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Communication

Dual-Boronic Acid Reagents That Combine Dynamic and Covalent Bioconjugation

Michael J. Swierczynski, Yuxuan Ding, and Zachary T. Ball*

Cite This: *Bioconjugate Chem.* 2022, 33, 2307−2313

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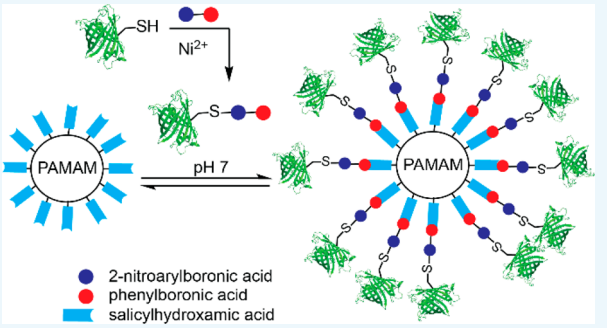
ABSTRACT: Boronic acids and boronate esters find appreciable use in chemical biology. Molecules containing orthogonal boronic acid pairs can be utilized for sequential metal-catalyzed cross-couplings for facile preparation of complex bioconjugates including protein-protein conjugates. In this paper, we expand bis-boronic acid reagents for tandem covalent and dynamic bioconjugation. Sequential cross-coupling of 2-nitroarylboronic acid with cysteine residues and condensation of phenylboronic acid with salicylhydroxamic acids (SHA) readily afforded bioconjugates under physiological conditions with dual covalent and dynamic linkages. Both small molecule− and macromolecule−protein conjugates were amenable with this approach and reversible upon addition of excess unfunctionalized SHA or reactive oxygen species. These investigations provide new insights into the kinetic stability of SHA adducts.



The ability to build bioconjugates with increasing complexity, specificity, dynamics, and reactivity enables a host of important applications in diverse fields, such as chemical biology, biomaterials engineering, and targeted therapeutic development. Molecular reagents with multiple biorthogonal reactive groups can utilize the subtle interplay of different reactivities to allow the construction of multiprotein conjugates[1] and trifunctional protein bioconjugates,[2] and other more complex bioconjugate structures and topologies. While bioconjugation most typically involves irreversible covalent bond formation, there is also intense interest in dynamic, reversible, or metastable linkages, which in turn allows for the design of "smart," responsive, or adaptive biomaterials, with applications such as stimuli-responsive enzyme inactivation[3] and payload release.[4]

Boronic acids have a long and varied history in biological and medicinal chemistry. Boronic acids are often stable in biological environments, but have important chemical properties that has led to their use for bioconjugation,[5−8] enzyme inhibition,[9,10] and reactive oxygen species (ROS) sensing,[11,12] among other concepts. We have established a research program examining unique reactivity and selectivity of boronic acid reagents in biological contexts, focusing initially on transition-metal-catalyzed cross-coupling reactions for C−X bond formation. These efforts uncovered remarkable structure−reactivity relationships among boronic acid reagents, and the information gleaned from these studies allowed the development of boronic acid pairs for sequential covalent bioconjugation, including for the preparation of protein−polymer−protein structures. The method combines different catalytic reactivities for sequential bioconjugation at cysteine (with an Ni^{II} catalyst) and at pyroglutamate-histidine sites (with a Cu^{2+} catalyst).[13] In this work, we extend the ideas of orthogonally reactive bis-boronic acid reagents for sequential bioconjugation processes that allow both stable cross-coupling linkages and dynamic covalent conjugation via boronate ester and related structures (Figure 1). To demonstrate the potential for sequential covalent and dynamic bioconjugation with boronic acids, this work focuses on cysteine as the target for covalent cross coupling. While many methods for targeting cysteine exist,[14,15] metal-catalyzed arylation provides electronically perturbed cysteine modifications, and provides the metal catalyst as an additional tool for spatiotemporal control of reactivity, and may serve as a first step toward other boronic-acid targets for covalent modification, such as pyroglutamate-histidine motifs.[13] Cysteine arylation and heteroarylation also provides one useful approach to access bioconjugates with increased serum stability relative to traditional Michael addition approaches.[16,17]

By testing the selectivity questions inherent in these polyfunctional environments, we hope to provide bioconjugate structures suitable for multiple modes of late-stage bioconjugation.

Received: October 30, 2022
Revised: November 17, 2022
Published: November 29, 2022

https://doi.org/10.1021/acs.bioconjchem.2c00508
Bioconjugate Chem. 2022, 33, 2307−2313

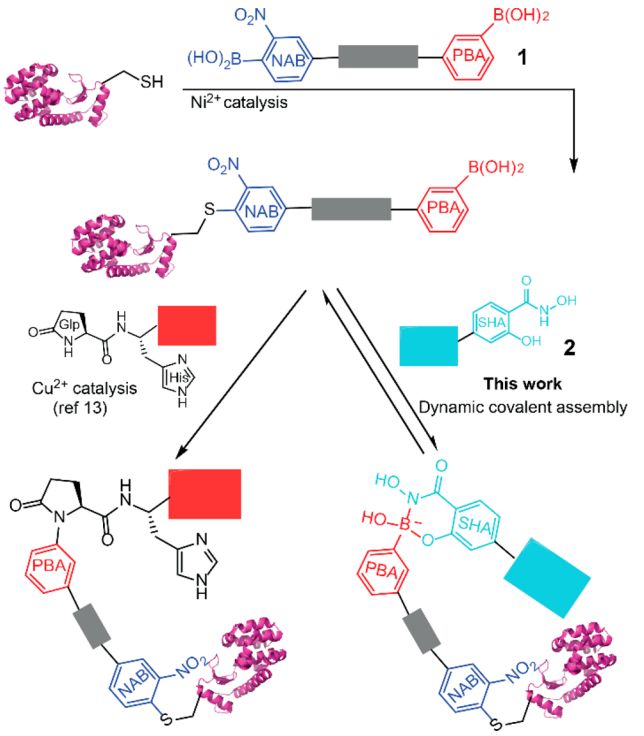


Figure 1. Bioconjugation strategies with bis-boronic acids.

Boronic acid condensation to afford boronate esters and similar structures has been used extensively for constructing assemblies with polyols, even in water.[18,19] Recently, salicylhydroxamic acid (SHA) has emerged as one of several structures that exhibit improved boronic acid binding.[19−27] The SHA−boronic acid linkage has been utilized for traceless protein delivery,[28] peptide dimerization,[29] and antibody purification.[24]

To better understand the structural requirements for SHA binding, we examined two boronic acid structures previously demonstrated to be orthogonal (unreactive) in Ni^{II}-catalyzed coupling with 2-nitroaryl boronic acid structures, but suitable for Cu-mediated coupling:[6,30] phenylboronic acid (PBA) and (*E*)-alkenylboronic acid (EAB). To evaluate binding on protein surfaces, lysozyme conjugates of PBA and EAB were prepared via amine modification with boronic acid NHS esters (average modification 1.5:1 boronic acid/protein, see SI for details). SHA binding was evaluated in an alizarin competition binding assay.[31] Alizarin Red S (ARS) is a catechol containing dye that upon reaction with a boronic acid to give a boronic ester product, a substantial shift in absorbance wavelength (consistent with a visible color change from red to yellow), as well as increased fluorescence (emission maximum 590 nm), is observed. Displacement of ARS in the complex with SHA results in release of the dye, which can be observed as a decrease in fluorescence and a blueshifted absorbance.[31]

While a model protein (lysozyme) functionalized with either EAB or PBA boronic acids exhibited evidence of SHA binding, the phenylboronic acid PBA structure demonstrated a significantly improved fluorescence turn-on response (Figure 2b). Upon addition of 1 equiv of salicylhydroxamic acid, a decrease in ARS fluorescence was observed for both conjugates, indicating formation of Lyso-SHA (Figure 2b). Interestingly, initial fluorescence of the EAB conjugate was lower, resulting in a smaller change upon treatment with SHA.

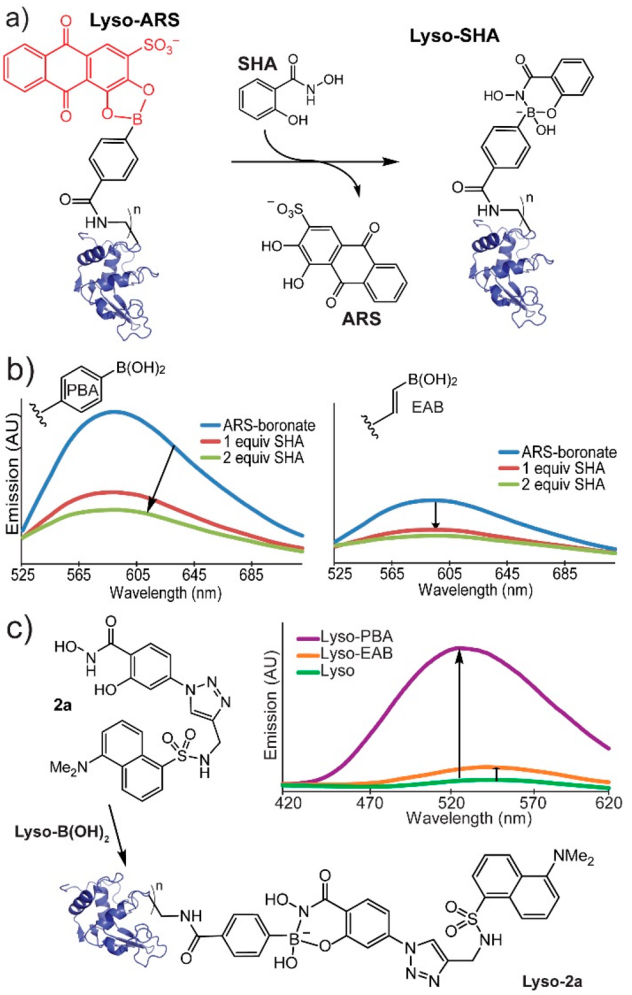


Figure 2. Lysozyme-boronic acid binding to salicylhydroxamic acid (SHA). (a) Scheme of alizarin competition binding assay of lyso-boronic acids with SHA. Addition of SHA causes dissociation of ARS-boronate complex and loss of fluorescence. (b) Emission spectra of SHA titration into alizarin complexes of lyso-PBA (left) and lyso-EAB (right). (c) Lyso-boronic acid binding to dansyl-salicylhydroxamic acid **2a**. Structure of **2a** (left) and emission spectra (right) of reactions after removal of unbound **2a** by ultrafiltration. Conditions: Protein (30 μM), **2a** (150 μM) in phosphate buffer (20 mM, pH 7.4) at room temperature (rt) for 30 min.

Protein conjugation with SHA was stable enough to survive purification by ultrafiltration to afford purified protein−SHA conjugates, but only in the case of phenylboronic acid (PBA) linkages (Figure 2c). To assess binding stability, a dansyl-functionalized SHA (**2a**) was prepared in three steps, taking advantage of the orthogonal reactivity of the azide group to allow preparation of the key trityl-protected salicylhydroxamate **5** (Scheme 1). EAB linkages proved kinetically labile, and most SHA was washed away during ultrafiltration (Figure 2c). In contrast, Lyso-PBA retained a robust dansyl fluorescence after ultrafiltration. Given the greater stability to routine handling, we focused on PBA as our boronic acid linkage of choice for additional studies.

Having established PBA as a suitable moiety for stable and dynamic SHA conjugation, we designed and synthesized a trifunctional, bis-boronic acid reagent **1**, containing two uniquely reactive boronic acids together with a desthiobiotin handle (Scheme 2), which provides chemoselective blot

https://doi.org/10.1021/acs.bioconjchem.2c00508
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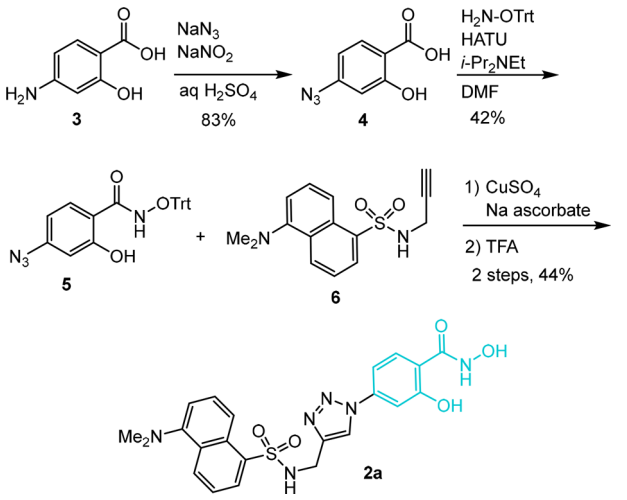
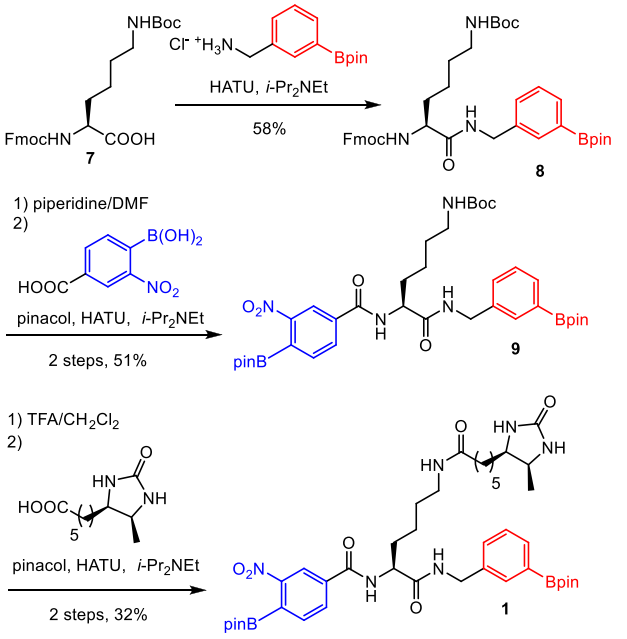
Scheme 1. Synthesis of Dansyl-Salicylhydroxamic Acid 2a



Scheme 2. Synthesis of Bis-Boronic Acid 1[a]



[a]The trifunctional reagent contains SHA-reactive phenylboronic acid (red), cysteine-reactive 2-nitrophenylboronic acid (blue), and desthiobiotin affinity handle (black).

imaging capabilities, among other attributes. The synthesis hinges on late-stage introduction of the desthiobiotin handle, which minimizes purification challenges in the presence of chemically sensitive boronic acid groups.[13,32]

Three model proteins bearing a single cysteine residue; T4 lysozyme V131C (T4L),[33] sfGFP S147C (GFP),[33] and CAL PDZ domain (CALP),[34] were treated with **1** in the presence of $Ni(OAc)_2$ to afford phenyboronic-acid-labeled proteins (Figure 3). Reactions of T4L and GFP proceeded to full conversion by SDS-PAGE and could be purified by simple ultrafiltration. The integrity of the PBA moiety was confirmed by ARS binding; ARS exhibited a profound color change (red to yellow) upon mixing with modified T4L (**T4L-1**) (Figure 3b), while no change was observed with unmodified T4L.

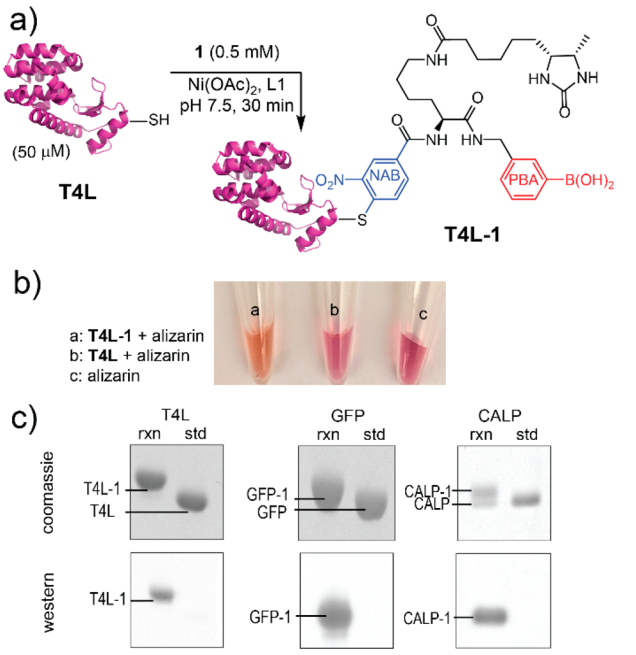


Figure 3. Single-phenylboronic acid protein modification. (a) Conjugation of single-cysteine proteins with bis-boronate ester **1**. Condns: Protein (50 μM), **1** (500 μM), tris(2-carboxyethyl)-phosphine (TCEP) (500 μM), $Ni(OAc)_2$ (1 mM), and 6,6′-dimethyl-2,2′-bipyridine (L1) (1 mM) in NMM buffer (50 mM, pH 7.5) at 37 °C for 30 min. (b) Confirmation of boronic acid integrity by Alizarin Red S (ARS) binding of conjugate **T4L-1**. (c) Gel and antibiotin blot images for protein conjugation.

With site-selective phenylboronic-acid-labeled proteins in hand, we investigated their reaction with salicylhydroxamic acid reagents to afford dynamic covalent conjugates. As above, an alizarin displacement assay was first used to assess conjugation based on absorbance spectra after peak deconvolution. Addition of SHA reagents containing a variety of functional handles (**2a**–**2c**) displaced ARS and formed conjugates with **T4L-1** as indicated by absorbance spectroscopy (Figure 4c) and fluorescence quenching (Figure S6). Additional information about dynamic covalent modification with the SHA moiety could be obtained by assessing fluorescence of the dansyl moiety in reagents **2a** and **2b**. Due to the solvochromatic nature of the dansyl fluorophore,[35,36] fluorescence spectra of **2a** and **2b** exhibited a wavelength shift (λ_{em} 558 to 531 nm) in the presence of the boronate–protein **T4L-1** (Figure 4d). Raw fluorescence spectra of proteins modified with bis-boronate **1** (e.g., **T4L-1**) are complicated by an additional fluorescence attributed to the nitroaryl linkage from the NAB moiety (λ_{em} 460 nm), and peak deconvolution was performed to isolate effects on the dansyl emission (see SI for details). Emission of reactions mixing a dansylated molecule without the SHA group with **T4L-1** gave no significant change in dansyl emission (Figure S8). Consistent with expectations, these data suggest that PBA–SHA linkages form readily, even between two macromolecular reagents as in the reaction of **T4L-1** with PEG polymer **2b**, and that SHA successfully out-competes a catechol for covalent conjugation at an arylboronic acid. The stoichiometric shift of dansyl fluorescence observed in 1:1 mixtures of **T4L** and dansyl–SHA reagents **2a** or **2b** demonstrates the orthogonal nature of PBA boronic acid

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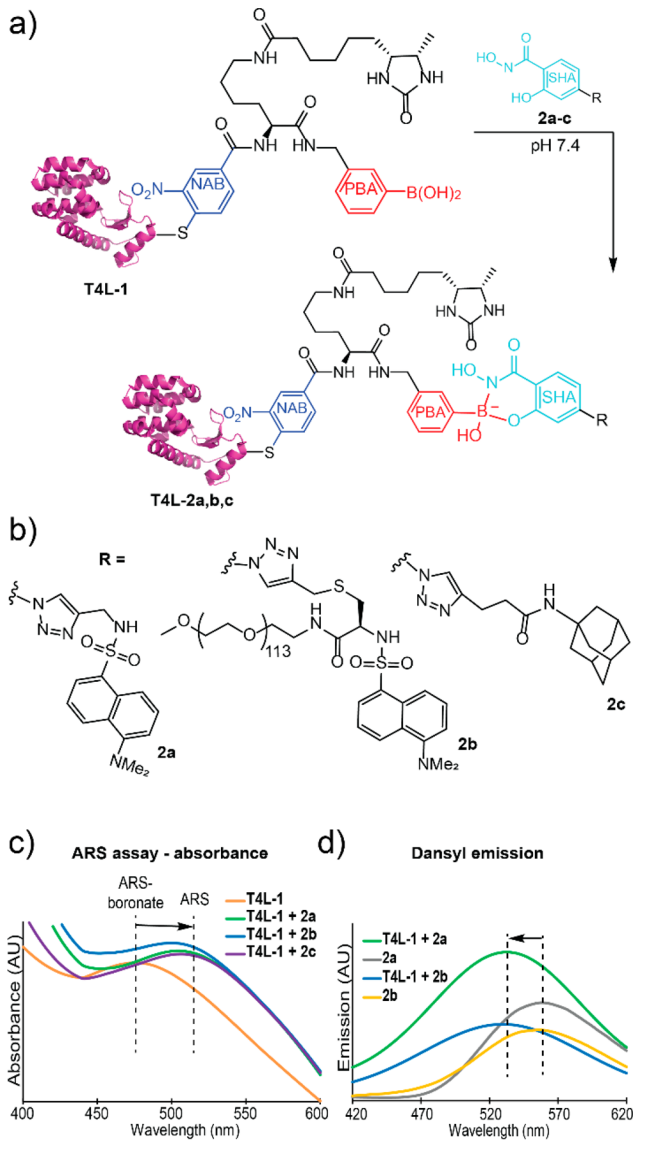

Figure 4. Binding evaluation of **T4L-1** to salicylhydroxamic acids. (a) Scheme of conjugation. (b) Structures of dansyl− (**2a**), dansyl-PEG5k− (**2b**), and adamantyl− (**2c**) SHA. (c) Absorbance spectra of ARS competition assay of **T4L-1** (30 μM) with SHA **2a**−**c**. (60 μM). (d) Fluorescence emission spectra of **2a** and **2b** upon treatment with **T4L-1**. Conditions: **T4L-1** (30 μM) and SHA **2a** or **2b** (30 μM) in phosphate buffer (20 mM, pH 7.4) at rt.

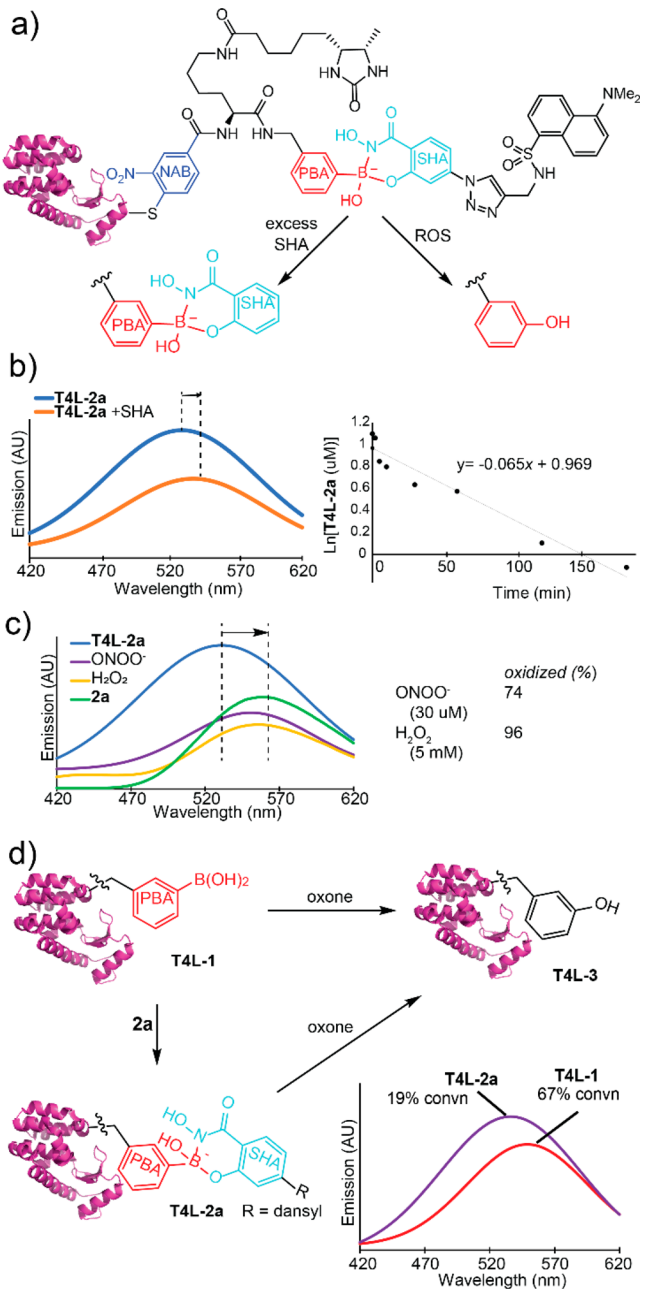

Figure 5. Release of dansyl-SHA **2a** from **T4L-2a**. (a) Scheme of release of **2a** from **T4L-2a**. (B, C) Kinetics of SHA release. Spectra (left) and plot (right) of release of **2a** from **T4L-2a** (3 μM) in the presence of excess SHA. Percent **2a** released was determined from the shift in λ_{em}. (c) Dansyl emission spectra of peroxynitrite- ($ONOO^-$) and hydrogen peroxide (H_2O_2)-mediated oxidation of **T4L-2a**. (d) Reaction scheme and dansyl emission spectra of oxone-mediated oxidation of **T4L-1** and **T4L-2a**. Conditions: **T4L-1** or **T4L-2a** (30 μM) and oxone (60 μM) in phosphate buffer (20 mM, pH 7.4) at 0 °C for 5 min. SHA **2a** (30 μM) was added to **T4L-1** reaction after oxidation to quantify emission.

structures in the initial nickel-catalyzed bioconjugation process to form **T4L-1**.

The thermodynamics of dynamic covalent reactivity of boronic acids with SHA is reasonably well established; a microscale calorimetry measurement in a related system determined K_d = 8 μM.[21] However, relatively little is known about the reaction kinetics, especially within complex protein structures. In particular, understanding the release rate (k_{off}) is essential for tailoring the properties of drug release systems, stimulus-responsive materials, and other applications. The release of dansyl **2a** from **T4L-2a** could be measured from the solvatochromic shift in dansyl emission upon release from protein, for reactions monitored in the presence of excess SHA to trap released boronic acid. We observed a linear plot of ln[**T4L-2a**] vs time (Figure 5b) under these conditions, consistent with a first-order, dissociative SHA-exchange process. The initial rate was unchanged by increasing [SHA]. We measured an apparent first-order rate constant of 6.5 × 10^{-3} s^{-1} and half-life of 90 min for SHA release from the PBA-functionalized protein (Figure 5b).

Reaction with ROS is another common means of cleaving arylboronate linkages, and this property has been studied with boronic esters and SHA−boron linkages for a variety of

https://doi.org/10.1021/acs.bioconjchem.2c00508
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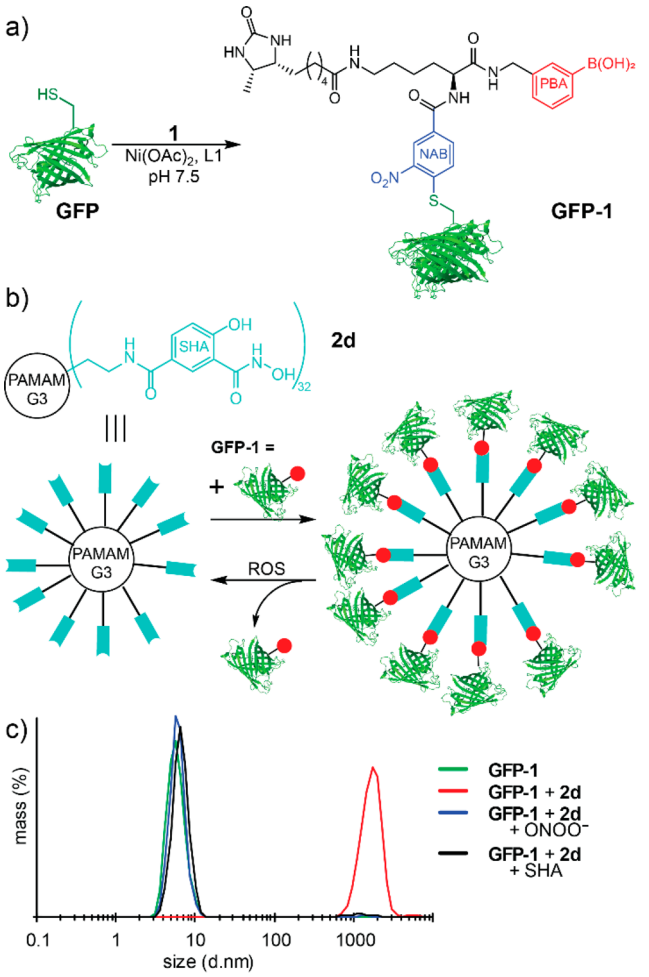

Figure 6. Formation and release of protein-dendrimer complex. (a) Modification of **GFP** with bis-boronate **1**. (b) Schematic of the formation of PAMAM-protein conjugate **GFP-2d**. (c) DLS of conjugation/release of **GFP-1** to **2d**. Conditions: **GFP-1** (30 μM), **2d** (1.5 μM) in phosphate buffer (20 mM, pH 7.4) at rt for 1 h. For release, peroxynitrite (150 μM) was added at rt for 10 min or salicylhydroxamic acid (2 mM) for 30 min.

interesting applications.[12,37,38] Peroxynitrite[39] and hydrogen peroxide[40] in particular oxidize boronic acids under biological conditions. In the present context, boronic acid−SHA conjugates provide an opportunity to build conjugates and materials that are responsive to their redox environment. To assess the redox stability of the boronate−SHA linkages, conjugates were subjected to peroxynitrite or hydrogen peroxide (Figure 5c). Consistent with expectation, treatment of **T4L-2a** with either peroxynitrite or hydrogen peroxide resulted in release of **2a**, following presumed oxidation of the boronate species, as assessed by absorbance spectroscopy. Interestingly, kinetics studies indicated that peroxynitrite or hydrogen peroxide indiscriminately oxidizes the boronate−SHA linkage of **T4L-2a** at rates indistinguishable from that with the boronic acid **T4L-1** (Figure S10). In contrast, the boronate−SHA linkage of **T4L-2a** was somewhat more stable toward oxidation with oxone (Figure 5d). Nonetheless, despite the tetravalent nature of the boron in boronate−SHA complexes, oxidation with a variety of species occurs at predictable rates similar to that of free boronic acids.

Finally, we investigated the potential for sequential boronate cross-coupling and subsequent boronate−SHA conjugation to build more complex polyvalent macromolecular assemblies, such as protein-dendrimer conjugates,[41,42] with dynamic covalent properties (Figure 6). An SHA-functionalized G3 PAMAM dendrimer (**2d**) was synthesized, containing 22 terminal SHA groups. The dendrimer **2d** was mixed with SHA-functionalized protein **GFP-1** in a 20:1 protein/dendrimer ratio. Dynamic light scattering (DLS) analysis indicated complete formation of a monomodal new assembly with apparent diameter of 2 μm(Figure 6, red trace), which may indicate some supramolecular assembly of dendrimers. No change was observed for reaction with unfunctionalized GFP (Figure S13). The dynamic covalent nature of this assembly was readily demonstrated by treatment with an excess of free SHA, resulting in a reversion to wholly free monomeric **GFP-1** (Figure 6, black trace). The dendrimer−protein assembly was also redox-sensitive; free monomeric GFP protein was readily released upon exposure of the assembly to peroxynitrite (blue trace).

In conclusion, bis-boronic acid reagents with orthogonally reactive organoboronate groups can be useful tools to construct protein conjugates and materials. Sequential nickel-mediated cross-coupling, selective for cysteine thiol groups, followed by dynamic covalent reaction of a second boronate group with SHA moieties, provides access to bioconjugates with defined stable and dynamic linkages. These properties are made possible by the effectively orthogonal reactivity of 2-nitroarylboronic acids and simple phenylboronic acids. The dynamic nature of the boronate−SHA linkage implies that a boronate−SHA linkage might be used as a temporary or transient material that can further react in subsequent boronic acid-based processes, including oxidation or copper-mediated cross coupling with diverse reagents.[6,30,40] The approach expands the potential for boronic acid reagents to selectively build dynamic biomaterials in an efficient and predictable manner.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.bioconjchem.2c00508.

General experimental details, methods for protein modification and product analysis, and characterization of new compounds (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Zachary T. Ball − *Department of Chemistry, Rice University, Houston, Texas 77005, United States;* orcid.org/0000-0002-8681-0789; Email: zb1@rice.edu

Authors

Michael J. Swierczynski − *Department of Chemistry, Rice University, Houston, Texas 77005, United States*

Yuxuan Ding − *Department of Chemistry, Rice University, Houston, Texas 77005, United States;* orcid.org/0000-0002-4201-2689

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.bioconjchem.2c00508

Notes

The authors declare no competing financial interest.

https://doi.org/10.1021/acs.bioconjchem.2c00508
Bioconjugate Chem. 2022, 33, 2307−2313

■ ACKNOWLEDGMENTS

Funding for this research was provided by the NSF under award numbers CHE-1904865 and CHE-2203948 and by the Robert A. Welch Foundation Research Grant C-1680 (Z.T.B.). We thank Raphael Hofmann and Jeffrey Bode (ETH Zurich) for supplying the plasmids for T4L(V131C) and sfGFP(S147C) and Nicholas Gill and Dean Madden (Dartmouth) for expression and purification of the CALP proteins.

■ ABBREVIATIONS

PBA, phenylboronic acid; EAB, (*E*)-alkenylboronic acid; NAB, nitroarylboronic acid; SHA, salicylhydroxamic acid; NMM, *N*-methylmorpholine; T4L, T4 lysozyme; GFP, green fluorescent protein; PEG, poly(ethylene glycol)

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https://doi.org/10.1021/acs.bioconjchem.2c00508
Bioconjugate Chem. 2022, 33, 2307−2313