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Article

Boronic Acid Resin for Selective Immobilization of Canonically Encoded Proteins

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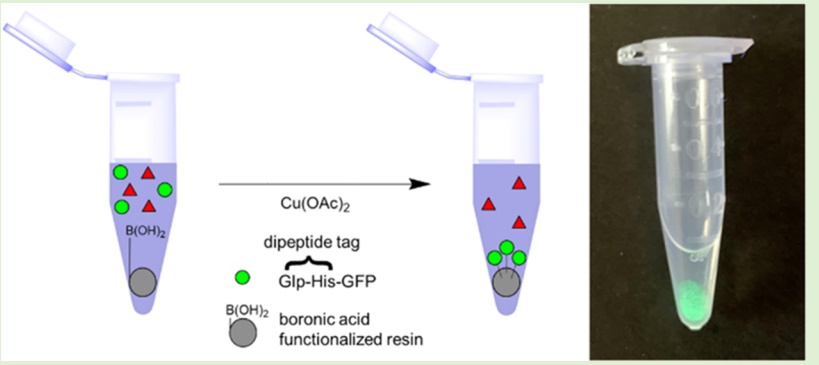

ABSTRACT: The use of transition-metal-mediated boronic acid chemistry presents a novel method of protein immobilization on a solid support. This is a one-step method that site-selectively immobilizes pyroglutamate-histidine (pGH)-tagged proteins. Herein, we describe the synthesis of alkenylboronic acid-functionalized poly(ethylene glycol) acrylamide (PEGA) resin and its subsequent reactions with pGH-tagged proteins to produce covalent linkages. The selectivity of immobilization is demonstrated within fluorescent studies, model mixtures, and lysates.

INTRODUCTION

Complex hybrid biomaterials combine the properties of biomacromolecules, such as proteins, with particles, polymers, or other nano-objects, producing new materials and structures with unique attributes and capabilities.[1,2] These materials find diverse uses in molecular sensing,[3,4] drug delivery,[5,6] separations science,[7,8] and surface coating and biocatalysis,[9] among many others. In this paper, we describe a boronic acid-functionalized resin that enables site-specific immobilization of individual proteins from complex mixtures based on a simple, canonically encoded dipeptide tag.

A variety of methods have been pursued for the construction of hybrid biomaterial nanostructures, and the requirements of diverse applications necessitate an array of complementary methods.[10] However, workflow and characterization can be challenging, requiring, in some cases, protein purification before a "primary" bioconjugation to incorporate a unique reactive site for later secondary bioconjugation with a nano-object.[11] Covalent immobilization strategies most commonly utilize nonspecific linkages to one side-chain type, such as lysine acylation,[12] which creates a random ensemble of orientations and linkage topologies and which, in turn, results in an ensemble of protein functional abilities.[13−15] Several different residue-selective methods have been pursued,[16−18] and cysteine, in particular, has advantages as a target of immobilization, as a rare residue with unique reactivity.[19]

Site-specific immobilization approaches aim to improve the homogeneity of immobilized protein molecules and to provide improved predictability over protein function. Significant efforts have developed a variety of interesting strategies for site-specific immobilization.[13−15] The N-terminal amine can function as a unique handle for immobilization[20] or unique handles have been incorporated via a separate primary bioconjugation reaction by chemical or enzymatic means prior to a subsequent immobilization step (Figure 1a,b).[11]

An ideal immobilization method would allow site- and protein-selective immobilization directly from complex mixtures, such as cell lysate. Toward that end, unnatural amino acids (UAAs) can be incorporated by amber codon suppression (Figure 1c);[21] alternatively, natural "tag" sequences or domains, such as hexa-histidine[23] or the spycatcher domain (Figure 1d),[22] can be engineered to provide unique reactive sites. However, challenges remain to develop robust, site-specific methods for the immobilization of individual

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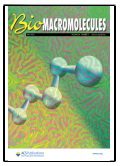

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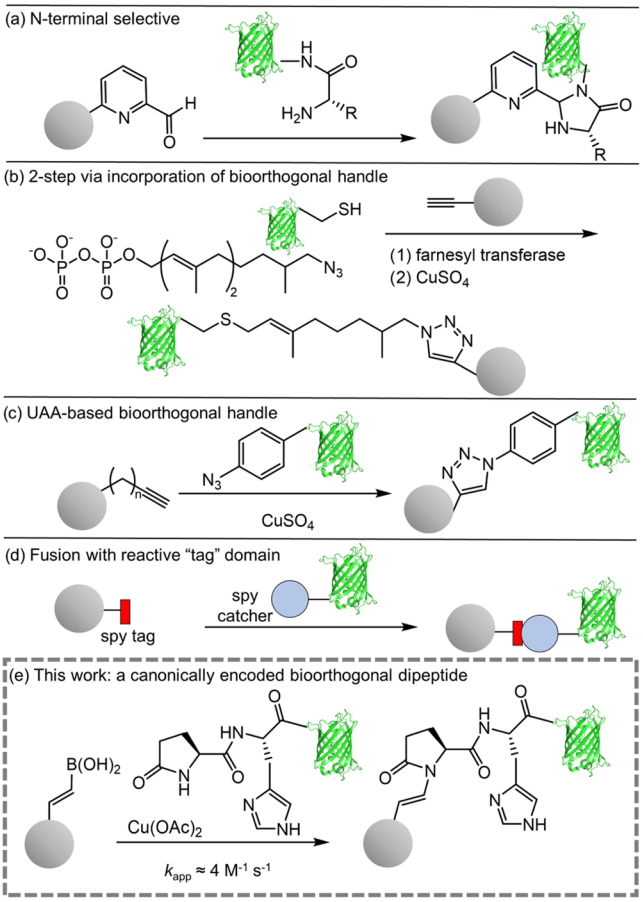

Figure 1. (a–d) Prior art aimed at immobilization involving N-terminal imidazolidinones (a),[20] farnesyltransferase (b),[11] unnatural amino acids (UAAs) (c),[21] and reactive domains (d).[22] (e) This work employs a pyroglutamate-histidine dipeptide tag.

proteins from complex mixtures, such as lysate, that allow general and scalable protein production and immobilization from a variety of cell/organism types based on reactive handles that are minimally disruptive to protein size, structure, and sequence.

EXPERIMENTAL SECTION

Materials. The chemicals were purchased from commercial suppliers and used without further purification. *N*-Methylmorpholine (NMM) buffer was prepared by adding NMM (TCI, M0370) to Milli-Q water and adjusting the pH with aq HCl (1 M) or aq NaOH (1 M). Gel imaging was performed on a Fujifilm LAS-4000 imager. Coomassie-stained gels were imaged with a differential in-gel analysis (DIA) light source. NMR experiments were conducted on a Bruker AVANCE 600 Spectrometer. Fluorescence intensity scans were performed with a TECAN Infinite 200 Plate Reader. Fourier transform infrared (FT-IR) spectra were performed with a Thermo Nicolet iS5. Extracted ion chromatogram (EIC) traces were performed with an Agilent MSD XT Single-Q LC–MS. The design and construction of pGH-tagged protein vectors along with purification from *Escherichia coli* cell lysate follow procedures from Ohata et al.[24]

Procedure for Synthesis of Resin 2. To a spin-column (1.2 mL bed volume, Bio-Rad) was added resin **1** (0.7 g wet mass). The resin was washed with dimethylformamide (DMF) (3 × 1 mL). Glutaric anhydride (0.1 g, 1.0 mmol) and 4-dimethylaminopyridine (20 mg, 0.16 mmol) were dissolved in DMF/pyridine (1:1, 1 mL) and the solution was transferred to the spin-column. The vessel was placed on a nutating shaker at rt for 18 h. The resin was washed with DMF (3 × 1 mL) and water (3 × 1 mL). The resin was then acidified with aq HCl (2 M) for 1 h by mixing on a nutating shaker. The resin was then washed with water (3 × 1 mL) and DMF (3 × 1 mL). The washed resin was stored in DMF at 4 °C and tested with ninhydrin. The resin was also characterized by FT-IR. This preparation was adapted from a previous report, and characterization data was consistent with the previous report.[20]

Procedure for Synthesis of Resin 3. To the spin-column containing resin **2** (0.70 g wet mass) were added 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (0.10 g, 0.50 mmol) and 4-dimethylaminopyridine (DMAP) (3.0 mg, 0.025 mmol) in DMF/Et_3N (1:1, 0.4 mL). The cysteine-derived alkenylboronic acid (0.04 mmol) was dissolved in DMF/Et_3N (1:1, 0.1 mL) and added to the reaction container. The reaction vessel was mixed with a nutating shaker at rt for 18 h. The resulting mixture was washed with DMF (3 × 1 mL) and CH_2Cl_2 (3 × 1 mL) and then again with DMF by gravity filtration before storing at 4 °C. The resin was subsequently tested with alizarin to indicate the presence of boronic acid based on staining.

General Procedure of Immobilization of Tagged Protein. To an Eppendorf tube was added boronic acid-functionalized resin (5 mg wet mass, stored in DMF). The resin was washed with NMM buffer (3 × 100 μL, 50 mM, pH 8.0, 0.5% Triton). Then, a 25 μL mixture of pGH-tagged protein(s) (10 μM) and $Cu(OAc)_2$ (100 μM) in NMM buffer was added to the resin and incubated at rt on a rotating wheel for 18 h. The resin was then subjected to washes (3 × 100 μL water, 1 M NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.2% Triton). Finally, the resin was analyzed by the fluorescence intensity scan to confirm attachment of pGH-tagged fluorescent peptide/protein to the resin **3**.

Procedure for Trypsin Digest of Immobilized ^{tag}sdAb–GST. To an Eppendorf tube was added boronic acid-functionalized resin (5 mg wet mass, stored in DMF). The resin was washed with NMM buffer (3 × 100 μL, 50 mM, pH 8.0, 0.5% Triton). Then, a 25 μL mixture of ^{tag}sdAb–GST (10 μM) and $Cu(OAc)_2$ (200 μM) in NMM buffer was added to the resin and incubated on a rotating wheel at rt for 18 h. The resin was then subjected to washes (3 × 100 μL water, 1 M NaCl, 1 mM EDTA, 0.2% Triton). Next, the resin was incubated with 100 μL of denaturing buffer (8M urea, 50 mM tris, 5 mM dithiothreitol (DTT), pH 8) on a rotating wheel at rt for 1 h. Then, iodoacetamide was added (15 mM), reactions were covered, and samples were placed on a rotating wheel at rt for 30 min. Samples were then diluted with tris-base (300 μL, pH 8), and 1 μg of trypsin was added. The samples were placed in a shaking incubator at 37 °C overnight. The same protocol was performed using free pGH-VHH-GST in the same concentrations. Samples were analyzed by liquid chromatography–mass spectrometry (LC–MS).

RESULTS AND DISCUSSION

Transition-metal catalysis provides new tools for the spatiotemporal control of reactivity in complex biological systems and thus has emerged as a complement to traditional organic reactivity for bioconjugation with natural protein sequences. As part of a program aimed at exploring selectivity questions in transition-metal catalysis in biological environments, we reported remarkably selective cross-coupling of pyroglutamate-histidine dipeptide sequences with boronic acid reagents.[24] Solution-phase studies demonstrated selective bioconjugation of the pyroglutamate-histidine dipeptide sequence in proteins with an apparent second-order rate constant, $k_{app} \approx 4\ M^{-1}\ s^{-1}$.[24] Product structures were established by isolation and characterization of a peptide substrate.[25] The reaction tolerates all canonical amino acids and exhibited a half-life ($t_{1/2}$) <5 min under typical reaction conditions.[24] Pyroglutamate-histidine sequences can be viewed as minimalist tags, encoded by natural machinery[26] via coexpression of a glutaminyl cyclase and exhibiting bio-

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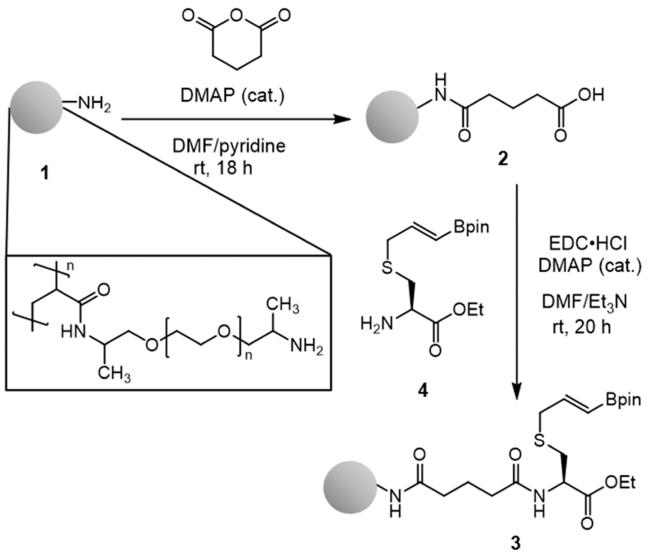
Scheme 1. Synthesis of PEGA−$B(OH)_2$ Resin



orthogonal reactivity,[27−29] yet with a tiny size that is minimally disruptive of natural function.[24] The uniquely selective reactivity of this dipeptide sequence, together with the biocompatibility and simple preparation of boronic acid-containing materials, led us to consider developing a strategy for boronic acid-based site-selective immobilization of a protein of interest from protein mixtures in a single step.

We constructed a boronic acid-functionalized resin using two synthetic steps from an amine-terminated poly(ethylene glycol) acrylamide (PEGA) resin-scaffold. This resin consists of a poly(ethylene glycol) acrylamide copolymer terminated with primary alkylamines. Diimide coupling with boronic acid−amine **4** afforded the boronic acid-functionalized resin after the initial reaction with glutaric anhydride (Scheme 1). Resin functionalization was assessed by FT-IR, ninhydrin test, and an alizarin test for boronic acid functionality (see the Supporting Information (SI) for details).[20,30,31]

Table 1. Selected Examples of Variation of Conditions for ^{tag}GFP Immobilization

entry	change from std.	rel. immob. % efficiency
1[a]		100
2	50 μM Cu^{2+}	58
3	1 h	64
4	no surfactant	58
5	no surfactant, pH 7.4	60

[a]Std. condns: 50 mM NMM, 0.5% Triton, 10% glycerol, 100 μM $Cu(OAc)_2$, pH 8.0 for 20 h at rt.

To assess copper-catalyzed covalent immobilization of pGH-tagged structures onto boronic acid-functionalized resin **3**, we employed a fluorescence assay to assess the reactivity and selectivity of a mixture of two fluorescent species. Mixtures of cyan fluorescent protein (CFP) and a fluorescein isothiocyanate (FITC)-labeled pGH-containing peptide (^{tag}FITC) were mixed with resin in NMM buffer in the presence of $Cu(OAc)_2$ (100 μM). The resin was then filtered and washed. The modified resin exhibited a strong emission at 517 nm, attributed to the FITC-labeled peptide, but no emission for

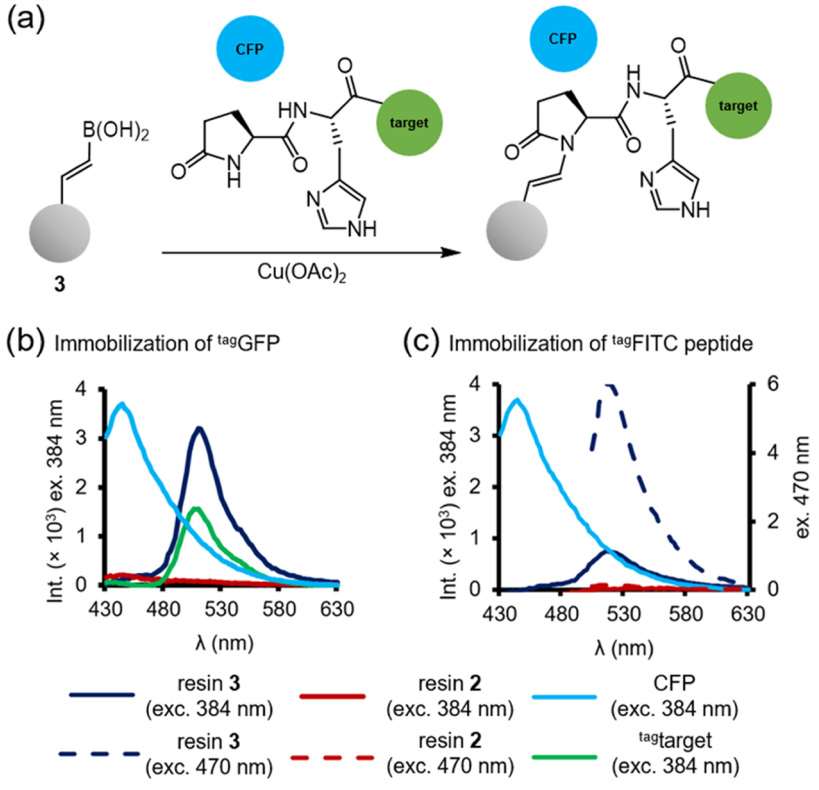


Figure 2. (a) Immobilization of pGH-tagged target protein/peptide with resin **3** in the presence of CFP. Reaction conditions: ^{tag}target (10 μM), $Cu(OAc)_2$ (100 μM), NaCl (100 mM), and 5 mg PEGA resin (wet mass) in 50 mM NMM, 0.5% Triton, pH 8.0 for 20 h at rt (25 μL total volume). (b, c) Fluorescence after reaction of resin **3**, resin **2** (negative control), and CFP at 384 nm ex. with (b) ^{tag}GFP (c) and ^{tag}FITC as the target.

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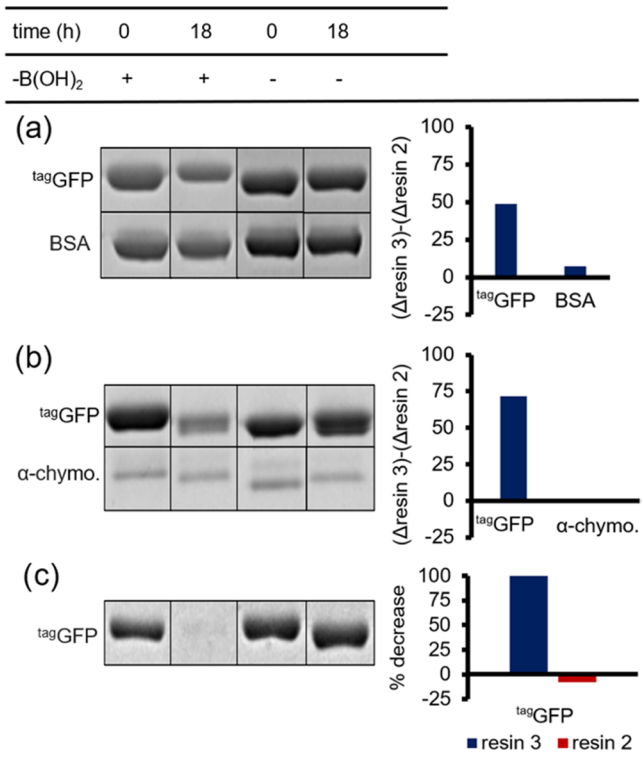

Figure 3. Immobilization of (a) ^{tag}GFP in the presence of BSA, (b) ^{tag}GFP in the presence of α-chymotrypsinogen, (c) ^{tag}GFP in dilute lysate. Coomassie Brilliant Blue (CBB) stain of ^{tag}GFP and untagged proteins. Reaction conditions: (a, b) ^{tag}GFP (10 μM), untagged protein (10 μM), $Cu(OAc)_2$ (100 μM), NaCl (100 mM), and 5 mg resin **3** (wet mass) in 50 mM NMM, 0.5% Triton, pH 8.0 for 20 h at rt (25 μL total volume). (c) ^{tag}GFP (10 μM), $Cu(OAc)_2$ (100 μM), and 5 mg resin **3** (wet mass) in dilute lysate for 20 h at rt (25 μL total volume). Protein % decrease is determined by band densitometric analysis with ImageJ software.

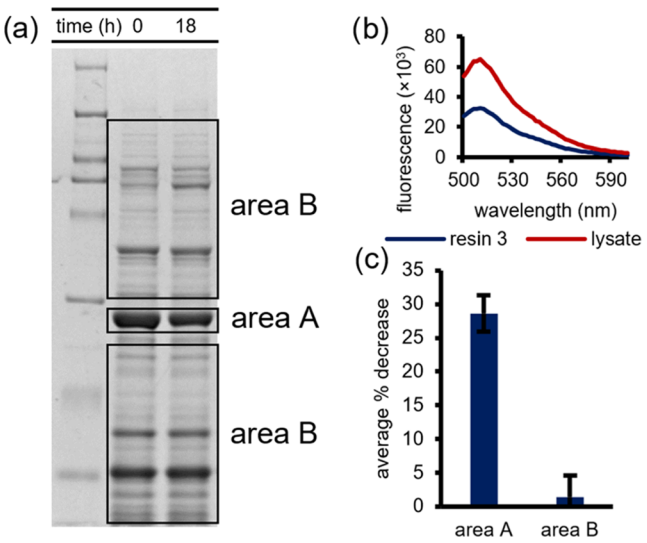

Figure 4. (a) SDS-PAGE gel analysis of soluble protein levels during immobilization of ^{tag}GFP in lysate. (b) Fluorescence of lysate and resin **3** after immobilization in lysate, indicating ^{tag}GFP fractionation onto resin. (c) Quantification of a decrease in soluble protein level for ^{tag}GFP (area A) and for other lysate proteins (area B). Data represents an average of three replicates.

CFP (446 nm), consistent with the selective pull-down of the pGH-labeled peptide. Fluorescence quantification at an excitation of 470 nm revealed immobilization of 0.044 nmol·mg^{-1} of resin (89% yield) of pGH-labeled green fluorescent protein (^{tag}GFP), consistent with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, indicating complete disappearance of ^{tag}GFP from solution (see the SI). Control experiments with the carboxylate resin **2** showed no incorporation of any fluorescence under the reaction conditions.

Similar results were obtained with mixtures of two fluorescent proteins, CFP and ^{tag}GFP: after the reaction and washing, the resin exhibited a strong GFP emission at 510 nm, without any emission signal attributable to CFP (Figure 2). It is noteworthy that we used His_6-tagged CFP in these studies and found that the tag, known to interact strongly with metals, is neither itself modified nor does the His_6-tag affect immobilization of ^{tag}GFP. Immobilization efficiency was moderately tolerant to variations in the immobilization conditions, as judged by the fluorescent emission of the product resin (Table 1). Reactions run for 1 h achieved 64% of the immobilization observed over 20 h (entry 3). Without a 0.5% triton surfactant, similar drops in efficiency were observed (entry 4), while pH 8.0 or 7.4 was similarly effective (entries 4 and 5).

We next examined immobilization efficacy and selectivity in more complex mixtures. Immobilization of the ^{tag}FITC peptide directly from*E. coli* lysate gave results similar to the competition experiments with CFP (see the SI). Encouraged by these results, we examined immobilization of ^{tag}GFP in the presence of several other proteins. Reaction efficacy was monitored by fluorescence of the resin product and by SDS-PAGE analysis of the unreacted filtrate. Fluorescence analysis again demonstrated immobilization of the ^{tag}GFP, while SDS-PAGE analysis could be used to quantify the immobilization. Protein immobilization, determined by SDS-PAGE, was 66% for ^{tag}GFP in the presence of α-chymotrypsinogen and 47% in the presence of bovine serum albumin (BSA) (Figure 3), and this immobilization was confirmed by fluorescence analysis of the product resin (see the SI). In both cases, levels of the control protein competitor in solution were unchanged relative to control.

Immobilization of ^{tag}GFP from whole lysate was also explored. Again, the characterization of reaction efficiency was assessed by fluorescence and SDS-PAGE analysis. Quantification of SDS-PAGE indicated a 29% pull-down efficiency of ^{tag}GFP, with no net change in other protein levels. Fluorescence analysis of lysate and resin confirmed similar levels of immobilized protein (Figure 4). The selectivity of immobilization in lysate suggests that pGH tag is a practical tool for immobilization in demanding and complex polyfunctional environments.

Expanding beyond fluorescent protein targets, we engineered and produced a pGH-tagged camelid single-domain antibody (^{tag}sdAb) as a fusion with GST. The ^{tag}sdAb−GST fusion was immobilized with resin **3** using standard conditions. On-resin digestion and LC−MS analysis allowed a measure of immobilization efficiency (Figure 5a,b). Quantification of peptide fragment peaks indicated immobilization and digestion of 0.032 nmol·mg^{-1} (64%) based on the released fragments of ^{tag}sdAb−GST by trypsin digest. Control experiments with carboxylate resin **2** yielded no peptide fragments upon trypsin treatment. The activity of the immobilized ^{tag}sdAb−GST was

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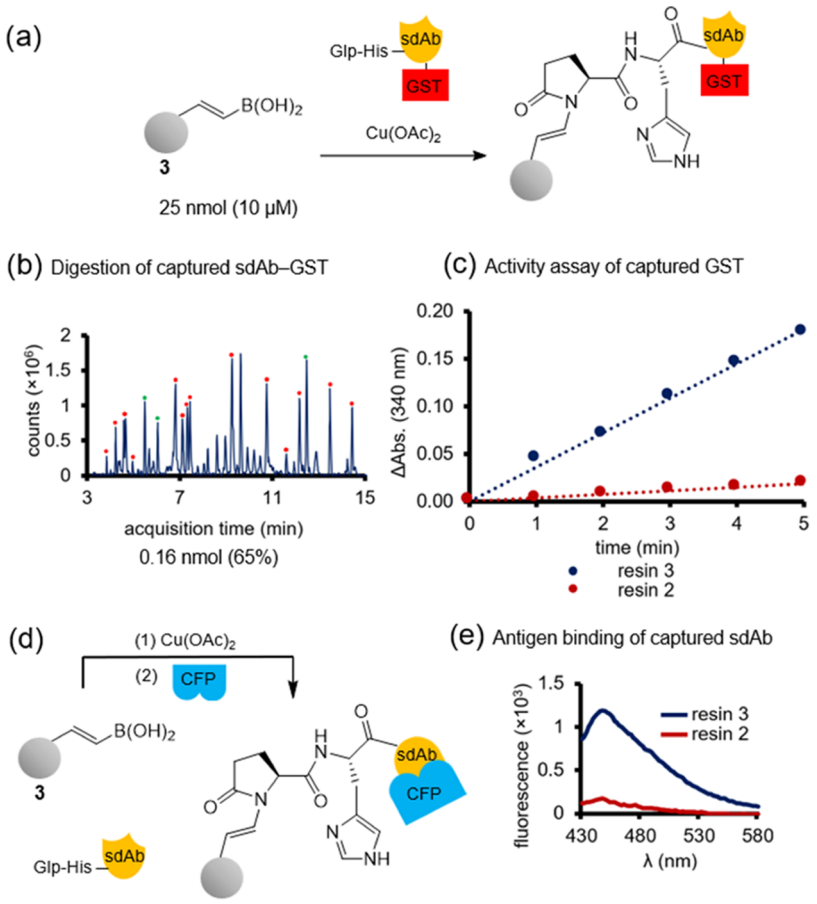

Figure 5. (a) Immobilization of ^{tag}sdAb−GST followed by (b) on-resin trypsin digestion and LC−MS analysis. Peaks in chromatograms labeled with a dot indicate the expected trypsin fragments. Green dots indicate peaks used for quantification. (c) Change in absorbance at 340 nm from the GST activity assay for resins **2** and **3** after being subjected to immobilization conditions and washed. (d) Immobilization of ^{tag}sdAb and subsequent immobilization of CFP by antigenic interaction with ^{tag}sdAb attached to PEGA. (e) Fluorescence of resins **2** and **3** after immobilization of ^{tag}sdAb and incubation with CFP.

determined with a GST activity assay (Sigma-Aldrich, CS0410) (Figure 5c). It was found that after immobilization and washing, resin **3** activity was 0.27 ± 0.02 μmol·min^{-1}·mg^{-1}. The control process with carboxylate resin **2** produced resin with no detectable activity. The immobilized fusionless ^{tag}sdAb protein also maintained its antigen-binding function (Figure 5d,e). Incubation of the antibody−resin with CFP resulted in antigen binding, as observed by fluorescence analysis of the resin. Control experiments with carboxylate resin **2** exhibited minimal antigen fluorescence. Nanobodies have limited stability with respect to denaturation, and the success of an antigen-binding assay demonstrates that the immobilized proteins can retain natural function.

CONCLUSIONS

A protein immobilization method has been developed based on a boronic acid-functionalized resin material. The pGH tag provides a minimalist alternative to other tagging approaches that is readily incorporated with natural machinery with high efficiency. The ability to produce bioconjugate materials in a single step from as-expressed canonical proteins, without the need for purification, is a significant advantage. Relative to enzymatic bioconjugation approaches, the use of a simple metal salt catalyst has advantages in cost, handling, and tolerance of diverse reaction conditions. At the same time, this work demonstrates remarkable selectivity and efficiency of a transition-metal-catalyzed coupling reaction of a natural pyroglutamate posttranslational modification. The reaction succeeds between two large and complex macromolecular structures within a diverse and complex mixture. The reactivity explored here should be amenable to the preparation of a variety of complex bioconjugate materials. Future challenges include the extension of this reactivity to the immobilization of proteins from higher organisms, including glycosylated proteins and/or immobilization of proteins at lower endogenous expression levels.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.biomac.3c00096.

Synthesis and characterization of organic compounds, experimental details of protein expression and purification, LC−MS data, fluorescence measurements, and SDS-PAGE analysis of protein immobilization experiments (PDF)

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Notes

The authors declare no competing financial interest.

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