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"Tag and Modify" Protein Conjugation with Dynamic Covalent Chemistry

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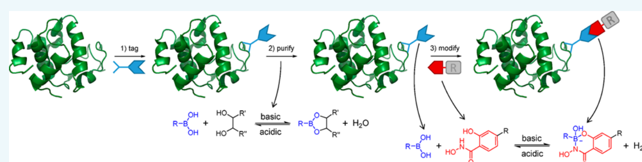
Supporting Information

ABSTRACT: The development of small protein tags that exhibit bioorthogonality, bond stability, and reversibility, as well as biocompatibility, holds great promise for applications in cellular environments enabling controlled drug delivery or for the construction of dynamic protein complexes in biological environments. Herein, we report the first application of dynamic covalent chemistry both for purification and for reversible assembly of protein conjugates using interactions of boronic acid with diols and salicylhydroxamates. Incorporation of the boronic acid (BA) tag was performed in a site-selective fashion by applying disulfide rebridging strategy. As an example, a model protein enzyme (lysozyme) was modified with the BA tag and purified using carbohydrate-based column chromatography. Subsequent dynamic covalent "click-like" bioconjugation with a salicylhydroxamate modified fluorescent dye (BODIPY FL) was accomplished while retaining its original enzymatic activity.



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■ INTRODUCTION

The site-selective modification of native proteins with new synthetic entities that confer desired functions offers immense opportunities for addressing challenges in basic biological and medical applications.[1−3] The attachment of, e.g., chromophores or polymers at distinct sites is nowadays widely used to study protein dynamics in live cells or to stabilize and deliver therapeutic proteins in vivo.[4,5] In recent years, there have been great advances in the identification of efficient synthesis routes to modify native proteins in a site-directed fashion under physiological conditions.[6] However, the separation of the successfully modified proteins from their native precursors can be challenging, particularly if a small substituent has been attached.[7,8] Therefore, the "tag and modify" strategy first proposed by Davis and co-workers[9] has opened new avenues in protein chemistry. It has been subsequently expanded to separate product mixtures by affinity chromatography,[10] for example, biotin−(strept)avidin[11,12] or metal−ligand[10,13] interactions can be exploited and introduce functionalities at the newly introduced reactive site. Still, many currently used binding tags have inherent limitations such as their bulkiness (i.e., LacZ or Galactose-binding protein),[14] binding constants too low to allow further modifications (hydrophobic peptide tags),[15] or excessively strong binding (biotin tags),[11] which require harsh conditions during elution. Therefore, it would be highly desirable to introduce a small reactive tag that serves both as affinity tag as well as reactive handle for further modifications and that allows cleavage under controlled physiological conditions.

In this context, dynamic covalent chemistry has been employed to instill these features[16] and the assembly of bioconjugates via C−N,[17−19] C−S,[19,20] and B−O[21] bonds has been demonstrated successfully. The latter case seems to be especially interesting because of its versatility and tunability of the interaction strength by selection of the binding partner and pH control. Herein, we introduce a new, synthetic approach to incorporate the boronic acid (BA) tag, which subsequently undergoes bioconjugation based on dynamic covalent bond formation providing both weak (K_d in mM range) and stable (low μM range) covalent linkages, serving simultaneously as purification and modification sites that are cleavable under pH-controlled conditions. BAs are eminent candidates for such applications due to their small sizes, bioorthogonality, bond stability, and reversibility that readily react with diols and salicylhydroxamate groups.[22] The BA-diol reaction provides a smart and easy-to-cleave platform for affinity purification based on BA carbohydrate interactions.[21,23] Subsequent site-directed functionalization of BA with salicylhydroxamates yields stable bioconjugates that remain intact in cell media but could be cleaved by slight pH changes[24,25] thus fulfilling the requirements for a "tag and modify" strategy.[9]

Lysozyme was selected as a model protein to demonstrate the applicability of BA as dynamic covalent tag for purification and post-modification reactions. Lysozyme is an O-glycosyl

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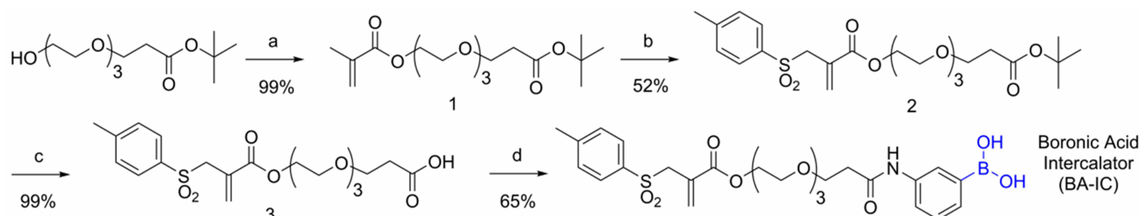
Scheme 1. Synthesis of Boronic Acid Intercalator (BA-IC)[a]



[a]a. methacryloyl chloride, Et_3N, CH_2Cl_2, 99%; b. 1. I_2, sodium tosylate, CH_2Cl_2; 2. Et_3N, EA, 52%; c. TFA, CH_2Cl_2, 99%; d. HBTU, DIEA, 3-aminophenylboronic acid hydrochloride, DMF, 65%.

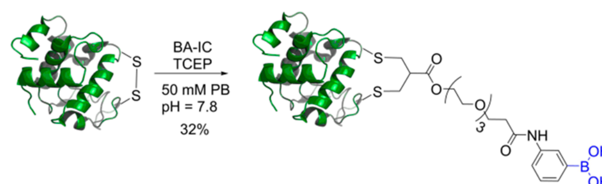
hydrolase that widely occurs both in plants and in animals where it participates in antibacterial defense by disintegrating cell walls of Gram-positive bacteria. An additional interesting feature of lysozyme is its ability to form amyloid fibers[26] rendering it attractive for the fabrication of bionanomaterials.[27−29] It has been shown previously that only one disulfide bond of lysozyme can be reacted with thiol alkylating reagents.[30] Therefore, we have combined the known allyl sulfone bioconjugation motif that reacts with accessible disulfides[31] of cyclic peptides and proteins with the BA "tag and modify" anchor group. The new BA-IC reagent allows purification of BA-lysozyme retaining its structural integrity and catalytic activity[6] as well as subsequent covalent bioconjugation with a salicylhydroxamate functionalized BODIPY-FL dye. Therefore, the approach could be broadly applicable as disulfides occur in many pharmaceutically relevant proteins like antibodies[32,33] or hormones[34] and the BA tag could in principle also be combined with other modification strategies such as N-terminal,[35] lysine,[36] or tyrosine[37] conjugations, as well as react with different ligands such as Schiff bases for further functionalization.[38,39] We believe that BA tags provide a versatile platform to further expand the scope for protein modifications for designing, e.g., responsive protein−drug conjugates or for building dynamic protein architectures based on stable covalent bonds in cellular environments.[40]

■ RESULTS AND DISCUSSION

Synthesis and Purification of BA-Tagged Lysozyme. The BA-containing allyl sulfone intercalator (**BA-IC**, Scheme 1) was designed to react with the accessible disulfides of peptides and proteins and introduces a single BA by reacting in a sequence of Michael addition and elimination steps.[31] **BA-IC** was obtained in a five-step synthesis starting from *tert*-butyl protected, monocarboxylic acid functionalized, triethylene glycol condensation with methacryloyl chloride yielding the corresponding methacrylate **1**. A tosyl group was introduced by an iodosulfonylation−dehydroiodination reaction to obtain the allyl sulfone **2**,[41] which was subsequently deprotected to liberate the corresponding carboxylic acid. Carboxylic acid rebridging reagent **3** was conjugated with 3-aminophenylboronic acid hydrochloride to transform it into boronic acid reagent **BA-IC** with a yield of 65% and an overall yield over all five reaction steps of 33% (Scheme 1).

Lysozyme was selected as model protein as it contains four disulfide bonds, of which three are buried within the molecule and only one (C_6−C_{127}) is close to the surface[42] and available for chemical modifications. The accessible disulfide bond was reduced to free thiols using 1.2 equiv of tris(2-carboxyethyl)-phosphine hydrochloride (TCEP) and subsequently reacted with **BA-IC** (Scheme 2).

Scheme 2. Incorporation of BA-IC into the Lysozyme



The reaction was conducted at room temperature in an aqueous solution for 24 h. BA-modified lysozyme (BA-lysozyme) was obtained after purification by fast protein liquid chromatography (FPLC) and desalting in 32% yield. Notably, additional 23% of native lysozyme was recovered from the reaction mixture. BA-lysozyme was lyophilized and characterized by MALDI-TOF MS.

Purification of BA-Lysozyme was accomplished by exploiting the interactions of BA with diols of the cross-linked agarose and dextran matrix of Superdex 200 10/300 GL FPLC column. Separation of BA-lysozyme from native lysozyme was achieved by affinity chromatography and not size exclusion, which allows more efficient isolation of the modified protein even from complex mixtures. Low binding affinity of sugar-based ligands like glucose or galactose, ranging from 1 mM to 200 mM in basic conditions, allows BA-lysozyme to be more strongly retained on the carbohydrate-based column compared to lysozyme and at the same time, allows facile elution without acidification.[43] Noteworthy, already a single BA group has a large impact on the retention volume, which increased from 21.46 to 23.48 mL (retention time: 42.92 to 49.96 min) using isocratic flow with basic (pH = 8.6) buffer (Figure 1).

To identify the modification site, tryptic digestion followed by MALDI-TOF-MS characterization was performed. Fragment-containing modified C_6−C_{127} disulfide bond was found ($[M+DHB+H]^+$ m/z = 1695.7441, calculated m/z = 1695.7234) and further analyzed via MSMS confirming the expected sequence. Additionally, unmodified fragments containing the remaining disulfide bonds (C_{30}−C_{115}, C_{64}−C_{180}, and C_{76}−C_{94}) were identified indicating site-selectivity of the reaction (Figure 2). Preserved enzymatic activity of modified proteins is crucial for all further applications.[6]

The catalytic activity of BA-lysozyme was assessed by recording turbidity changes of *Micrococcus lysodeikticus* lyophilized cells suspension in time. Here, the absorbance decreases when lysozyme hydrolyzes bacterial cell walls, which is proportional to its activity.[44] Enzymatic activity of BA-lysozyme was compared to native lysozyme and retained

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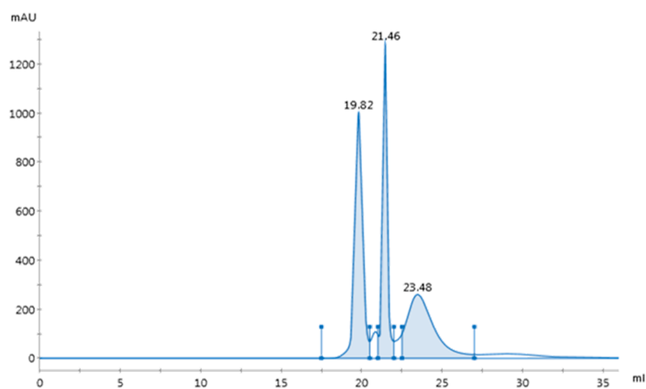


Figure 1. FPLC chromatogram of the reaction mixture with visible separation of lysozyme, modified lysozyme, and a higher molecular weight side product (retention volume = 19.82 mL).

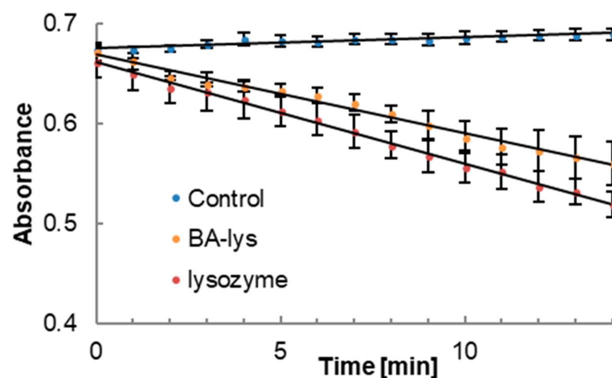
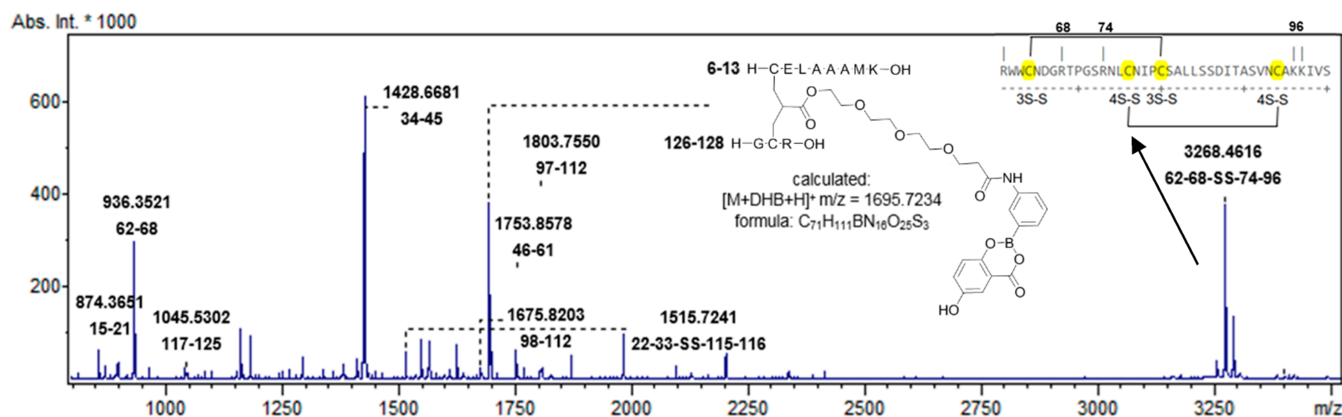


Figure 3. Rate of decrease in absorbance (slope) is proportional to lysozyme activity. Clearance of cell suspension turbidity proceeds with similar pace in case of both BA-modified (orange) and native lysozyme (red). Data are plotted as $n = 4 \pm$ standard errors of the mean (SEM).

activity of about 80 ± 4% was observed from the hydrolysis of bacterial cell walls (Figure 3). In comparison, mono- or statistical modifications of lysozyme often resulted in drastic losses of its enzymatic activity. For example, statistical modifications of lysine residues (K_1, K_{13}, K_{33}, K_{96}, K_{97}, and K_{116}) reduced activity even though none of the lysine residues is located within the active site.[45]

Also other modifications such as hexa-, penta-, and tetra-acetylations[46] or multiple phosphopyridoxylations[47] reduced lytic activity to less than 5% even though there was no impact on the conformation of lysozyme.[46] It was speculated that subtle changes in the surface charges had an impact on the interactions of lysozyme with bacterial membranes due to

a)



b)

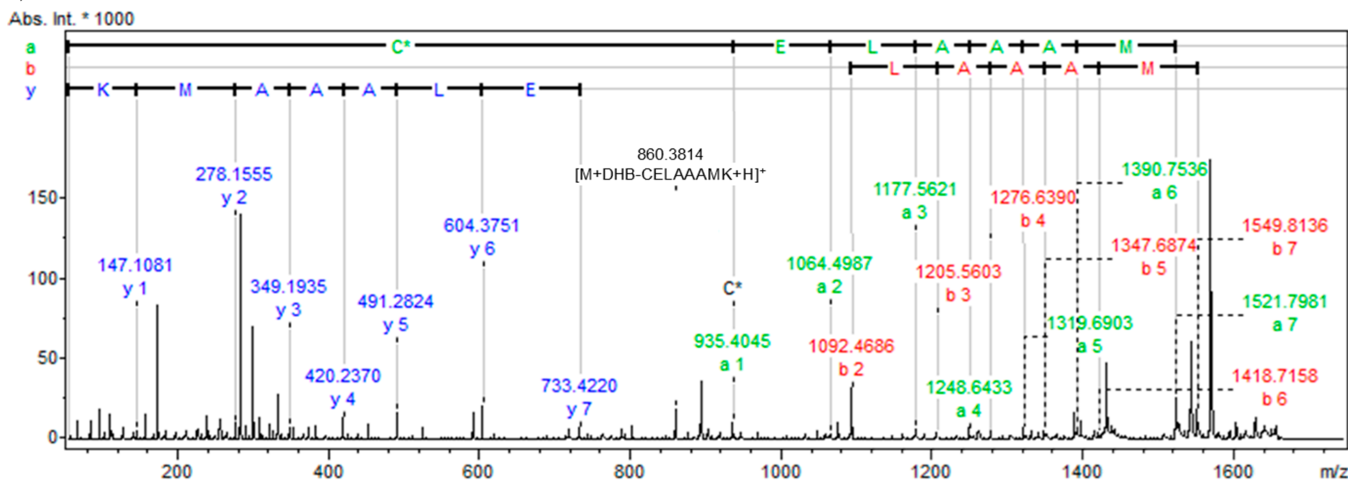


Figure 2. (a) MALDI-TOF-MS interpretation of BA-lysozyme digest. Peak corresponding to modified fragment was selected for MSMS. 2,5-Dihydrobenzoic acid (DHB) was used as a matrix. (b) Interpretation of BA modified fragment's MSMS spectrum. To simplify, only a, b, and y ion series for 6−13 fragment are annotated. Annotations for a-17, b-17 ion series and 126−128 fragment are included in the Supporting Information.

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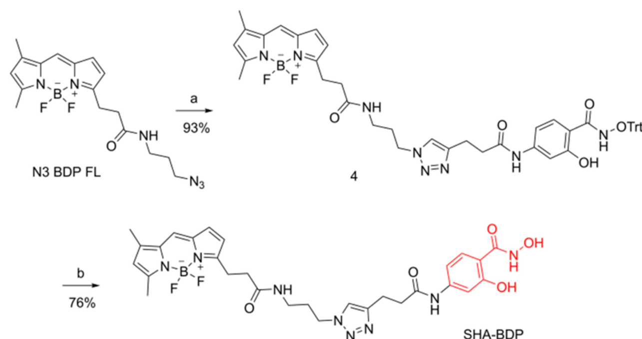
reduced electrostatic interactions by lysine residues. Therefore, BA-monofunctionalization by alkylation of surface accessible reduced disulfides, followed by rebridging, represents an attractive biorthogonal alternative that does not alter surface charges of peptides and proteins.

Reversible Functionalization of BA-Lysozyme. In the previous section, the weaker BA−diol interaction was exploited for purification of BA-lysozyme. However, for post-modification reactions, the formation of more stable bioconjugates would be highly desirable. Therefore, salicylhydroxamate (SHA) residues were selected for fast and efficient modification of BA-lysozyme. Bioconjugation proceeded by simply mixing the reagents in slightly alkaline aqueous buffer. BODIPY-FL (BDP-FL) was selected as fluorescent probe because of its high quantum yield and pH stability and the respective SHA-dye was prepared in a convenient two-step synthesis as depicted in Scheme 3.

Scheme 3. Synthesis of SHA-BDP[a]

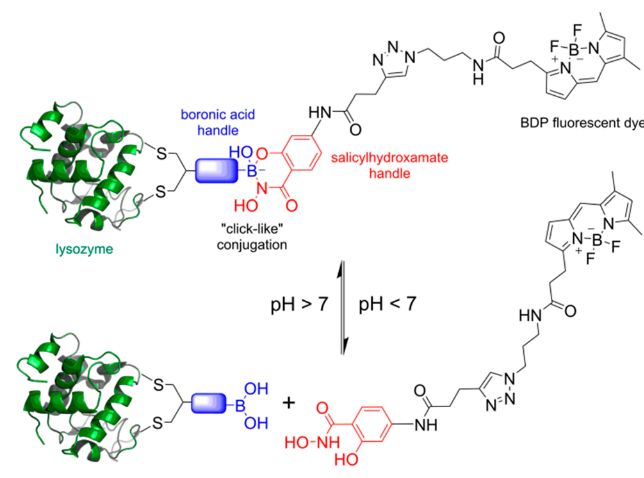


[a]a. $CuSO_4$, sodium ascorbate, DMSO, 93%; b. BF_3OEt_2, MeOH/CH_2Cl_2, 76%.

Commercially available BDP-FL azide was first conjugated to 4-azido-2-hydroxy-*N*-(trityloxy)benzamide using copper catalyzed azide−alkyne cycloaddition. Unprotected SHA derivatives are known to coordinate copper ions such as cytotoxic Cu(I).[48] Here, the trityl protection group ensures complete Cu(I) removal through HPLC purification. Detritylation proceeded under mild conditions with boron trifluoride diethyl etherate as Lewis acid[49] and the final product was purified by the HPLC yielding SHA-BDP in 71%. For characterization, ^{1}H NMR and HPLC-ESI-MS have been recorded, which are given in the Supporting Information.

Functionalization of BA-lysozyme with SHA-BDP and the stability of the obtained lysozyme-BDP dye conjugate (Scheme 4) were then studied using microscale thermophoresis (MST), which requires rigorous purification of the SHA-BDP dye for accurate assessment of the dissociation constant. SHA-BDP dye was titrated to BA-lysozyme and a dissociation constant K_d of about 8.0 ± 2.0 μM was obtained in phosphate buffer at high ionic strength (50 mM, pH = 8), which corresponds to previous findings.[50] BA-lysozyme binds and releases SHA-BDP in a dynamic, pH-controlled fashion. The dissolved bioconjugate rapidly responded to slight pH changes such as acidification to pH ∼6, and no binding of SHA-BDP was observed under these conditions (Figure 4). As negative control, native lysozyme was used to exclude any impact of nonspecific interactions between the reagents. In this way, we could demonstrate that the comparatively small BA tag preserved its binding capacity even when employed in a large biomolecule serving as biorthogonal anchor for post-modifications.

Scheme 4. Dynamic Covalent Bioconjugation of BA-Lysozyme with SHA-BDP and pH-Induced Cleavage of the Bioconjugate



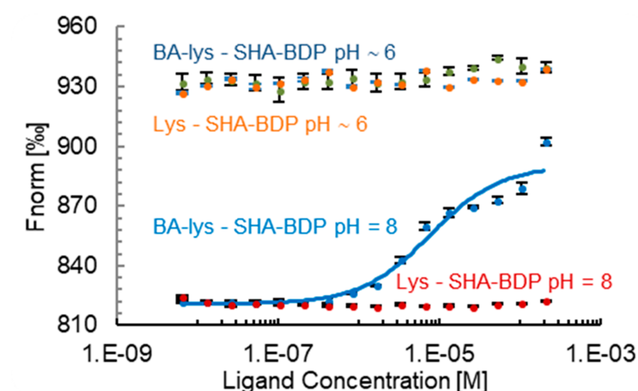


Figure 4. Microscale thermophoresis confirms assembly of BA-lysozyme with SHA-BDP (blue line) and release of SHA-BDP upon acidification (gray dots). No binding was detected for the control (red and green dots). Data are plotted as n = 3 ± SEM.

CONCLUSION

We introduce BA as a small, bioorthogonal tag that allows straightforward purification and functionalization of the model protein lysozyme. A novel allyl sulfone bioconjugation reagent with a single BA group (**BA-IC**) was prepared that modifies accessible disulfides in a site-directed fashion and imparts the new BA moiety. In this way, lysozyme was equipped with a single BA group that significantly changed the retention time allowing purification by affinity chromatography from carbohydrate-based resins. The new BA modification did not influence catalytic activity of the model protein lysozyme that usually is very sensitive to surface modifications. In the next step, the BA group on lysozyme served as reactive anchor for introducing an SHA-modified fluorescent BODYPY dye in a biorthogonal and dynamic covalent "click-like" conjugation reaction. The formed conjugate revealed a K_D of about 8.0 ± 2.0 μM at high phosphate buffer strength and remained stable in cell media.[24] However, after slightly reducing the pH to 6, instantaneous cleavage of the bioconjugate occurred.

In comparison to other available protein tags that were summarized by Kimple et al. recently,[14] the BA affinity tag

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provides comparable small size (0.4 kDa) as biotin (~0.2 kDa) and it is substantially smaller than the hexahistidine tag (~1.0 kDa). Small tag sizes are highly desirable for studying protein structure and dynamics by Förster resonance energy transfer (FRET) to minimize the influence of the tag on the protein.[51] Additionally, large tag dimensions can distort natural processes of the protein such as their trafficking or their conformational changes, which would limit their applications in FRET imaging.[52] Biotin as purification and bioconjugation tag usually requires the application of (strept)avidin that is bulky and its cleavage can be challenging without denaturation. In contrast, BA reacts with diols for purification and SHA groups for stable functionalization by forming covalent bonds that respond to slight pH changes within the physiological range. BA derivatives[53] further provide high biocompatibility, which represents an additional advantage for in vitro and in vivo usage compared to, e.g., toxicity arising from the metal or transition metal containing reagents complexing to the His-tag.

We envision that dynamic "tag and modify" markers are particularly attractive for drug delivery applications when drug molecules remain attached at neutral pH via stable covalent bonds but are released under the more acidic endosomal compartments inside cells. Therefore, such dynamic covalent interactions would also qualify for tag exchange capitalizing on the different stabilities of BA with catechol and SHA groups, which would be highly attractive for building dynamic protein architectures.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.8b00358.

Full experimental procedures and characterization data for new compounds (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

BA, boronic acid; BODIPY (BDP), boron-dipyrromethene; K_d, dissociation constant; SHA, salicylhydroxamate.

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