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Tyrosinase-Mediated Oxidative Coupling of Tyrosine Tags on Peptides and Proteins

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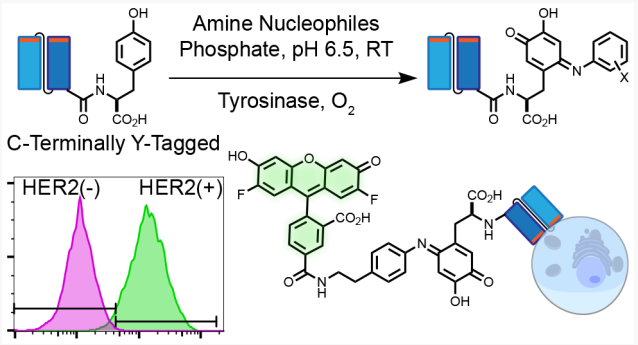
ABSTRACT: Oxidative coupling (OC) through *o*-quinone intermediates has been established as an efficient and site-selective way to modify protein N-termini and the unnatural amino acid *p*-aminophenylalanine (*p*aF). Recently, we reported that the tyrosinase-mediated oxidation of phenol-tagged cargo molecules is a particularly convenient method of generating *o*-quinones in situ. The coupling partners can be easily prepared and stored, the reaction takes place under mild conditions (phosphate buffer, pH 6.5, 4 to 23 °C), and dissolved oxygen is the only oxidant required. Here, we show an important extension of this chemistry for the activation of tyrosine residues that project into solution from the N or C-termini of peptide and protein substrates. Generating the *o*-quinone electrophiles from tyrosine allows greater flexibility in choosing the nucleophilic coupling partner and expands the scope of the reaction to include C-terminal positions. We also introduce a new bacterial tyrosinase enzyme that shows improved activation for some tyrosine substrates. The efficacy of several secondary amines and aniline derivatives was evaluated in the coupling reactions, providing important information for coupling partner design. This strategy was used to modify the C-termini of an antibody scFv construct and of Protein L, a human IgG kappa light chain binding protein. The use of the modified proteins as immunolabeling agents was also demonstrated.



INTRODUCTION

Site-selective protein bioconjugation reactions are critical for the construction of biological probes,[1] targeted delivery agents,[2] and well-defined biomaterials.[3,4] In particular, the conjugation of fluorophores, drugs, and other small molecules to antibodies and other binding proteins has become a standard approach for synthesizing immunodetection reagents,[5] improving the therapeutic indices of toxic drugs through antibody-drug conjugates (ADCs),[6,7] and directing molecular tools to target antigens.[8] These chemistries are also critical for attaching proteins to surfaces in defined orientations, enabling the miniaturization of diagnostics and the construction of electronic devices interfaced with living systems via proteins.[9,10] Often, surface cysteine residues[11,12] or unnatural amino acids[13] are used as attachment points, and several approaches have taken advantage of the unique reactivity of the N-terminal positions to create protein conjugates.[14−17] While these strategies target functional groups that can be installed conveniently in many cases, there remain instances in which the introduction of new nucleophilic residues can lower expression yields or hinder protein function. As an example, the N-termini of immunoglobulins are typically on the same face of the protein as the antigen binding loops, which can limit the utility of these sites as points of attachment for large cargo or surfaces.

The C-terminus can provide another site for modification, although it has historically been more difficult to functionalize. C-terminal decarboxylative alkylation was achieved recently with photoredox chemistry using a water-soluble photocatalyst by taking advantage of the lower oxidation potential of this site relative to side-chain carboxylates.[18] Intein trans splicing is a commonly used technique for attaching peptide sequences to C-termini, though this method requires the genetic fusion of bulky constructs and leaves a cysteine residue at the ligation site.[19] As another alternative, the widely used sortase enzyme links cargo groups bearing polyglycine (n = 1−5) motifs to protein C-termini after the threonine residue in an LPXTG recognition sequence.[20,21] butelase,[22,23] subtiligase[24] farnesyl transferase,[25,26] and SpyTag/SpyCatcher,[27,28] work similarly by recognizing and ligating specific peptide sequences. Tubulin

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tyrosine ligase[29,30] and AnkX[31,32] (a bacterial phosphocholine transferase) can also append synthetically modified small molecule substrates to proteins based on peptide recognition sequences. Each of these methods has advantages and disadvantages to consider when choosing a conjugation strategy, including the size of the recognition motif; the residual sequence left behind after modification; the required pH and buffer conditions; the synthetic accessibility of the coupling partners; and the kinetics, availability, stability, and selectivity of the enzyme catalyst.

Our lab has developed a set of oxidative coupling chemistries that involve the reaction of *o*-quinone and *o*-iminoquinone intermediates with N-terminal proline residues and aniline groups (Figure 1a).[33] This set of techniques can be used to conjugate a wide range of small molecules, nucleic acids, nanoparticles, surfaces, and additional proteins to specific locations on biomolecular substrates. However, two features of the initial versions of this this reaction have limited its use. First, the use of strong oxidants, such as sodium periodate, can result in collateral oxidation of cysteine and methionine side chains, and even protein-tolerant oxidants like ferricyanide ion need to be removed from the protein solution following the reaction. Second, some of the *o*-quinone precursors require synthetic expertise to make and can be difficult to store due to oxidation by atmospheric oxygen (although the storage issue can be addressed using *o*-methoxyphenol substrates[34]). Recently, we reported that the tyrosinase enzyme from the common button mushroom *Agaricus bisporus* (abTYR) is capable of oxidizing simple phenols attached to cargo molecules to yield *o*-quinones in situ (Figure 1b).[14] The enzyme relies on two copper ions chelated in a deep active site pocket to transfer an atom from molecular O_2 to the ortho position of a bound phenol (Figure 1c–e).[35,36] The enzyme operates rapidly at 4 °C and room temperature at a mild pH of 6.5, is commercially available, and can be stored at −80 °C in solution for months. It readily oxidizes phenols that are appended with NHS-activated cargo molecules, allowing simple tyramine derivatives to be used as readily available and storable building blocks. Most importantly, in previous work,[14] we and others have found that it leaves native tyrosine residues untouched if they are within the secondary and tertiary structural elements of folded proteins.

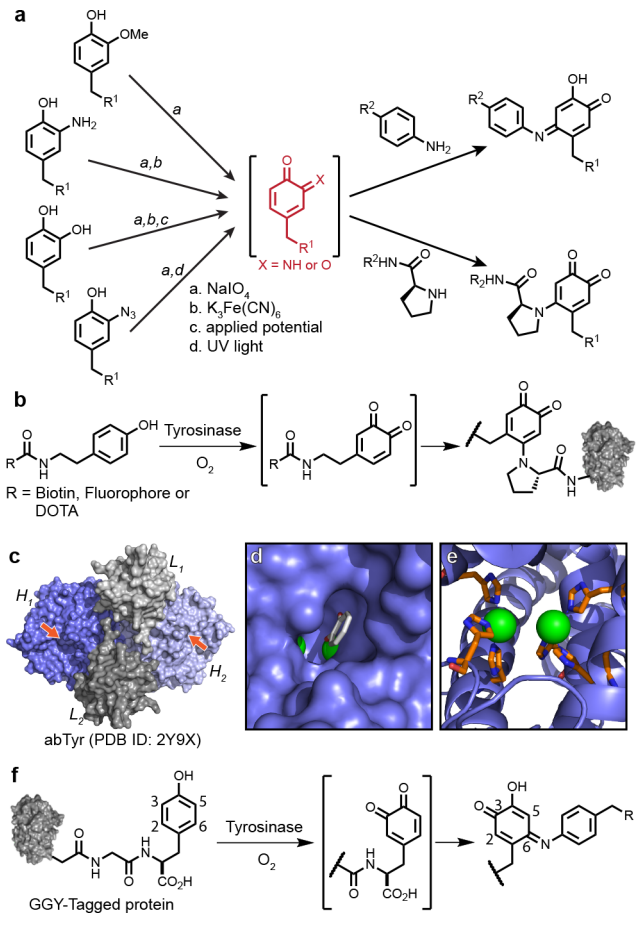

Figure 1. Summary of oxidative coupling strategies for site-selective protein modification. (a) Previously reported methods are shown for accessing *o*-quinones and *o*-iminoquinones for coupling to N-terminal proline residues and aminophenyl groups. (b) A recently reported tyrosinase-mediated oxidation of phenols allows coupling to N-terminal proline residues with oxygen as the stoichiometric oxidant. (c) The tyrosinase from *Agaricus bisporus* (abTYR) consists of a tetramer with two active sites, indicated by the orange arrows. (d,e) The copper ions that catalyze the oxidation reaction are located at the bottom of a deep pocket in the protein. As a result, tyrosine residues that are embedded in the protein structure cannot participate in these reactions. A tropolone inhibitor (gray) in (d) suggests the location of the tyrosine phenol during the reaction. (f) This work presents tyrosine-based tags on proteins for the selective tyrosinase-mediated generation of *o*-quinones at protein N or C termini. Once formed, these species couple readily with exogenous amine nucleophiles.

The ability of tyrosinases to generate *o*-quinones on proteins is well documented, at least at high substrate concentrations. Tyrosinases and phenol oxidases have been known for decades to mediate cross-linking between proteins in meat,[37] whey,[38] and flour[39] via nonselective tyrosine-tyrosine, tyrosine-cysteine, and tyrosine-lysine linkages. More recently, Long and Hedstrom described abTYR-mediated oxidation of multiple tyrosine residues in a hemagglutinin tag (YPYDVPDYA) and similar variants at the C-terminus of *E. coli* dihydrofolate reductase.[40] Although a hydrazide-functionalized dye could be attached to the oxidized tag, fragmentation of the sequence was a major side reaction. The many reports of tyrosinase-generated *o*-quinones modifying proteins via different linkages may have been a deterrent against pursuing these species as handles for selective modification in the past. As one example, Montanari et al. specifically sought to suppress *o*-quinone formation during tyrosinase-mediated oxidation of tyrosines to catechols for conjugation with boronic acids, out of a concern that these species would cause detrimental side reactions.[41] The most successful utilization of abTYR for tyrosine activation to date was by Delft and co-workers, who used it to prime a tyrosine-tagged antibody for strain-promoted Diels–Alder cycloaddition with a cyclooctyne-functionalized drug molecule.[42] This work highlights the potential of tyrosine residues to act as enzymatically activated attachment points in complex biomolecular substrates.

The field of organic chemistry is filled with examples of *o*-quinones reacting with phosphines,[43] thiols,[36,44] amines,[44] and other diverse nucleophiles. The challenge therefore becomes the identification of nucleophiles that can compete with undesired, sometimes intramolecular, pathways to afford clean bioconjugates without cross-linking. Here, we report that tyrosine residues encoded on linkers at protein N or C-termini (tyrosine tags or -GGY) can be readily oxidized by abTYR and by the smaller, readily expressed, *Bacillus megaterium* tyrosinase (bmTYR) for subsequent reaction with secondary amine and

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J. Am. Chem. Soc. 2020, 142, 5078–5086

aniline nucleophiles (Figure 1f). These reactions are demonstrated for a number of different proteins, including an antibody single chain variable fragment and immunoglobulin-binding Protein L. This work expands the range of protein substrates that can be modified using OC techniques, allows the modification of C-terminal positions on proteins, affords greater flexibility in the selection of nucleophilic coupling partners, and provides the field with a new tyrosinase with broader substrate scope.

RESULTS AND DISCUSSION

These studies began by examining whether the N-terminal *N*-acyl-tyrosine residue on the α-endorphin peptide would be sterically unencumbered enough to be oxidized by abTYR (Figure 2a). This was tested by combining this peptide at 100

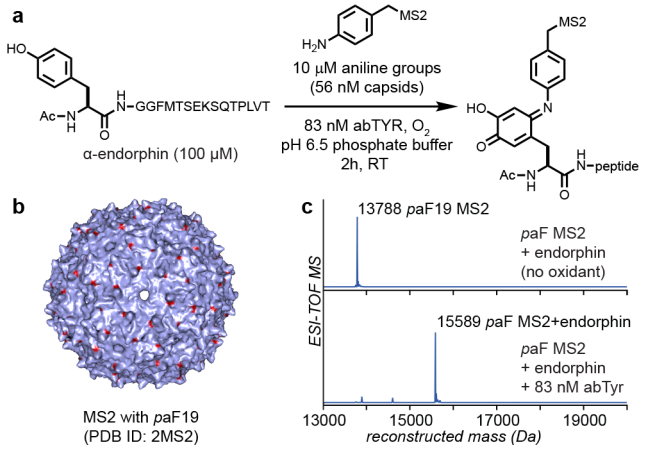

Figure 2. Tyrosinase activation of solvent-exposed tyrosine residues on α-endorphin. (a) The tyrosine residue at the N-or C-terminal end of the peptide was readily oxidized. (b,c) In this example, 180 copies of an α-endorphin peptide were added to *p*-aminophenylalanine (*p*aF) groups (red) introduced into MS2 viral capsid proteins.

μM with MS2 capsids (10 μM, based on monomer concentration) bearing *p*-aminophenylalanine (*p*aF) residues that were introduced using amber codon suppression.[45,46] In the presence of 83 nM abTYR, clean, nearly quantitative conversion was observed to the expected aniline addition product (Figure 2b). As there are 180 copies of the aniline groups on the surface of each capsid, this corresponded to the installation of >170 new peptide groups on the external surface of each assembly in 2 h at room temperature. It should be noted that acetylation of the α-endorphin N-terminus was essential to the success of the reaction, since a free N-terminal amine would readily attack the proximal *o*-quinone as it does upon the oxidation of free L-tyrosine during melanin biosynthesis.[36] We have confirmed this pathway for other peptides, and thus we recommend using peptides with acyl or glycine residues before the tyrosine site.

The identity of the product was confirmed for a small molecule model resulting from the tyrosinase-mediated addition of *p*-toluidine to *N*-acetyl-L-tyrosine in D_2O with abTYR. The reaction required more tyrosinase (350 nM corresponding to 42 U/mL) and an extended reaction time to proceed in D_2O, and turned purple. NMR analysis of the crude reaction mixture confirmed that the initially formed 5-anilino-1,2-catechol is subsequently oxidized back to the quinone state, presumably by adventitious oxygen in the solution (see NMR section of the Supporting Information). The 5.7 ppm proton signal attached to the 5 carbon of the oxidized tyrosine phenol gradually diminished over the course of several hours as it exchanged with deuterium in solution, consistent with enol tautomerization. This is in agreement with a previous report by our group for products generated from *o*-aminophenols,[47] which showed that this species exists as the *p*-iminoquinone tautomer in solution. The mass change of the MS2-peptide conjugate corresponded exactly to that predicted by the analogous small molecule product.

To test the abTYR OC reaction on a more challenging protein substrate, a -GGY tag was appended to the C-terminus of a single chain variable fragment (scFv) of the Trastuzumab antibody. Trastuzumab is an FDA approved monoclonal antibody for the treatment of HER2(+) breast cancer[48] and is widely used as a model construct for testing bioconjugation reactions.[20,42,49,50] Trastuzumab is also commonly used in an scFv format, which is of interest because of its improved tissue and tumor penetration relative to full length antibodies[51,52] and its potential to be used for constructing bispecific antibodies.[53] We chose to use an established *E. coli* periplasmic expression protocol to produce the Trastuzumab scFv with a C-terminal -GGY tag,[54,55] Figure 3a–c. One potential challenge presented by this substrate is that, of its 15 tyrosine residues, 8 are located on the antigen binding site with their phenol side chains oriented toward the bulk solution. Given the potential for off target oxidation of the internal tyrosine residues, we were pleased to find that the C-terminally -GGY tagged version of the scFv coupled in nearly quantitative yield to aniline (Figure 3c), while a nontyrosine tagged version was untouched under the reaction conditions (see the Supporting Information, Figure S1).

This substrate was next used for the evaluation of several parameters of the reaction. To ensure meaningful comparisons, different abTYR samples used in these experiments were first assayed for reactivity by following the oxidation of L-tyrosine and subsequent dopachrome formation[36] with UV–vis spectroscopy. Enzyme concentrations in subsequent reactions were adjusted such that the same final U/mL of enzyme was used for each. This was necessary because equal masses of different batches of commercially obtained abTYR were found to vary somewhat in their enzymatic activity. Typically, 1 mg/mL tyrosinase stock solutions had activities between 900 and 1200 U/mL, corresponding to 83 to 111 nM enzyme in a typical 12 U/mL reaction.

Varying the concentration of aniline from 25 to 750 μM showed that maximum conversion was achieved at a concentration of 150 μM with at least 8 U/mL abTYR (Supporting Information, Figure S2). At lower abTYR concentrations, higher concentrations of aniline appeared to inhibit the enzyme, as evidenced by residual unoxidized starting material in these reactions. The fact that residual uncoupled protein was fully oxidized in reactions with at least 8 U/mL tyrosinase suggests that the coupling partner has a limited time to intercept the *o*-quinone intermediate before it is quenched by a competing process (likely an internal nucleophile on the protein). In a delayed nucleophile addition experiment, aniline was added to a series of scFv-GGY OC reactions 5, 10, 20, 40, or 60 min after abTYR. This experiment showed that, while the starting protein was completely oxidized in every case, the amount of product formed depended inversely on the delay until the coupling partner was added (Supporting Information, Figure S3). Thus, the

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J. Am. Chem. Soc. 2020, 142, 5078–5086

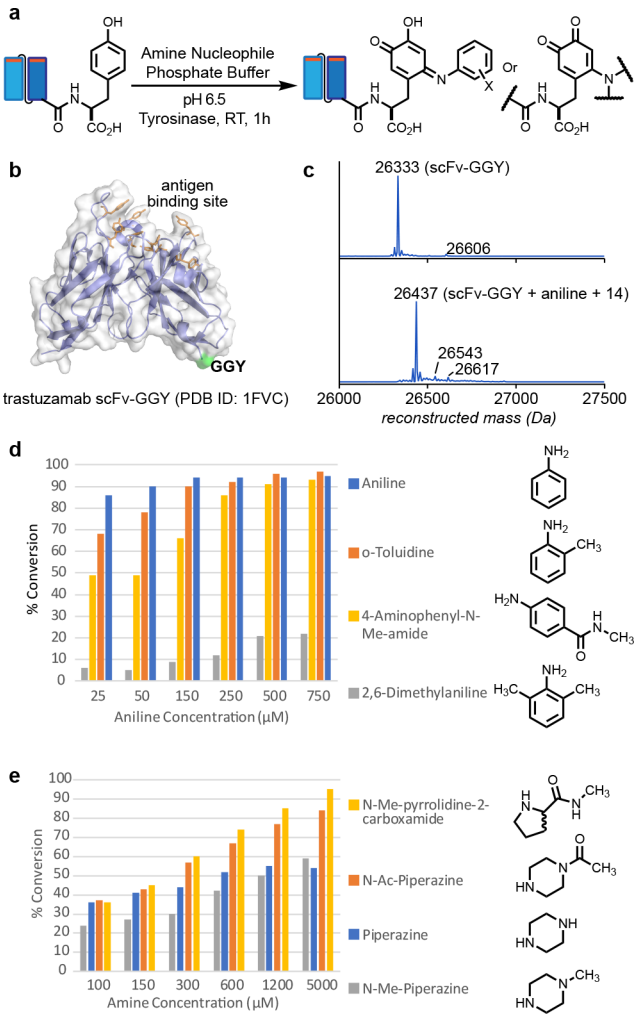

Figure 3. Efficiency of abTYR-mediated coupling with amine nucleophiles (a) A C-terminally -GGY tagged Trastuzumab scFv was used as a coupling partner. (b) A structural rendering shows the position of the C-terminus relative to the antigen binding regions. Solvent-exposed tyrosine residues that do not participate in oxidation reactions are shown in orange. (c) Representative mass spectra of scFv-GGY are shown before and after coupling (at 3.8 μM protein) with 150 μM aniline and 12 U/mL abTYR for 1 h at RT. (d) Aniline-derived nucleophiles were screened from 25 to 750 μM. (e) Pyrrolidine- and piperazine-derived nucleophiles were similarly screened from 100 to 5000 μM. In both cases, the extent of reaction conversion was estimated through integration of LC-TOF MS spectra following charge ladder reconstruction. Integrations included only peaks within 1000 Da of main starting material and product peaks; peaks attributable to aggregation or dimerization were significant in some reactions with inefficient nucleophiles or low nucleophile concentration. See the Supporting Information, Figures S4, S5, and S6 for representative spectra.

choice of nucleophilic coupling partner is of key importance, and it should be present from the start of the reaction before adding tyrosinase to intercept the electrophile as rapidly as possible.

The coupling efficiencies of aniline derivatives were evaluated to further optimize the reaction. Compounds including *o*-toluidine and 2,6-dimethylaniline were investigated as nucleophiles that might not occlude the tyrosinase active site as readily as aniline; however both proved to react more sluggishly with the *o*-quinone, giving lower conversions overall (Figure 3d and Supporting Information, Figure S4). Additionally, 4-aminophenyl-*N*-methylamide proved to be an efficient nucleophile, while *p*-anisidine appeared to be oxidized during the reaction, as evidenced by development of orange color and substrate degradation. This result was not unexpected given our past experience using *p*-anisidine derivatives as oxidative coupling electrophiles.[56] Various piperazines and a racemic *N*-methyl pyrrolidine were also assessed (Figure 3e and Supporting Information, Figures S5 and S6). These proved to be less reactive nucleophiles, and required much higher concentrations to achieve good conversion. Racemic proline-*N*-methylamide provided the best conversion of the cyclic secondary amine nucleophiles, consistent with our previous findings that proline N-termini are efficient oxidative coupling partners.[14,16,57]

To confirm that the abTYR-mediated modification of GGY tagged Trastuzumab scFv did not perturb the binding activity of this antibody fragment, the fluorescent dye Oregon Green 488 (OG 488) was derivatized with an aniline nucleophile and oxidatively coupled to the scFv (Supporting Information, Figure S7a). The reaction proceeded optimally with 25 μM aniline-OG 488 and 12 U/mL abTYR to give the scFv-GGY-OG 488 construct with 84% conversion in 1 h (Figure 4a,b). Flow cytometry showed that this construct could recognize the HER2(+) SK-BR-3 breast cancer cell line selectively over HER2(−) MDA-MB-468 breast cancer cells (Figure 4c).

The stability of the aniline conjugate to Tyr-tagged scFv was monitored at 4 °C over the course of 7 days in a standard phosphate and NaCl protein storage buffer with 15% glycerol (pH 7.4). The linkage was found to remain intact throughout this period (Supporting Information, Figure S8). The addition of 10 mM dithiothreitol (Supporting Information, Figure S9) or glutathione (Supporting Information, Figure S10) resulted in the formation of thiol adducts, presumably into the new aromatic ring, but did not result in cleavage of the linkage between the protein and the small molecule. In the case of glutathione, this further modification occurred quantitatively within 24 h. Moreover, subsequent treatment of the scFv-GGY-aniline-glutathione product with DTT for 24 h showed that the initial glutathione adduct was not released competitively. This indicates that the pre-addition of glutathione could be used to avoid the addition of other thiols in serum, if required (Supporting Information, Figure S11).

Protein L is an IgG binding protein that recognizes variable regions of human kappa light chains. Discovered in pathogenic *Peptostreptococcus magnus*, it consists of five binding domains[58] connected in tandem by short 9−10 amino acid linkers[59] (Figure 5a). The avidity of the combined interactions of these five domains allows the protein to achieve a K_d value of 130 nM at pH 8.[60] When expressed recombinantly, cell-wall anchoring domains of the wild type protein are typically omitted, and some truncated versions have only 3 or 4 light chain binding domains. Protein L is routinely used for the purification of scFv constructs[61] and has been employed as a universal flow cytometry marker for cells expressing chimeric antigen receptors (CARs),[62] which typically utilize scFv segments to recognize their targets. Because Protein L binds to variable light chains without interfering with the antigen recognition loops, it can be used as a "secondary" detection reagent for bound scFv's and IgG's.

We expressed Protein L in *E. coli* with flexible, C-terminal -GGY and -GGGGSGGY tags for abTYR-mediated modification with aniline-functionalized OG 488 to create secondary

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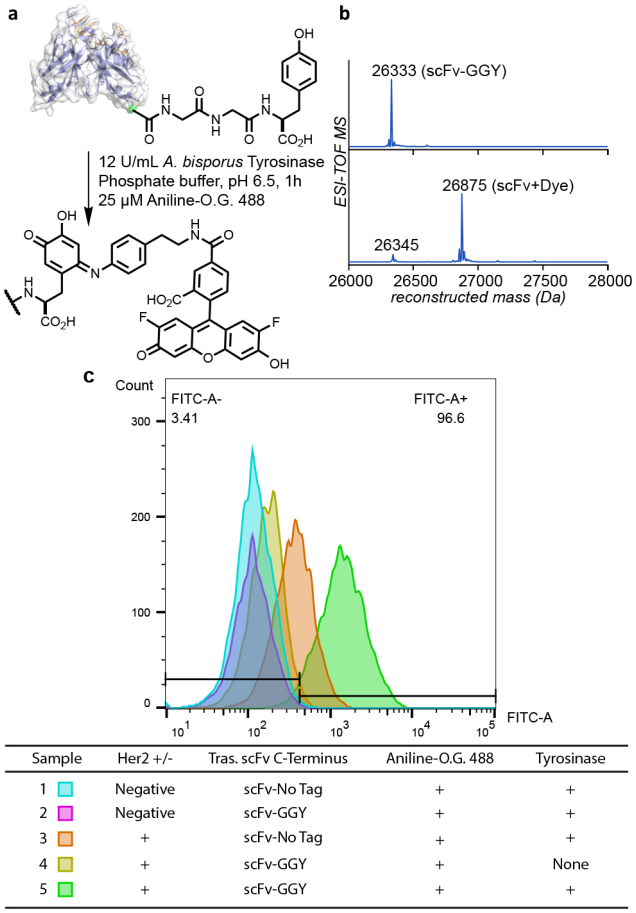
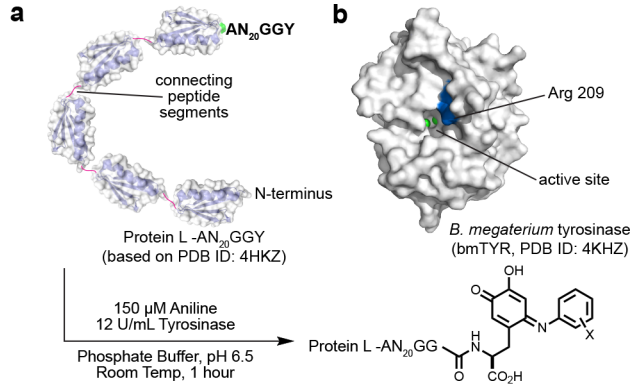


Sample	Her2 +/-	Tras. scFv C-Terminus	Aniline-O.G. 488	Tyrosinase
1	Negative	scFv-No Tag	+	+
2	Negative	scFv-GGY	+	+
3	+	scFv-No Tag	+	+
4	+	scFv-GGY	+	None
5	+	scFv-GGY	+	+

Figure 4. Flow cytometry study of fluorophore-coupled Trastuzumab scFv binding SK-BR-3 (HER2+) cells. (a) Oxidative coupling of GGY-tagged scFv with 12 U/mL *A. bisporus* Tyrosinase and 25 μM Aniline-Oregon Green 488. (b) ESI TOF-MS indicates that scFv-GGY was coupled with 84% conversion (see also the Supporting Information, Figure S7). A nontagged version of the scFv was unmodified (see the Supporting Information, Figure S1). (c) Flow cytometry fluorescence data of HER2(−) cells (MDA-MB-468) and HER2(+) (SK-BR-3) cells treated with the Trastuzumab scFv-GGY-O.G.488 construct or the indicated negative control. See the Supporting Information, Figure S14, for gating information.



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Variant	Type	Linker	Length	% Conversion
1	Flexible	-GGY	3	94
2	Flexible	-$(G_4S)_1$GGY	8	93
3	Flexible	-$(G_4S)_2$GGY	13	98
4	Flexible	-$(G_4S)_3$GGY	18	98
5	Alpha-helical	-$A(EAAAK)_2$AGGY	15	99
6	Rigid Ala/Pro	-$(AP)_3$GGY	9	99
7	Poly-Asn	-AN_{20}GGY	24	98
8	scFv C-Term. Seq.	-EIKRTGGY	8	97
9	ΔDomain 5	-(Domain 4-5 Linker)GGY	10	96
10	ΔDomain 5	-(Domain 4-5 Linker)G_4SGGY	15	95
11	None	None	0	0

Figure 5. Exploration of C-terminal linkers and utility of *B. megaterium* tyrosinase. (a) A variety of types and lengths of linkers were appended to the C-terminus of protein L, including two that utilized the natural interdomain linker sequence between domains 4 and 5 (ΔDomain 5). Protein L-variants were subject to the standard coupling reaction with *B. megaterium* tyrosinase. (b) Crystal structure of the 35.5 kDa bmTYR. (c) Conversion of protein L variants observed by TOF-LCMS after treatment with bmTYR (see the Supporting Information, Figures S12 and S13 for spectra). None of the variants could be modified by *A. bisporus* tyrosinase.

scFv detection reagents. Surprisingly, these constructs were resistant to oxidation by abTYR, most likely because the terminal tyrosine residue does not extend sufficiently from the bulk of the protein structure to reach into the sterically occluded abTYR active site. In an attempt to circumvent this problem, we generated a panel of Protein L variants with C-terminal linkers of various types and lengths preceding the tyrosine tag (Figure 5b and Supporting Information, Figures S12 and S13). In addition to the flexible $(G_4S)_{1-3}$ linkers, we extended Protein L with an α-helical $(EAAAK)_2$ repeat sequence,[63] a non-hydrogen bonding, rigid $(AP)_3$ repeat,[64] a poly asparagine (N_{20}) sequence, and the C-terminal sequence of the easily modified Trastuzumab scFv (EIKRTGGY). Additionally, the C-terminal fifth light chain binding domain was deleted and the native linker between the fourth and fifth light chain binding domains was extended with -GGY and -G_4SGGY tags. Disappointingly, none of these variants could be oxidized by abTYR.

We recognized that the steric bulk of the 120 kDa tyrosinase protein likely makes access to the active site difficult for many protein substrates. While in principle it should be possible to generate an abTYR-oxidizable variant by continuing to extend the C-terminal linker, longer linkers increase the risk of the tag interfering with protein function, and are more difficult to install during gene construction, dampening the convenience of the tyrosine tagging approach. We were therefore motivated to express and test the much smaller *Bacillus megaterium* tyrosinase (bmTYR).[65,66] This 35.5 kDa protein is robustly expressed in BL21 (DE3) *E. coli* with a yield of up to 160 mg/L and has a much more accessible active site in comparison to abTYR. Gratifyingly, all tyrosine-tagged Protein L variants exposed to bmTYR were quantitatively oxidized and reacted with 150 μM aniline with over 90% conversion in 1 h, while the nontagged variant remained untouched (Figure 5b and Supporting Information, Figures S12 and S13).

With a solution to Protein L modification in hand, we next performed the bmTYR mediated OC with 25 μM aniline-OG 488 on the -AN_{20}GGY tagged Protein L variant. The desired conjugate was obtained with 87% conversion in 1 h (Figure 6c and Supporting Information, Figure S7b). This construct was then applied to HER2(+) SK-BR-3 or HER2(−) MDA-MB-468 breast cancer cells that had been pretreated with the nontyrosine tagged trastuzumab scFv. Only the Protein L construct that had been modified with aniline-OG 488 under the bmTYR-mediated OC conditions was able to label the HER2(+) cells after exposure to the scFv (Figure 6d). Cells

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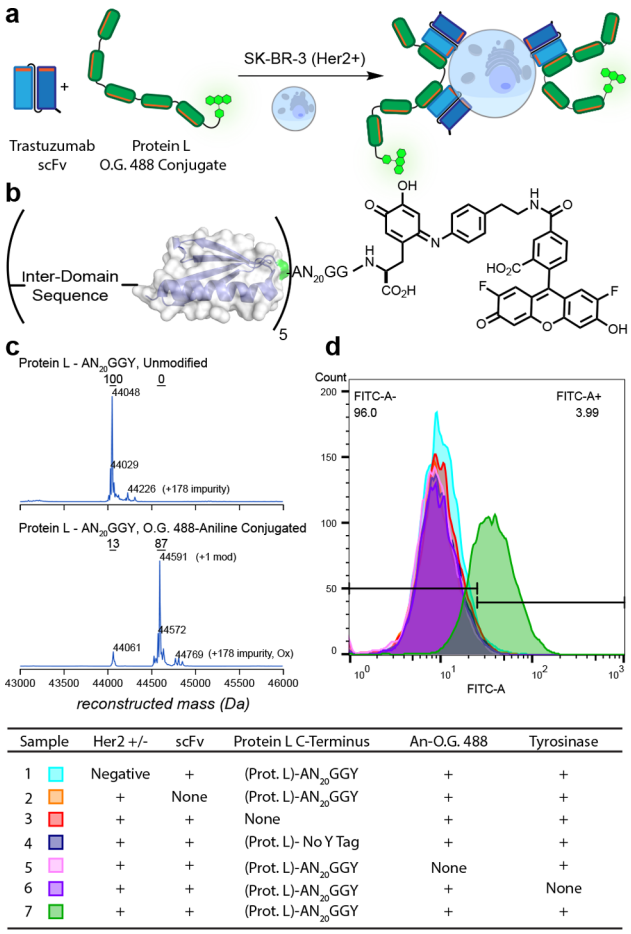
J. Am. Chem. Soc. 2020, 142, 5078−5086



Sample	Her2 +/-	scFv	Protein L C-Terminus	An-O.G. 488	Tyrosinase
1	Negative	+	(Prot. L)-AN$_{20}$GGY	+	+
2	+	None	(Prot. L)-AN$_{20}$GGY	+	+
3	+	+	None	+	+
4	+	+	(Prot. L)- No Y Tag	+	+
5	+	+	(Prot. L)-AN$_{20}$GGY	None	+
6	+	+	(Prot. L)-AN$_{20}$GGY	+	None
7	+	+	(Prot. L)-AN$_{20}$GGY	+	+

Figure 6. Detection of HER2(+) cells using a Protein L - O.G. 488 conjugate "secondary" affinity reagent. (a) Detection scheme: nontyrosine tagged trastuzumab scFv binds to HER2(+) SK-BR-3 cells and is recognized by O.G. 488-modified Protein L. (b) Secondary affinity reagent was made from an -AN$_{20}$GGY terminated Protein L variant using 25 μM O.G. 488-Aniline, with *B. megaterium* tyrosinase. (c) Mass spectra of the Protein-L-AN$_{20}$GGY before and after modification. (d) Flow cytometry flourescence data of SK-BR-3 cells treated according to the scheme above and negative controls. MDA-MB-468 cells were used as the HER2(−) control. See the Supporting Information, Figure S15, for gating information.

without HER2 or samples with a component omitted from the coupling and labeling workflow were untagged.

The reaction was also tested on C-terminally tyrosine tagged sfGFP with several different linkers between the protein and the GGY tag (Supporting Information, Figures S16 and S17). Oxidation of the tyrosine tags was achieved with both abTYR and bmTYR, with bmTYR proving to be more general and achieving greater substrate oxidation. Coupling was achieved in high (>88%) conversion with an aniline nucleophile. In this case, the product protein exhibited an additional −17 Da peak relative to the expected mass. We anticipate that this resulted from condensation of the *p*-iminoquinone product with nearby amines, yielding Schiff bases with lysine side chains or the N-terminal amine group. Nonetheless, stable reaction products were still formed in single positions using the oxidative coupling strategy. The 17 Da loss appears to be substrate dependent, and current work is focusing on understanding and mitigating instances of its occurrence.

It is noteworthy that the substrates presented herein do not have surface-exposed cysteine residues, as we have found that these can also participate in efficient couplings between peptide and protein substrates. This work is reported in a separate publication.[67] If this interferes with the addition of aniline and cyclic amine nucleophiles, we have shown in previous studies[16] that thiol nucleophiles can be conveniently capped through disulfide formation with Ellman's reagent. Following the oxidative coupling reaction, these groups can be removed through brief exposure to an appropriate reducing agent.

Based on our experience with this method, we suggest the following initial conditions for new protein substrates: Extend protein C-termini with flexible -GGY or $(G_4S)_{1-3}$GGY tags and prepare cargo functionalized with 4-ethylaniline (such as the aniline-OG 488 construct used here). Screen 4 μM of the protein substrate with several concentrations of nucleophile between 25 and 500 μM at pH 6.5. Attempt coupling with 12 U/mL of commercially available abTYR for 2 h. In cases where this fails, try bmTYR for 2 h as an alternative. Assay tyrosinase by monitoring L-tyrosine oxidation to gauge activity[14] before use.

CONCLUSION

In this work, we have expanded the capabilities of oxidative coupling chemistry by exploiting the ability of tyrosinase enzymes to generate *o*-quinones on sterically exposed C or N-terminal tyrosine residues. These groups can then be coupled to aniline or cyclic-amine nucleophiles, which compete favorably with internal nucleophiles in the protein itself. The chemical modification of native amino acids is an appealing approach to protein bioconjugation because it avoids the increased complexity and lower expression yields that can be associated with noncanonical amino acid incorporation.

Most native amino acid targeting strategies, including the established oxidative coupling approaches, modify chemically distinct groups through the use of carefully calibrated electrophiles added exogenously. The enzymatic tyrosine oxidation strategy reverses that common paradigm by unmasking a latent electrophile on the protein substrate with a restrained oxidizing agent, giving the user flexibility in choosing the nucleophilic coupling partner. This shift eliminates the problem of off-target modification by the small molecule, which is difficult to avoid because bioconjugation reactions on proteins typically use a large excess of the small molecule component. The presence of the protein substrate at much lower concentrations (nanomolar to low micromolar) minimizes off-target protein−protein coupling as well, yielding cleaner coupling products overall.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.9b12002.

Experimental procedures, full mass spectra of key proteins before and after coupling, NMR spectra of the small molecule oxidative coupling product, flow cytometry gating schemes and statistics, and other relevant data (PDF)

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Notes

The authors declare no competing financial interest.

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