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Communication

One-Step Protein–Polymer Conjugates from Boronic-Acid-Functionalized Polymers

Michael J. Swierczynski and Zachary T. Ball*

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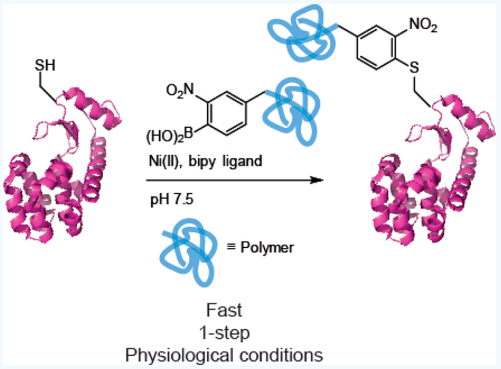
ABSTRACT: Polymer–protein conjugates are hybrid materials with interesting and useful properties. Methods to prepare diverse diblock materials of this sort often struggle to deal with the complexity and size of reagents, and so polymer–protein conjugation represents a stringent testing ground for nontraditional bioconjugation methods, such as metal-catalyzed arylation. This work demonstrates a simple Ni^{2+}-promoted arylation of cysteine residues with end-functionalized polymer–boronic acid reagents, and explores some molecular and physical properties possible in these hybrid structures.



Macromolecule–macromolecule conjugates, such as protein–polymer conjugates, are fascinating hybrid materials with unique properties, including stability to environmental stressors,[1,2] broad solubility profiles,[3,4] unique therapeutic potential,[5−8] and thermoresponsive behavior such as temperature-dependent micellization.[9−11] Synthetic methods to prepare these compounds include the use of prefunctionalized proteins for reaction with reactive proteins or polymerization from a protein macromonomer.[12,13] Direct polymer–protein conjugation of natural proteins is an alternative synthetic approach that limits the number of required chemical manipulations on protein reagents.[14,15] However, the low concentration of macromolecular reactive end-groups with a sea of functional groups[16] makes this a relatively daunting challenge.

Site-specific arylation promoted by transition metals has seen significant interest recently,[17] due to generally unique and potentially orthogonal reaction conditions, as well as the ability to create linkages that are electronically, structurally, and sterically different from those available with traditional reagents. Efforts to employ arylation methods in ever more complex environments are still in a nascent stage.[18,19] We recently described[20] a nickel-catalyzed cysteine arylation reaction with boronic acid reagents. The synthetic ease and efficiency of this process led us to imagine that boronic acid polymer end groups might serve as useful handles for direct attachment of polymers to cysteine groups. At the same time, this concept allows us to explore chemoselectivity questions in nickel catalysis in new contexts and to assess reaction efficiency for coupling pairs at quite low concentration. Herein, we report the adaption of boronic acid cysteine arylation chemistry for selective macromolecule–macromolecule bioconjugation.

RESULTS

Polymer–boronic acid reagents are readily prepared from suitably reactive boronic acid reagents and end-functionalized polymers. Poly(ethylene glycol) (PEG) was chosen as the initial polymer. A 5-kDa PEG-nitrophenylboronic acid (**1a**) was easily synthesized by coupling 4-carboxy-2-nitrophenylboronic acid with an amine-terminated 5-kDa PEG. The V131C mutant of T4 lysozyme (T4L) was chosen as a model protein, containing a single solvent-exposed cysteine.[21,22] T4L was treated with varying amounts of PEG–boronic acid **1a** under our previously optimized conditions with Ni^{2+} and a bipyridine-derived ligand **L1**.[20] In 30 min, a mass shift was observed by SDS-PAGE corresponding to mono-PEGylated T4L (Figure 1b). Similar experiments with a protein lacking a cysteine residue showed no reactivity.[20]

PEGylation was further confirmed by barium iodide staining, which selectively stains for PEG,[23] and size exclusion chromatography (Figure 1b,c). Conjugation occurred with as little as 2.5 equiv of polymer–boronic acid, and nearly full conversion (>90%) upon increasing the amount of boronic acid (Figure 1b, lanes 1–3).

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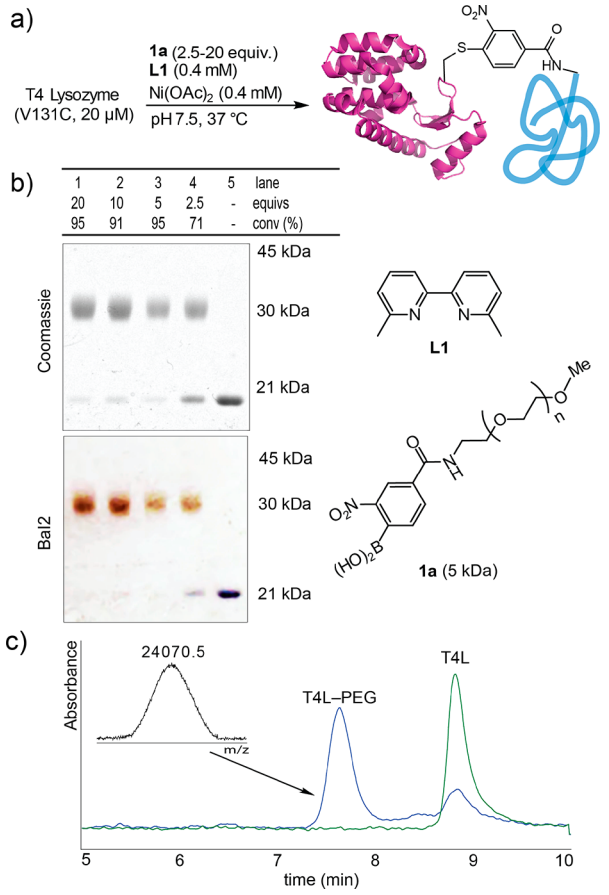

Figure 1. (a) Modification of T4L with **1a**. (b) Left: Coomassie (top) and BaI_2 (bottom) stained gels of T4L modification with varying equiv of **1a**. Conditions: T4L (20 μM), TCEP (0.2 mM), **1a** (50−400 μM), **L1** (0.4 mM), $Ni(OAc)_2$ (0.4 mM) in NMM buffer (50 mM, pH 7.5) at 37 °C for 30 min. Conversion measured by gel densitometric analysis (ImageJ). Right: Structures of PEG 5k-nitrophenylboronic acid **1a** and 6,6′-dimethyl-2,2′-bipyridine (**L1**) ligand. (c) SEC analysis of T4L (green) and PEG−T4L (blue). PEG−T4L verified by MALDI-MS (inset).

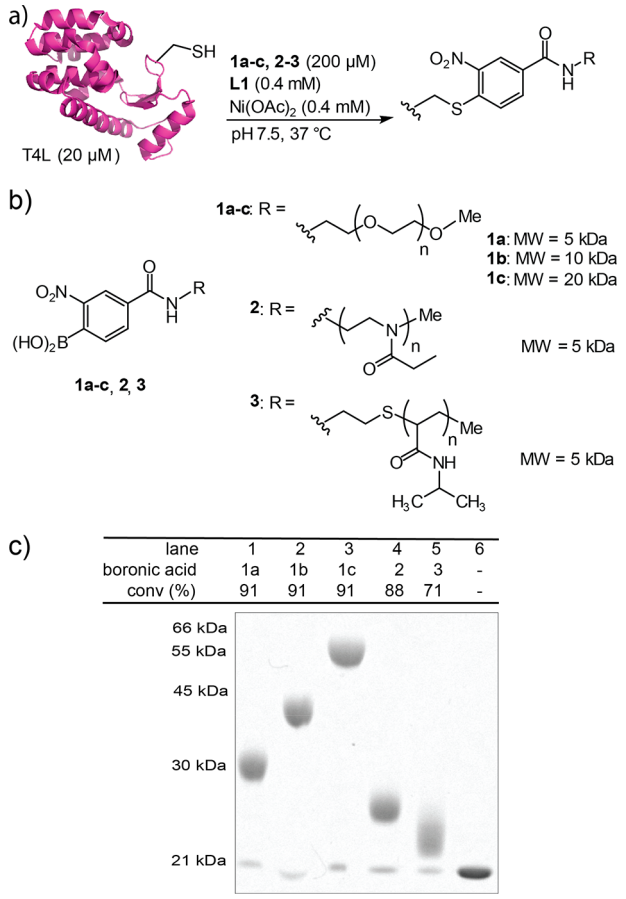

Figure 2. Modification of T4L. (a) Scheme of T4L modification with boronic acids. (b) Structures of PEG (**1a−c**), PEOZ (**2**), and PNIPAM (**3**) nitrophenylboronic acids. (c) Gel image of T4L polymer modifications. Conditions: T4L (20 μM), TCEP (0.2 mM), boronic acid (200 μM, 400 μM for **3**), **L1** (0.4 mM, 0.8 mM for **3**), and $Ni(OAc)_2$ (0.4 mM, 0.8 mM for **3**) in NMM buffer (50 mM, pH 7.5) at 37 °C for 30 min. Conversion measured by gel densitometric analysis (ImageJ).

Encouraged by the successful conjugation of a 5-kDa PEG, PEG-nitrophenylboronic acids with increasing molecular weights were synthesized bearing 10-kDa (**1b**) and 20-kDa (**1c**) PEG chains. To test our chemistry beyond the conjugation of PEG, we also synthesized boronic acid-functionalized variants of other useful biocompatible polymers: poly(2-ethyl-2-oxazoline) (PEOZ, **2**) and poly(*N*-isopropylacrylamide) (PNIPAM, **3**). T4L was reacted with polymer−boronic acids **1a−c** and **2−3** under the optimized conditions. Excellent conversion was achieved with all polymer−boronic acids except PNIPAM **3** (Figure 2c, lanes 1−4). PNIPAM is a thermoresponsive polymer and becomes insoluble at elevated temperatures. With this in mind, lowering the temperature (to 30 °C from 37 °C) afforded conditions for efficient production of a T4L−PNIPAM conjugate as well (Figure 2c, lane 5).

The conjugation was further explored on other proteins to establish the scope of the catalytic process. The S147C mutant of superfolder green fluorescent protein (sfGFP), containing a single, solvent-exposed cysteine,[22] was chosen as the next protein. Under conditions previously developed, the polymer−boronic acids **1a−c** and **2−3** all reacted efficiently with sfGFP to provide arylated cysteine conjugates (Figure 3a). Conjugation was also tested on the CAL PDZ[24,25] domain (CALP). Unlike previous proteins studied, polymer conjugation with CALP was sluggish under our initial conditions, possibly reflecting limited solvent accessibility of the natural CALP cysteine residue. This limitation is commonly observed in cysteine conjugation chemistries, and can be overcome under denaturing conditions.[26−28] In the case at hand, urea was found to be completely compatible with the nickel-catalyzed coupling, and protein conjugates were readily produced under these conditions (Figure 3b). We were pleased to observe that nickel-catalyzed polymer conjugation was also observed with CALP bearing a His-tag, a common purification tag that strongly binds to nickel ions (Figures 3b and S3).

Other polymer architectures proved amenable to this approach. Symmetric bis-boronic acid polymers, such as **4** (Figure 4) would allow direct access to synthetic protein dimers with PEG linkers, analogous to ABA-type triblock copolymers.[29−31] Linked protein homodimerization is a useful approach for enhanced structural properties and greater biological functionality.[32−35] A 2:1 ratio of protein and polymer **4** was employed to further assess reaction efficacy. At protein concentrations of 100 μM, the PEG-linked dimer was the major product observed within 1 h with both T4L and

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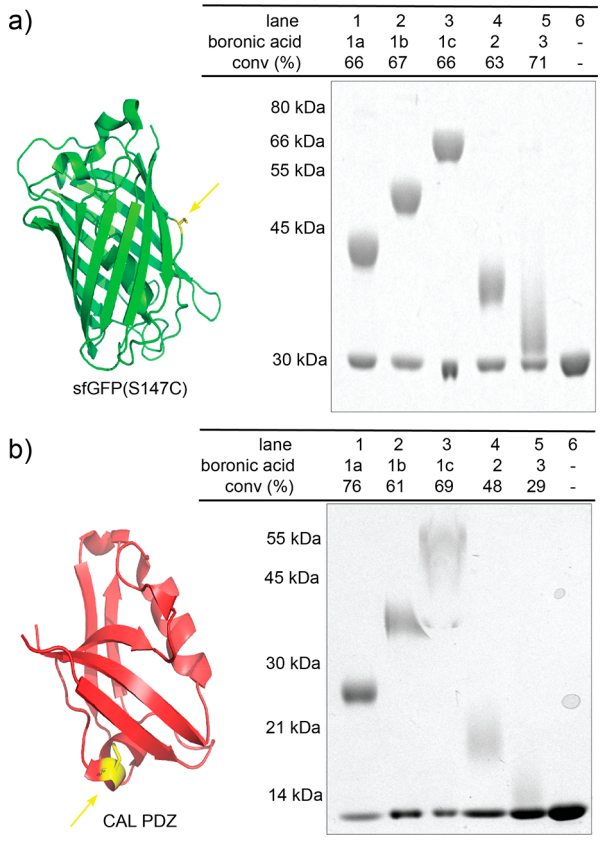

Figure 3. (a) Modification of sfGFP(S147C). (b) Modification of CAL PDZ domain (CALP). Free cysteines denoted by yellow arrow. Conditions: proteins (20 μM), TCEP (0.2 mM), boronic acid (200 μM, 400 μM for **3**), **L1** (0.4 mM, 0.8 mM for **3**), $Ni(OAc)_2$ (0.4 mM, 0.8 mM for **3**) in NMM buffer (50 mM, pH 7.5) at 37 °C for 30 min. Buffer contained 8 M urea for CALP. Conversion measured by gel densitometric analysis using ImageJ.

sfGFP, along with minor amounts of incomplete single-conjugation (Figure 4b,c "Mono").

Protein−polymer conjugation is a convenient way to engineer chemical and physical properties in a predictable way. PNIPAM exhibits a lower critical solution temperature (LCST) phase transition, which can be exploited for responsive materials with properties such as thermoresponsive micellization.[36−38] Differential scanning calorimetry (DSC) confirmed a clean phase transition for the diblock T4L−PNIPAM around 55 °C in buffer. Dynamic light scattering (DLS) also indicated a desolvation-driven aggregation event around 55 °C, with a significant increase in particle size. At low temperatures, a hydrodynamic radius of 7 nm was observed, consistent with monomeric T4L−PNIPAM. Above the phase transition, aggregates of average size 65 nm were observed, consistent with other reports of PNIPAM conjugates.[37,39] The diblock transition occurs ∼20 °C higher than that of the PNIPAM homopolymer (Figure S8).

Protein−polymer conjugates can also engender solubility in nonaqueous environments. Several enzymes have shown increased solubility and stability in organic solvents upon multisite PEGylation.[3] The diblock sfGFP−polymer conjugates were tested for enhanced solubility in acetonitrile. Unconjugated sfGFP was completely insoluble in acetonitrile, while both sfGFP−PNIPAM and sgGFP−PEOZ were soluble (Figure 5c).

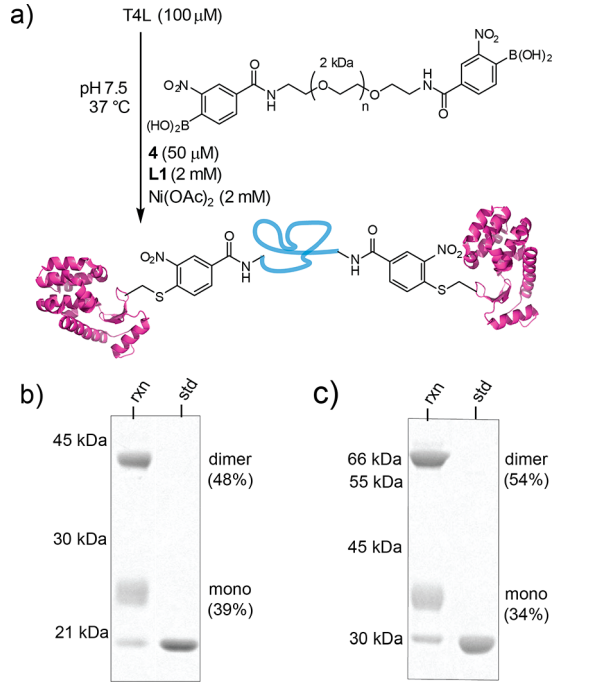

Figure 4. Dimerization of proteins with bis-boronic acid **4**. (a) Scheme of homodimerization of T4L. Gel images of homodimerization of T4L (b) and sfGFP (c). Conditions: proteins (100 μM), TCEP (1 mM), **4** (50 μM), **L1** (2 mM), and $Ni(OAc)_2$ (2 mM) in NMM buffer (50 mM, pH 7.5) at 37 °C for 1 h. Mono represents protein that did not dimerize. Conversion measured by gel densitometric analysis (ImageJ).

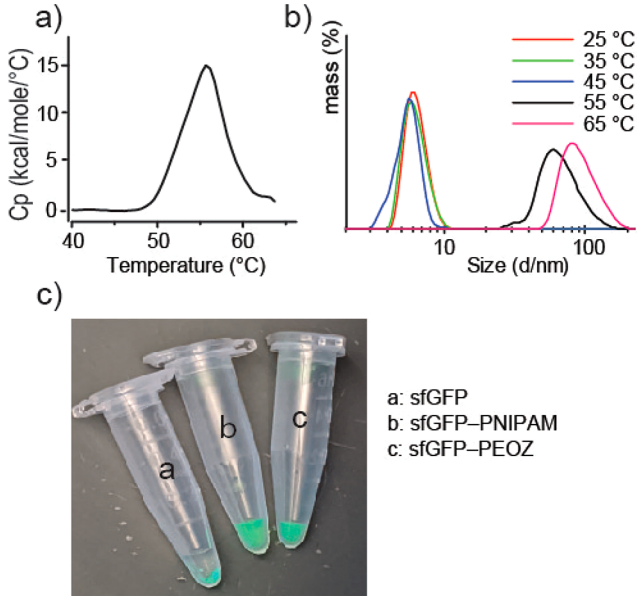

Figure 5. Analysis of polymer−protein conjugates. (a) DLS analysis of T4L−PNIPAM at various temperatures. (b) DSC analysis of PNIPAM-BOH and T4L−PNIPAM. (c) sfGFP solubility in acetonitrile. a: sfGFP, b: sfGFP−PNIPAM, c: sfGFP−PEOZ.

The chemistry presented here is a simple, selective method of creating site-selective polymer−protein conjugates and demonstrates a remarkably efficient catalytic process for Chan-Lam type coupling in water with two reagents at biologically relevant low concentrations. The chemoselectivity observed is all the more remarkable given that copper-

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Bioconjugate Chem. 2020, 31, 2494−2498

catalyzed hydroxylation of boronic acids with water typically occurs under mild conditions,[40] but is effectively out-competed here by thiol reactivity at concentrations roughly 6 orders of magnitude lower than that of the aqueous solvent. Polymer−boronic acids are easily prepared with a variety of polymers, and emergent hybrid properties can be demonstrated with these materials.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.bioconjchem.0c00516.

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

Author

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

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.bioconjchem.0c00516

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

NMM, *N*-methylmorpholine; T4L, T4 lysozyme; PEG, poly(ethylene glycol); PEOZ, poly(2-ethyl-2-oxazoline); PNIPAM, poly(*N*-isopropylacrylamide)

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