugates (AFCs) assembled via this chemistry.

To check the stability of the DAB construct without influence from instability due to other chemistries, we prepared an AFC that does not have any thiol-maleimide linkage on either the functionalized antibody or fluorophore. Tras HC-2fPBA, which is functionalized through the engineered cysteine residues using a maleimide crosslinker (Scheme 1), is therefore not suitable for this study. Tras LC-2fPBA is appropriate, as the 2fPBA functional group is attached to the antibody through a stable amide bond. Since Tx Red-am-zide contains a thiol-maleimide linkage, it was necessary to prepare an alternative payload for this study. We thus synthesized BODIPY-FL- α -amino-hydrazide (BODIPY-am-zide; Scheme S4), in which the fluorochrome and the reactive functionality are connected through an amide linkage. Analogous conditions shown in Scheme 1 were used to prepare the conjugate of Tras LC-2fPBA and BODIPY-am-zide (Tras LC-DAB-BODIPY) for serum stability evaluation.

As positive control, unmodified Tras LC was reduced and conjugated with BODIPY-FL-maleimide (BODIPY-mal) through the endogenous cysteine residues (Tras LC-mal-BODIPY). Size exclusion chromatographic analyses of the AFCs that had been incubated in human serum at 37 °C for different lengths of time were monitored by fluorescence to detect fluorophore-associated conjugates. As expected, the positive control displayed another peak arising over time that corresponds to a fluorophore conjugate with a lower molecular weight protein (Figure 4b). This is consistent with previous literature that demonstrated the transfer of a maleimide payload to serum albumin (~66 kDa).⁴⁶ Meanwhile, minimal exchange or deconjugation was observed for Tras LC-DAB-BODIPY during the 7-day incubation (Figure 4a; Figure S14), suggesting that the DAB conjugate is stable in human serum.

Immunoaffinity to HER2.

To examine whether immunoconjugates prepared by 2fPBA- α -amino-hydrazide ligation retain their antigen selectivity toward HER2, Tras LC-DAB-BODIPY and Tras HC-DAB-BODIPY were tested for their binding to HER2+ and HER2- cells. SK-BR-3 (HER2+) and MDA-MB-231 (HER2-) breast cancer cells were treated with the conjugates and their binding to HER2+ cells were detected by fluorescence via flow cytometry. An increase in fluorescence was observed in SK-BR-3 cells but not in MDA-MB-231 cells when treated with either of the AFCs (Figure S15). Furthermore, comparable affinity to HER2 was measured by ELISA for both of the conjugates relative to their respective unmodified antibody (Figure S16). Both results demonstrate that the function of these antibody conjugates remains unperturbed.

Multi-bioorthogonal labeling.

With the 2fPBA- α -amino-hydrazide coupling reaction deemed applicable for generating site-specific, stable, and functional immunoconjugates, we further examined the versatility of this chemistry to adapt to rising interests in the ADC field toward more complex conjugates, such as dual-payload and bispecific ADCs.^{29–33} The use of two different chemical approaches to site-specifically attach two different pay-loads or to couple two antibody fragments has previously been demonstrated, though many of the reported methods still necessitate sequential steps of conjugation due to cross-reactivity issues.^{29, 33}

To explore the potential for a more efficient one-step approach, we first set out to test the orthogonality of the 2fPBA- α -amino-hydrazide reaction to two of the most widely employed bioorthogonal reactions, SPAAC and Tz-TCO chemistries. SPAAC has demonstrated compatibility with aldehyde/ketone condensation,^{35, 48} increasing the likelihood of finding orthogonality here as well. Meanwhile, Tz-TCO ligation is well known for its fast kinetics (10^3 to 10^6 M⁻¹s⁻¹).⁴⁹ Orthogonality to this reaction will enable dual fast-reacting chemistries to be achieved in one setting.

Tras LC antibody was first functionalized with 2fPBA, azide (Az), or TCO using the TG method as described earlier (Scheme S6). Fluorophores with complementary functional groups, including Tx Red-am-zide, TAMRA-aza-dibenzocyclooctyne (TAMRA-DBCO), and BODIPY-FL-tetrazine (BODIPY-Tz), were used as payloads to conjugate with the 2fPBA-, Az-, and TCO-functionalized antibodies, respectively. To check for orthogonality,

Tras LC-2fPBA was allowed to react with Tx Red-am-zide in the presence of TAMRA-DBCO or BODIPY-Tz. Similarly, Tras LC-Az or Tras LC-TCO was allowed to react with TAMRA-DBCO or BODIPY-Tz, respectively, in the presence of Tx Red-am-zide. As demonstrated by SDS-PAGE and ESI-MS, no cross reaction was observed in all cases (Figure 5a; Figure S17–19). The results indicate that the 2fPBA- α -amino-hydrazide reaction is mutually orthogonal to both SPAAC and Tz-TCO chemistries.

It has been demonstrated that SPAAC and Tz-TCO ligations can be performed together by using select cyclooctyne and tetrazine derivatives.⁵⁰ In particular, bulky cyclooctynes such as DBCO do not cross-react with disubstituted tetrazines.⁵¹ Given the orthogonality of the 2fPBA- α -amino-hydrazide reaction to both of these chemistries, we reasoned that we could further exploit the reported orthogonality between SPAAC and Tz-TCO reactions to perform triple bioorthogonal labeling simultaneously. In one pot, a mixture of Tras LC-2fPBA, Tras LC-Az, and Tras LC-TCO was allowed to react concurrently with Tx Red-am-zide, TAMRA-DBCO, and BODIPY-FL-methyltetrazine (BODIPY-methylTz). BODIPY-methylTz was used in place of BODIPY-Tz, as monosubstituted tetrazine can potentially cross-react with cyclooctynes.⁵² By ESI-MS analysis, only three peaks corresponding to the expected conjugate products of each of the three chemistries were observed (Figure 5b; Figure S21). This represents a rare demonstration of *simultaneous* triple bioorthogonal conjugations. The few previous reports of triple labeling require at least one sequential step,^{53–55} which precludes the potential for real-time multi-labeling and analysis.

As an application to cater to emergent interest in multi-functional ADCs, the orthogonality of the 2fPBA- α -amino-hydrazide reaction to the Tz-TCO chemistry was further exploited to generate a site-specific dual-payload conjugate. The latter was selected as the concomitant chemistry because of its fast kinetics. To introduce two different reactive handles site-specifically onto one antibody, Tras LC was first modified with TCO on the light chain via TG. It is known that upon deglycosylation, the native Q295 residue on the heavy chain becomes available for recognition by TG,⁵⁶ providing a second site for modification on the antibody. Accordingly, Tras LC-TCO was deglycosylated by PNGaseF and then subjected to a second round of TG-mediated ligation to introduce the 2fPBA functionality on the heavy chain (Figure 5c). We note that TG was less efficient in transamidating the Q295 site with the amine-2fPBA crosslinker. In optimization of this step, we found that increased concentrations of the crosslinker and TG as well as prolonged incubation (2–4 days) were helpful in driving the heavy chain modification to completion (Table S1; Figure S22).

The dual-modified Tras LC-TCO/HC-2fPBA was then allowed to react concurrently with BODIPY-Tz and Tx Red-am-zide. SDS-PAGE and ESI-MS analysis confirmed site-specific conjugation of BODIPY-Tz to the TCO-modified light chain and Tx Red-am-zide to the 2fPBA-modified heavy chain (Figure 5c; Figure S23). Both reactions were essentially complete in less than 30 min (Figure S24), supporting the capability for dual rapid conjugations.

Overall, the result demonstrates successful collaboration between the 2fPBA- α -amino-hydrazide reaction and SPAAC and Tz-TCO ligations to enable simultaneous multi-protein labeling as well as dual-labeling within a single protein. Efforts have gone into discovering

such “orthogonal bioorthogonal chemistries”^{34, 51} and we anticipate the new additions to open up new possibilities in diverse multi-labeling applications.

CONCLUSIONS

The work presented here examines the use of the coupling reaction of 2fPBA and α -amino-hydrazide for fast and efficient assembly of site-specific bioconjugates, herein exemplified by immunoconjugates. Kinetically, the reaction is conducive to quantitative conversion at low reactant concentrations. Bioconjugation using this reaction is chemoselective in a complex medium. The zwitterionic nature of the DAB scaffold imparts minimal hydrophobicity to the overall bioconjugate. Antibody conjugates formed via the DAB linkage exhibit serum stability and *in vitro* selectivity to antigen-presenting cells, supporting the suitability of this chemistry to generate stable and functional biomolecule conjugates. The collective speed, bioorthogonal reactivity, hydrophilicity and stability that this reaction offers are befitting to the current demands in the ADC field and should be broadly applicable in time- and cost-efficient production of homogeneous and stable bioconjugates.

The present method for attaching a 2fPBA handle onto an antibody involves appending a 2fPBA-heterobifunctional crosslinker via chemical or enzyme-mediated approaches to an engineered site. Nevertheless, the outlook for genetically introducing this unique reactive functionality is also promising, as structurally similar analogs have been incorporated into proteins, including antibodies.^{6, 57–59} Moreover, a number of novel approaches have been developed recently that enable site-specific installation of a reactive handle onto a native antibody.^{60–63} Such methods may be applied to introduce the 2fPBA functionality without further protein engineering necessary.

Finally, we demonstrate the mutual orthogonality of the 2fPBA- α -amino-hydrazide reaction to both SPAAC and Tz-TCO ligations, enabling simultaneous triple bioorthogonal labeling and rapid one-pot construction of site-specific dual payload-antibody conjugates. We envision that our reported chemistry, in concert with other bioorthogonal chemistries, can be extended to couple assorted macromolecules and small molecules, or combinations thereof, for the generation of diverse bioconjugates and materials with precision and efficiency.

EXPERIMENTAL SECTION

Synthesis of bifunctional reagents.

Syntheses of the following molecules are described in the Supporting Information (SI): maleimide-PEG3–2fPBA; amine-PEG4–2fPBA; Texas Red- α -amino-hydrazide; BODIPY-FL- α -amino-hydrazide.

Antibody-fluorophore conjugation.

Tras LC and Tras HC were functionalized with 2fPBA at the engineered site as described in the SI. To demonstrate site-specific conjugation with Tx Red-am-zide via SDS-PAGE, 0.5 mg/mL Tras LC, Tras LC-2fPBA, Tras HC, and Tras HC-2fPBA were allowed to react with 26.7 μ M fluorophore at room temperature (RT) for ~4 h. Each reaction mixture was then treated with sample buffer containing 2-mercaptoethanol. All the samples were heated at

70 °C for 10 min before being subjected to SDS-PAGE. Fluorescent images of the gel were taken under a long wavelength UV lamp. The subsequent Coomassie-stained gel was imaged under white light.

To examine the kinetics of antibody-fluorophore conjugation, 1 mg/mL (~6.7 μ M) Tras LC-2fPBA or Tras HC-2fPBA was incubated with 26.7 μ M (two equivalents per 2fPBA group) or 133 μ M (ten equivalents) Tx Red-am-zide in Dulbecco's phosphate buffered saline (DPBS) containing 2% DMSO (v/v) at RT. At each time point, an aliquot (16 μ L) of the reaction mixture was reduced with 5 mM tris(2-carboxyethyl)phosphine (TCEP) (by adding 4 μ L of 25 mM TCEP stock made from 0.5 M TCEP, pH 7.0 - Sigma, 646547). After 5 min incubation at RT, the sample was injected into the LC-MS for analysis (see below for LC-MS conditions). The extent of fluorophore loading at each time point was calculated based on the peak intensity of unre-acted 2fPBA-functionalized light or heavy chain (P1) and the peak intensity of the conjugate of light or heavy chain (P2) as resolved on the deconvoluted mass spectrum. The equation used was $DAR = [P2 / (P1 + P2)] \times 2$. The calculated DAR was used to yield the plots shown in Figure 3.

Serum stability.

The stability study was performed according to a reported method³⁹ with minor revisions. Human AB serum (Omega Scientific, HS-20) was centrifuged at 157 xg for 5 min using an Eppendorf 5415C centrifuge and then filtered through a 0.2 μ m filter. The filtered serum was kept at -20 °C until use. Antibody-fluorophore conjugates, Tras LC-DAB-BODIPY and Tras LC-mal-BODIPY were prepared as described in the SI. Each AFC was added to the serum such that the final AFC and serum concentrations were 0.1 mg/mL and 90% (v/v), respectively. The 0 h samples were flash-frozen with liquid nitrogen within 5 min after introducing the AFC to the serum. The remaining AFC-serum mixtures were incubated at 37 °C in a 5% CO₂ incubator. At each time point, an aliquot was taken out, flash-frozen, and stored at -80 °C. The samples at different time points were analyzed collectively via size exclusion chromatography (SEC) using a Waters ACQUITY UPLC Protein BEH200 SEC 1.7 μ m 4.6 $\times$ 150 mm column connected to a Waters ACQUITY UPLC H Class system. The mobile phase was DPBS containing 15% acetonitrile, with a flow rate of 0.30 mL/min and a total run time of 26 min. The inline fluorescence detector of the UPLC system was set to excite at 480 nm and to collect emission from 520 nm to detect BODIPY fluorescence.

Triple bioorthogonal labeling.

Procedures to make Tras LC-2fPBA, Tras LC-Az, and Tras LC-TCO are described in SI. A mixture containing equal composition of all three antibodies was prepared and deglycosylated by PNGase F. Meanwhile, a mixed fluorophore stock was made that contained equal composition of Tx Red-am-zide, TAMRA-DBCO (Sigma Aldrich), and BODIPY-FL-methylTz (Broadpharm). The mixed fluorophore stock was added to the antibody mixture and incubated at RT. Final concentrations were 1 mg/mL (6.7 μ M) of each antibody and 133 μ M (10 eq. per reactive group) of each fluorophore in DPBS, 4% DMSO. The sample was reduced by 5 mM TCEP, pH 7 for 5 min prior to MS analysis.

Dual payload-antibody conjugation.

Tras LC-TCO/HC-2fPBA was prepared as described in the SI. For dual fluorophore labeling, 1 mg/mL (6.7 μ M) Tras LC-TCO/HC-2fPBA was allowed to react with ~26.7 μ M each of BODIPY-Tz (Jena Bioscience) and/or Tx Red-am-zide in DPBS, 2% DMSO at RT. For samples treated with both fluorophores, the fluorophore stocks were first mixed 1:1 and then simultaneously added to the antibody. After 1.5 h, the samples were treated with sample buffer containing 2-mercaptoethanol, heated for 10 min at 70 °C, and subjected to SDS-PAGE. The gel was imaged under a long wavelength UV lamp to observe fluorescent labeling. The same gel was Coomassie-stained and was imaged under white light.

LC-MS analysis of antibodies.

LC-MS analysis of antibodies was performed using a Waters LC-MS system equipped with a 2545 binary gradient module, a 2767 sample manager, a 2998 photodiode array detector, and a SQ detector 2. A Poroshell 300 SB-C8 2.1 $\times$ 75 mm, 5 μ m column (Agilent Technologies) was used, with the column temperature set to 80 °C. Each run was 5 min with 1 mL/min flow rate. The mobile phase was 95% solvent B from 2–4 min and 95% solvent A for other times. Solvent A was water with 0.05% formic acid and solvent B was acetonitrile with 0.05% formic acid. The raw mass spectrum was deconvoluted using MaxEnt1 on the MassLynx 4.1 software. Unless otherwise noted, antibodies were reduced by 100 mM TCEP (by adding from 0.5 M TCEP-HCl solution) prior to injection into LC-MS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Bioorthogonality of 2fPBA- α -amino-hydrazide coupling. (a) Reaction scheme of 2fPBA (**1**) and α -amino-hydrazide (**2**) and the calculated rate constants of each step. (b) Kinetics of DAB formation as monitored by the change in absorbance at 298 nm. 2fPBA (50 μ M) was mixed with one (black) or ten (red) equivalent(s) of **2a** in 0.1 M PBS, pH 7.4. (c) SDS-PAGE demonstrating chemoselectivity of labeling of α -amino-hydrazide-functionalized tubulin spiked in A549 cell lysate using BODIPY-FL-2fPBA. Left: fluorescence; right: Coomassie.

Figure 2. Site-specific antibody-fluorophore conjugation. Reducing SDS-PAGE of unmodified or 2fPBA-functionalized Tras LC and Tras HC that were allowed to react with Tx Red-am-zide. Left: fluorescence; right: Coomassie. MW = molecular weight marker.

(a) Tras LC-2fPBA + Tx Red-am-zide **(b) Tras HC-2fPBA + Tx Red-am-zide** **Figure 3.**

Kinetics of conjugation of 2fPBA-functionalized antibody (1 mg/mL; 6.7 μ M) with Tx Red-am-zide (26.7 μ M for two equivalents or 133.4 μ M for ten equivalents) in DPBS, pH 7.4, containing 2% DMSO. (a) Tras LC-2fPBA + Tx Red-am-zide. (b) Tras HC-2fPBA + Tx Red-am-zide. Each symbol/color represents a different trial. The first point of each plot corresponds to 5 min time point. The extent of loading was determined by ESI-MS. Representative mass spectra are shown in Figure S9–S10.

Figure 4. Serum stability of (a) Tras LC-DAB-BODIPY compared with (b) a hinge-cysteine conjugate, Tras LC-mal-BODIPY. Samples were incubated in human serum at 37 °C and evaluated by size-exclusion chromatography using fluorescence detection. AU = arbitrary unit.

Figure 5.

Multi-bioorthogonal labeling. (a) Reducing SDS-PAGE demonstrating the mutual orthogonality of the 2fPBA- α -amino-hydrazide reaction to SPAAC (left panel) and Tz-TCO ligation (right panel). The band region displayed corresponds to the light chain of the antibodies. Full images of both gels are shown in Figure S17. Top: fluorescence; bottom: Coomassie. Gray dotted lines in the top scheme indicates the absence of cross-reactivity, while colored double-head arrows indicate reactivity between the two connecting species. (b) Scheme of simultaneous triple bioorthogonal labeling and the overlaid mass spectra of a mixed antibody sample before (black) and after (blue) reaction with a mixture of functionalized fluorophores. Left panel: light chain region; right panel: heavy chain region. *The peak of 24597 Da corresponding to Tras LC-2fPBA coincides with the doubly-charged

ion of the deglycosylated heavy chain, which contributes to the signal after reaction. Mass spectrum of the sample without deglycosylation supported that Tras LC-2fPBA was consumed after the reaction (Figure S21). (c) Scheme of dual payload-antibody conjugation and reducing SDS-PAGE demonstrating site-specific conjugation of each payload to the expected subunit. Left: fluorescence; right: Coomassie. TG = transglutaminase; MW = molecular weight marker.

Scheme 1.

Site-specific functionalization of engineered trastuzumab antibodies with 2fPBA and the subsequent conjugation with an α -amino-hydrazide payload. TG = transglutaminase. The orange colored portion of the structure (middle panel) corresponds to part of the side chain of the amino acid residue where the crosslinker is attached: glutamine for Tras LC; cysteine for Tras HC.

Table 1.

Comparison of selected chemoselective chemistries for site-specific antibody-drug conjugation.

Chemistry ^a	Antibody ^b	Payload	Condition	Time ^c	Yield ^d	Ref.
Oxime	pAcPhe; 133 μ M	Alkoxyamine; 1.3 mM	100 mM Ammonium acetate, pH 4.5 at 37 °C	96 h	>95%	6
HIPS	fGly; 200 μ M	HIPS; 800 μ M	50 mM Sodium citrate, 50 mM NaCl, pH 5.5, 0.85% DMA, 0.085% Triton X-100 at 37 °C	72 h	>90%	7
Trapped - Knoevenagel	fGly; 25 μ M	Thiopyrazolone; 1 mM	100 mM Sodium citrate, 25 mM NaCl, pH 7, 1% DMA, 0.25% Triton X-100 at 37 °C	2 h	83%	8
SPAAC	AzK; 67 μ M	Aza-dibenzocyclooctyne; 200 μ M	PBS, 10% DMSO at 25 °C	16 h	>95%	44
IEDDA	CypK; 13 μ M	Tetrazine; 68 μ M	PBS, 10% ACN at 25 °C	3 h	>95%	10
DAB	2TPBA; 13 μ M	α -amino-hydrazide; 133 μ M	PBS, 2% DMSO at 25 °C	<0.5 h	>95% ^e	

^aHIPS: hydrazino-*iso*-Pictet Spengler; SPAAC: strain-promoted alkyne-azide cycloaddition; IEDDA: inverse electron demand Diels-Alder.

^bpAcPhe: *p*-acetylphenylalanine; fGly: formylglycine; AzK: N⁶-(2-azidoethoxy)carbonyl-L-lysine; CypK: cyclopropenyllysine. The concentration as shown took into account the number of reactive groups on the antibody. In all cases, the antibodies had 2 reactive groups per antibody, hence the concentration is twice the reported antibody concentration.

^cThe time corresponds to the incubation time as reported in the referenced articles.

^dThe yield corresponds to the percent conversion to the maximum possible DAR.

^eThe time and yield shown for DAB chemistry are based on the conjugation of Tras LC-2TPBA and Tx Red-am-zide.