Reaction Landscape and Bioconjugation Profile of Tyrosinase Generated Quinones

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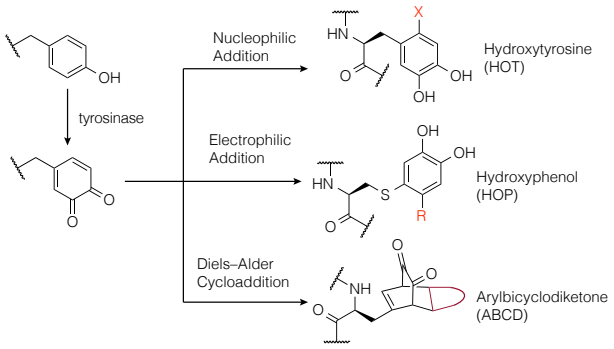
ABSTRACT

We describe a class of bioconjugation reactions that enables site-specific modification of proteins through enzymatic generation of *o*-quinone from either tyrosine residues or phenol reagents. The enzymatically generated *o*-quinone rapidly reacts chemically with numerous common nucleophiles and dienophiles, including thiols, anilines, alkoxyamines, cyclooctynes, and cyclooctenes. Nucleophilic chemoenzymatic reaction with engineered tyrosine residues creates a hydroxytyrosine (HOT) bridge; a similar reaction with phenols creates a hydroxyphenol (HOP). Diels-alder cycloaddition following *o*-quinone generation results in an arylbicyclodiketone (ABCD). The stability of each conjugate against physiological pH and temperature varies from less than one day to multiple months in vitro.



INTRODUCTION

Site-specific bioconjugation to native amino acids remains a challenge for chemists.[1] Discerning surface-abundant amino acids like lysine,[2] aspartic acid and glutamic acid from similar neighbors with chemical reagents is usually impossible; except in rare cases, the functional group of an amino acid side chain overwhelms any subtle variations in steric or electronic local environment that might lead to enhanced reaction kinetics at a single site. In contrast, installation of amino acids that are infrequently found on protein surfaces (cysteine in particular) can result in an island of unusual function, which can be leveraged for site-specific chemical reaction.[3]

A similar specificity is achieved by enzymes,[4] which have evolved to isolate functional groups in a unique steric and electronic context. Many enzymes react with amino acid sidechains. They can be used to install or remove post-translational modifications like phosphates, glycans, or lipids. Protein chemists can exploit the amino acid sequence specificity of enzymes[5] for bioconjugation, as has been demonstrated for transglutaminase (TG),[6] formylglycine-generating enzyme (FGE),[7] sortase,[8] phosphopantethienyl transferase (PPTase),[9] and laccase,[10] among others. Site-specific reaction is achieved through recombinant installation of the amino acid(s) recognized by the enzyme. Tyrosinase[11] is also in this class of enzymes.[12] Tyrosinase performs a two-step oxidation of tyrosine to dihydroxyphenylalanine (DOPA), and subsequently, the *o*-quinone of DOPA (dopaquinone).[13] In nature, dopaquinone is a precursor to both eumelanin and, upon combination with cysteine, pheomelanin, the aromatic skin and hair pigment polymers; in the lab, scientists can exploit this reactivity to modify recombinant proteins. Tyrosinase recognizes only the phenol sidechain of tyrosine and it is able to convert it to the *o*-quinone without requiring specificity from flanking amino acids. Importantly, tyrosine is quite rarely found on protein surfaces; in those few occurrences, its sidechain tends to be occluded by hydrophobic packing. This results in very few tyrosine residues for which the phenol is sufficiently extended to reach into the active site of tyrosinase. As such, it is possible to install tyrosine residues and achieve site-specific protein modification. This method has been put to use in a variety of examples, including conjugation of cytotoxic cargos through Diels-Alder cycloaddition,[14] protein–protein conjugation of Cas9,[15] and profiling the oxidative coupling of anilines.[16]

In this work, we have profiled many of the mechanistic considerations of site-specific chemoenzymatic modification of protein tyrosine residues installed by site-directed mutagenesis.

We have categorized the chemoenzymatic reactivity of *o*-quinones into three different reaction modes, which are analyzed in parallel, and we highlight their values and challenges. The first includes nucleophilic addition of exogenous nucleophiles to produce hydroxytyrosine (HOT) bridged conjugates. In the second, the reaction polarity is reversed, so that nucleophilic amino acids on the protein react with *o*-quinones generated from exogenous phenol reagents to produce hydroxyphenol (HOP) conjugates. Third, exogenous dienophiles are introduced to the *o*-quinone, resulting in diels-alder cycloaddition and the formation of arylbicyclodiketone (ABCD) bridged conjugates.

RESULTS

Formation and side reactions of dopaquinone generated in situ

Two previous reports demonstrate that human IgG1 framework antibodies do not contain any tyrosine residues sufficiently exposed to react with tyrosinase.[14, 16] Our experiments employed an antibody fragment (Fab) as a test substrate for bioconjugation. We first profiled the mechanism of tyrosinase-mediated *o*-quinone generation in the absence of any partner reagent. Incubation of a Fab engineered to contain tyrosine spaced by one glycine from C220 (EU numbering) at 1 mg/mL with 5% mol^{-1} of mushroom tyrosinase at pH 6, resulted in a mass shift of the Fab by 14 Da (Fig 1), and the appearance of a prominent UV band at 480 nm (Fig S1), which were assigned to *o*-quinone. The reaction is rapid with >80% conversion in 30 min. A control substrate containing a leucine in place of tyrosine does not result in any change in mass or UV spectrum under the same conditions. The formation of dopaquinone is possible from pH 5–7.5, with a range of efficiencies, as measured by changes in mass (Fig S2a) and UV spectra (Fig S2b), which is consistent with previous reports of tyrosinase activity.[17]

However, the formation of *o*-quinone product is in competition with two notable side reactions. First, we found that dopaquinone-containing Fabs form covalent dimers that form a shoulder on the RP chromatogram of the Fab, Fig S3a. The mass of the dimer is consistent with coupling of two *o*-quinone-containing Fabs, suggesting the reaction depends on lingering unreacted quinone, Fig S3b. In addition, the UV spectrum of the putative

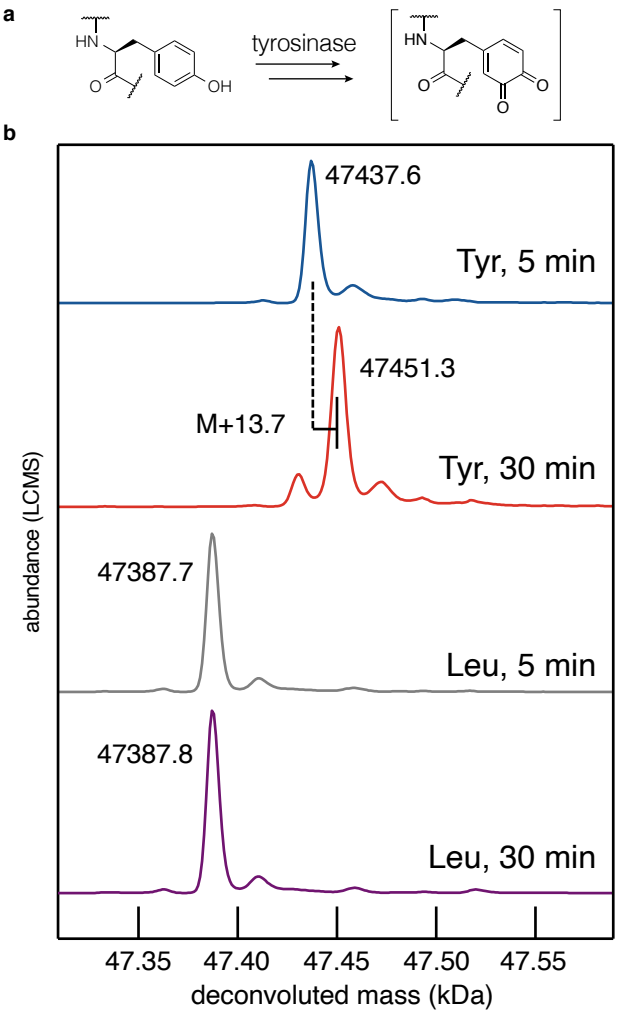


Figure 1. Transient generation of dopaquinone from tyrosine. Treatment of a human IgG1 Fab containing an engineered tyrosine with tyrosinase in pH 6 buffer at RT results in rapid, transient observation of a 14 Da mass shift.

dimer is blue shifted (to 410-420 nm) from the transient dopaquinone (480-500 nm), suggesting consumption of the quinone toward a more alkyl-like conjugate.

Second, we detected fragmentation of the Fab to a lower molecular weight. The mass of the fragment is consistent with cleavage on the amide backbone on the N-terminal side of the Tyr site, Fig S2a. The fragmentation is faster at higher pH, suggesting a deprotonation followed by rearrangement to eliminate a new C-terminus. Unfortunately, the mass accuracy for intact Fab (~1 Da) does not allow conclusive assignment of CT carboxylic acid or amide as the released product. As will be discussed below, both reactions can be avoided by including a partner reagent to outcompete side product formation.

In situ conjugation of exogenous cysteine to tyrosine depends on surface tyrosine accessibility

During pheomelanin production in living organisms, dopaquinone undergoes a Michael-type reaction with cysteine.[18] This natural pathway has inspired numerous examples of paring thiol nucleophiles with tyrosinase to crosslink and modify proteins in vitro, including the modification of sensors[19] and grafting proteins to surfaces.[20] To avoid potential reaction of the amine at the Cys N-terminus, we used *N*-(acetyl)-cysteine (*N*-Ac-Cys) as a test substrate. In a previous successful evaluation of tyrosinase for bioconjugation, for steric reasons, tyrosine was appended to a full-length IgG using a tetraglycine spacer. In another report, a variety of linkers were attempted for the modification of protein L at its N- and C-terminus.[16] Surprisingly, on protein L, even long extended linkers did not enable efficient conjugation. However, a smaller MW species of tyrosinase was able to efficiently produce the desired dopaquinone. In a similar vein, in this work, an effort to minimize the introduction of non-native amino acids in substrate proteins, we tested a panel of glycine linker lengths from 0–4 at the Fab C-terminus, Fig. 2. Reaction of 2 equiv. of *N*-Ac-Cys mol^{-1} of Fab–G_4Y resulted in efficient conjugation in the presence of tyrosinase. The product mass included both the addition of nucleophile and one oxygen atom, consistent with a hydroxytyrosine (HOT) thioether. Direct fusion of tyrosine to the CT was noticeably slower than tags with glycine spacers, with only 23% product formation in 1 h. In our hands, only one spacing residue was required to provide tyrosinase access to the Tyr sidechain, as GY, G_2Y, G_3Y and G_4Y tags all resulted in complete reaction within 1 h. It is unclear what sets protein L apart from Ab-like domains, and further investigation of incorporating Tyr sites into proteins is warranted. Nevertheless, with this result in hand, we designed a minimal Tyr tag based on the first two residues of the IgG1 hinge (DK), but replacing lysine with arginine in an effort to avoid potential intramolecular cross-reactivity of its sidechain amine with *o*-quinone. This Fab–DRY was used for subsequent experiments.

Compatibility of tyrosinase bioconjugation with a range of nucleophiles

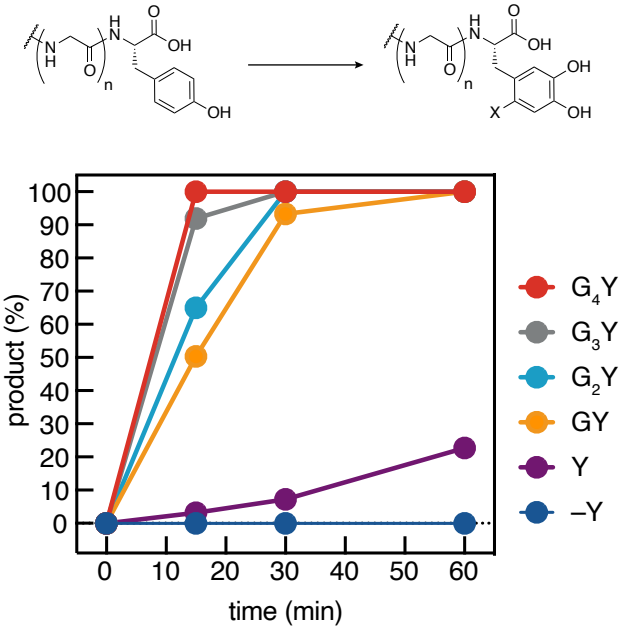


Figure 2. Distance dependence of tyrosinase-mediated bioconjugation. Conjugation of *N*-acetyl-cysteine (*N*-Ac-Cys) to Tyr-containing tags at the Fab C-terminus is faster with increasing numbers of glycine (n = 0, 1, 2, 3, 4) residues between the Fab and Tyr. When Tyr is not added to the Fab C-terminus, there is no observable conjugation.

We hypothesized that in addition to cysteine, synthetic thiols—and other nucleophiles—would be suitable reaction partners for *o*-quinone. We acquired or synthesized a panel of nucleophiles attached to discrete PEG_8 chains (for mass shift ID), including thiol, aniline, alkoxyamine, and primary amine functional groups. These were tested for their ability to react with *o*-quinone on Fab in situ, Fig 3. Addition of minimal excess reagent (1.1 equiv. of nucleophile mol^{-1} of Fab) allowed us to discern the relative reactivity of each reagent. As with the *N*-Ac-Cys reaction, the product masses include both the addition of nucleophile and one oxygen atom.

The reaction of PEG_8-SH is fast enough that we did not observe buildup of intermediate *o*-quinone, Fig 3b. Interestingly, even at this low stoichiometric ratio, we observed a small amount of two additions of PEG_8–SH to the Fab. To evaluate the mechanism of this second addition, we repeated the conjugation with 2, 5, and 10 mol. equiv. of thiol. We observed progressively more doubly-conjugated product, but still with only a single O-atom addition to the Fab mass, Fig S4. We con-

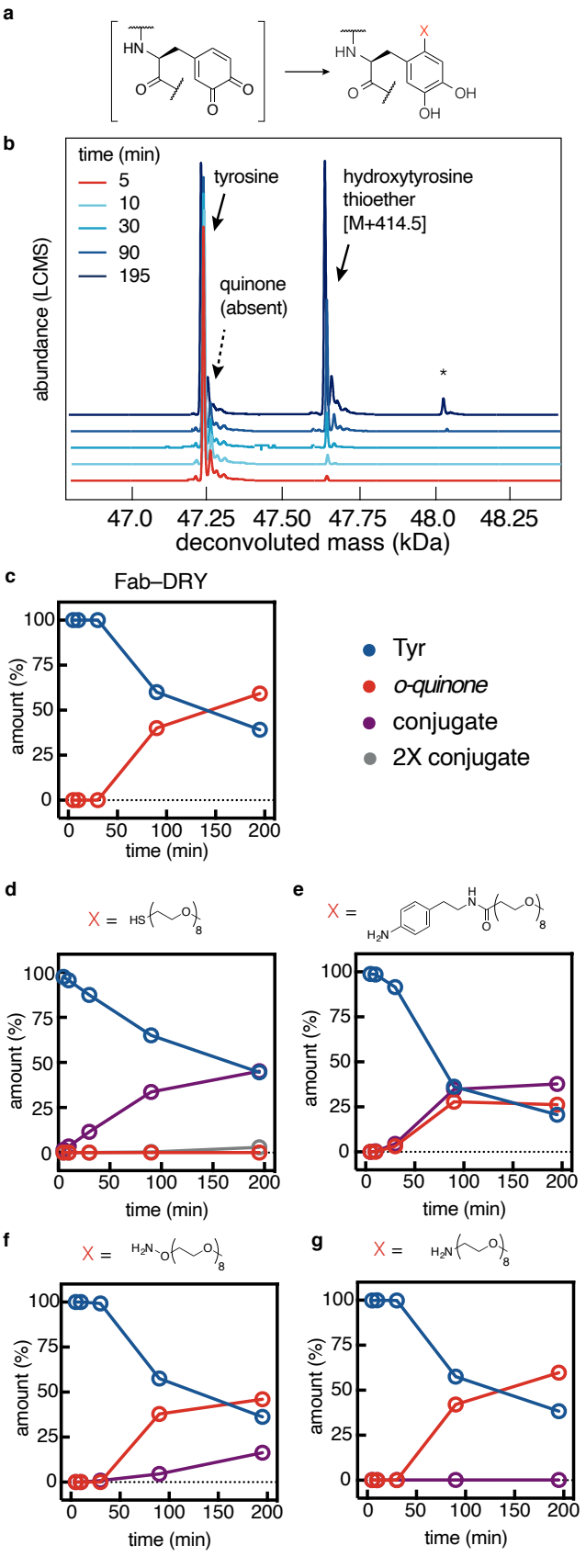


Figure 3. Reaction of nucleophiles with dopaquinone generated in situ under limiting conditions (1.1 mol. equiv. nucleophile). (a) Reaction scheme. (b) Mass spectrum time-course for reaction of PEG_8-SH and Fab-DRY in the presence of tyrosinase. Product formation timecourse in the absence (c) of reagent, and in the presence of PEG_8-modified thiol (d), aniline (e), alkoxyamine (f), and amine (g).

sidered that the second addition might correspond to free thiol reacting with the interchain disulfide. To account for this, a control reaction in which the same Fab lacking an engineered Tyr was incubated with PEG_8–SH and tyrosinase. This resulted in no change from starting material. This suggests that both equivalents of thiol react with the transiently generated dopaquinone.

As previously reported, conjugation of a PEG_8 aniline was also successful, Fig 3e. This mechanistic pathway is well-known as an "oxidative coupling" as popularized by Francis and co-workers.[21] In our experiment, product formation was commensurate with *o*-quinone generation. Interestingly, we also observed the *p*-amino aniline itself can react with tyrosinase to generate the *o*-iminoquinone (data not shown). In an attempt to decrease the reactivity of the aniline for tyrosinase while maintaining nucleophilicity, we prepared the *m*-amino form of the aniline. The side-reaction of the aniline reagent decreased, and after 5.5 h of reaction time, the protein was cleanly conjugated to ~50% with a mass corresponding to nucleophilic addition of the aniline reagent. Overnight reaction, however, resulted in a mixture of conjugate, double addition product, and tyrosine tag fragmentation, Fig S5. The stability of aniline conjugates is discussed below.

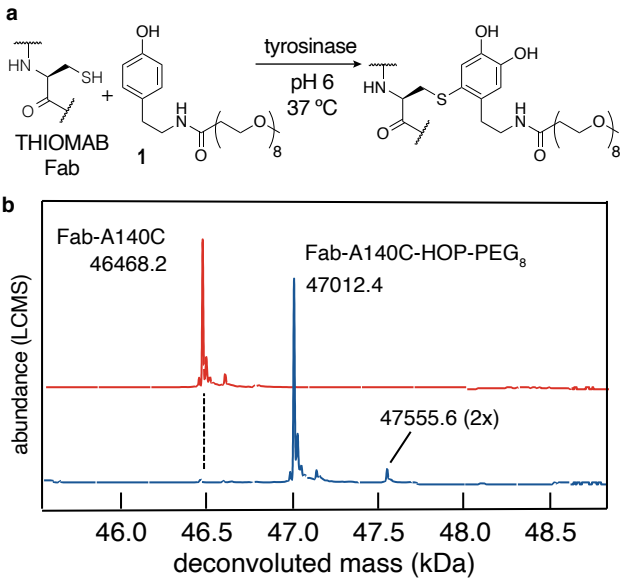
In the case of the alkoxyamine, product formation was slower, trailing *o*-quinone generation, Fig 3f. Increasing the ratio of alkoxyamine to substrate results in higher yield of conjugation, reaching >85% conjugated product in 24 h, Fig S6. This is in contrast to the thiol reaction, which reaches completion at stoichiometries higher than 1.1 mol. equiv. in under 1 h. Unlike the aniline and thiol, however, the alkoxyamine product is a mixture of two peaks, the expected mass from nucleophilic addition, and one that is 18 Da less. This may imply the initial reaction of alkoxyamine is followed by a dehydration reaction. This 18 Da peak is present throughout the reaction progress, and after 24 h,

the monoconjugate and a small amount of doubly conjugated product are both accompanied by this satellite mass. The overnight mixture also reveals a small amount of backbone fragmentation product. Taken together, the addition of alkoxyamines into the dopaquinone seems to be reversible, and the equilibrium can result in deconjugation even in the presence of excess nucleophile.

Unlike the nucleophiles above, a primary PEG_8 amine did not form measurable amounts of conjugated product at pH 6. Given the higher pKa of primary amines, we considered that the nucleophilicity would depend on pH, with higher reactivity when deprotonated. To our surprise, neither increasing the pH (6.75, 7.25 and 8) nor the stoichiometry (5 equiv. mol^{-1}) of exogenous amine resulted in any appreciable product formation, Fig S7. This is in contrast to secondary amines that are known to participate in dopaquinone modification, including N-terminal proline[22] and piperazine-type scaffolds.[16] The inability of primary amines to form conjugates with dopaquinones is puzzling. It may indicate rapid reversibility of conjugates, or sluggish nucleophilicity of alkyl amines.

Exogenous phenol reagents act as "on-demand" electrophiles for nucleophilic amino acids on proteins

In addition to the reaction of dopaquinone on proteins with nucleophiles reagents, we wished to evaluate the "reverse" reaction in which quinone reagents are added to nucleophilic amino acids. A commonly used reaction for site-specific modification of proteins is maleimide addition to proteins containing engineered surface Cys residues. In a similar manner, enzymatic generation of *o*-quinone from a phenol reagent can be thought of as an "on-demand" electrophile for reaction with surface accessible Cys residues. Indeed, this has been reported to work for peptide conjugation to site-specifically engineered Cas9.[15] In our department, we have designed THIOMAB™ antibodies and antibody fragments to contain one or more engineered cysteine residues incorporated into the constant domain of the IgG1 framework.[23] Optimal locations for Cys incorporation were discovered by screening expression and purification yield against conjugate stability.[24] For this experiment, we generated three THIOMAB™ antibody fragments. One was prepared by truncation of the native IgG1 Fab sequence after C_{226} in the hinge. Two others were made by site-specific cysteine mutations at HC-A140C or LC-K149C, two sites known to have stability against maleimide deconjugation. This panel of Fabs were reacted with a synthetic PEG_8-phenol in the presence of tyrosinase. We observed rapid hydroxyphenol (HOP) conjugate formation with near quantitative yield in < 1h, Fig 4. As with the nucleophilic reactions, the product masses include a single oxygen atom in addition to the conjugate mass. In contrast to the nucleophilic reactions, we discovered two of the three THIOMAB™ Fabs were modified with multiple HOP conjugates at a level of 5–10%. The site of these off-target reactions are as yet unknown, but given the poor reactivity of amines shown above, they are unlikely to be lysine. Site K149C



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Fab	SM	1	2	3
HC-C226	0.6	87.6	10.0	1.8
HC-A140C	1.9	93.3	4.8	-
LC-K149C	0.2	99.8	-	-

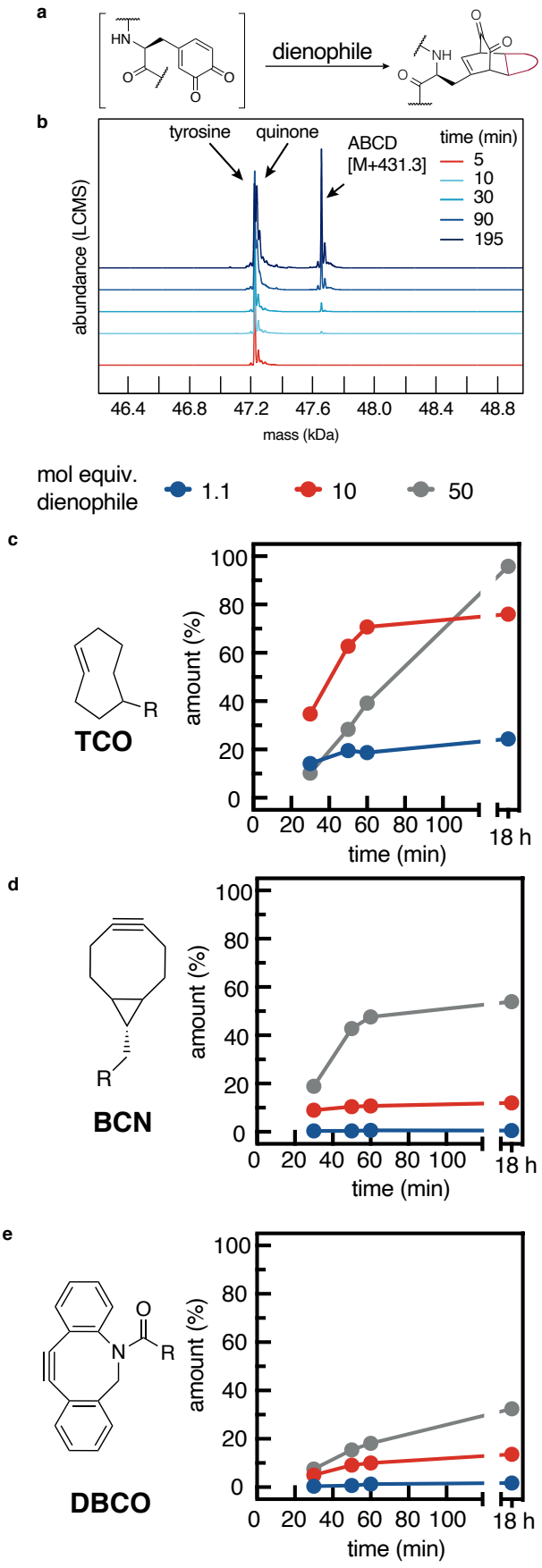
Figure 4. Reaction of *o*-quinone-containing electrophiles with antibody fragments containing engineered cysteines. (a) Reaction scheme between Cys-containing THIOMAB antibody fragments and PEG_8-phenol. (b) LCMS of starting material and product of the reaction between Fab–A140C and PEG_8-phenol in the presence of tyrosinase. (c) Table of product distributions for conjugation of PEG_8-phenol for three THIOMAB sites.

Figure 5. Conjugation of dienophiles with dopaquinone in situ. (a) Reaction scheme. (b) mass spectrum timecourse for reaction of TCO–PEG$_3$-COOH and Fab–DRY in the presence of tyrosinase. Product formation timecourse of (c) TCO, (d) BCN, and (e) DBCO reagents toward dopaquinone generated in situ.

did not result in any multiple conjugate addition.

Diels-Alder cycloaddition to enzymatically generated dopaquinone

In the realm of total synthesis, it is well known that the highly electron deficient diene of the *o*-quinone is primed for Diels-Alder cycloaddition with a variety of both dienes and dienophiles.[25] This reactivity is more challenging to harness in aqueous settings, but in one previous example, this reactivity was successfully translated into bioconjugation using the strained bicyclo[6.1.0]nonyne (BCN) bearing a pendant monomethyl auristatin E (MMAE).[14] We hypothesized that a variety of other strained dienophiles might be compatible with chemoenzymatic Diels-Alder cycloaddition to tyrosine. Numerous reagents are now available for strain-promoted azide-alkyne cycloaddition, primarily through an inverse-electron demand Diels-Alder (iEDDA) cycloaddition. We reasoned that similar reactivity would be possible using the transiently generated *o*-quinone. Leveraging commercial iEDDA reagents, we determined the reactivity of cycloaddition under reagent limited conditions (1.1 equiv mol^{-1}), as well as higher levels of reagent (10 and 50 equiv. mol^{-1}). We found that product formation rates correlated with reagent concentration, and that yields in an overnight reaction generally continued that trend. Between different cycloaddition partners, the rate and yield of each was trans-cyclooctene (TCO) > bicyclononane (BCN) > dibenzocyclooctyne (DBCO), Fig 5. Importantly, only TCO as a partner was able to reach near complete reaction. In all cases, product formation consisted of enzymatic O-atom addition followed by diels-alder cycloaddition to generate the arylbicyclodiketone (ABCD) conjugate. In contrast to nucleophilic thiol addition, for each ABCD reaction the secondary chemical reaction was slower than enzymatic *o*-quinone generation. The kinetics of the second reaction step are critical – we observed residual unreacted dopaquinone, Fig S8, and competing Fab dimer formation, Fig S9,



for all reactions except TCO with 50 mol equiv. of reagent.

Stability of HOT, HOP, and ABCD bioconjugates

An important role of a bioconjugate is to remain intact as a function of chemical and thermal stress. We synthesized and purified HOT, HOP, and ABCD conjugated Fabs for stability testing. After affinity chromatography, each conjugate was formulated and stressed in a simple aqueous system (PBS pH 7.4, 37 °C) as a "minimum bar" for initial triage. Stability was determined by LCMS monitoring of deconjugation, following free Fab and, when possible, any released small molecule(s). The deconjugation rate of Fab and reagent of each conjugate type is outlined in Figure 6. The stability of the alkoxyamine conjugate was not tested because equilibrium-driven deconjugation was already apparent during synthesis. The relative stabilities of each type of linkage are dependent upon the identity of the linkage.

Aniline conjugates were rapidly unstable; we observed multiple new peaks, some attributable to addition of H_2O, reaching 30% deconjugation after 36 h, Fig S10, which continued until the end of the experiment at 60 h. This may be thermally driven, as a previous report shows storage of a similar conjugate in PBS at 4 °C does not result in deconjugation.[16] HOT-thioethers were also unstable over time, with conjugate decreasing almost 40% in 24 h, Fig 6a, with multiple new species forming as a result, Fig S11, including Tyr fragmentation, as described above.

For THIOMAB™ Fabs with HOP conjugates, we found a time-dependent increase in 1 and 2 O-atom additions on the conjugated Fab prior to deconjugation, Fig S12. After deconjugation, the O-atom modification remained on the THIOFAB™ fragment, suggesting oxidation on the Fab precedes deconjugation. The rate of aryl thioether ox-

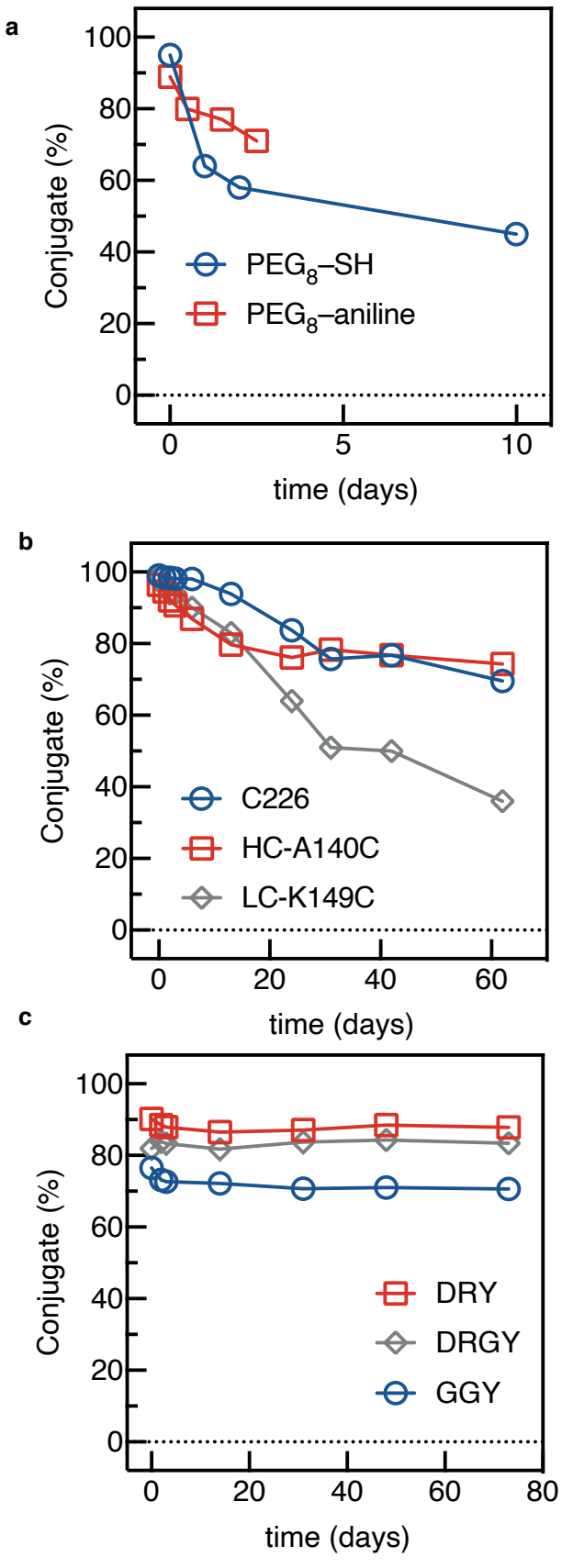


Figure 6. Stability of tyrosinase-mediated bioconjugates against PBS, pH 7.4, 37 °C. Conjugates were purified using KappaSelect affinity chromatography, formulated in PBS by dialysis, and stored at 37 °C. Deconjugation was monitored by LCMS. Remaining conjugate was calculated as a % of deconvoluted mass peak abundances. The timecourse of intact conjugate remaining are shown for (a) nucleophilic (HOT), (b) electrophilic (HOP), and (c) Diels-Alder (ABCD) attachments, respectively.

idation was dependent upon local environment, as A140C, K149C and C226 demonstrated significantly different oxidation and deconjugation rates. Interestingly, we found that the amount of HOP-cysteine deconjugation plateaued after ~30-60 d, Fig 6. This suggested that continued deconjugation might depend upon consumption of dissolved oxygen in the buffered system.

In contrast to the nucleophilic conjugates, ABCD linkages prepared from TCO reagents appeared to be indefinitely stable under these mild conditions. We did not observe any subsequent modification or deconjugation of TCO ABCD conjugates at any timepoints up to 90 d, Fig 6c.

DISCUSSION

Tyrosine is most often found buried in hydrophobic pockets of proteins, a core component of packing interactions that define protein folding; it is rarely found accessible on protein surfaces. If tyrosine is engineered into an accessible site it can be specifically modified chemoenzymatically with tyrosinase and exogenous nucleophiles or dienophiles. Similarly, alkyl phenols used as reagents can also serve as "on demand" electrophiles, generated in situ by enzymatic transformation to *o*-quinones, that react with rapid kinetics to modify cysteine.

The reaction conditions described meet all the requirements of mild protein bioconjugation: dilute substrate (μM), neutral pH (6–7.5), absence of co-solvent, room temperature, and low stoichiometry of exogenous reagents (1–5 equiv mol^{-1}). The catalyst (tyrosinase) is readily available from commercial sources in sufficient purity and activity, and it can be produced and activated recombinantly from bacterial expression.[26]

Competing reactions during chemoenzymatic generation of dopaquinone

The rapid reactivity of dopaquinone and *o*-quinone highlights an intrinsic liability of this conjugation method. If the secondary chemical reaction is insufficiently rapid, the *o*-quinone functional group can find alternative reaction pathways that do not lead to product formation. A well-known example is *o*-quinone homodimerization. A second non-productive route is hypothesized to be proton abstraction followed by *o*-quinone methide elimination from tyrosine, resulting in protein backbone fragmentation.

Our results demonstrate that, for antibody fragments, the phenol of tyrosine can be accessed with minimal extension from the protein surface. While direct fusion of tyrosine onto a protein terminus was modestly reactive, a single amino acid spacer is sufficient to allow access to the tyrosinase active site. In this way tyrosine can be thought of as an alternative to standard site-directed cysteine installation for bioconjugation, in that it doesn't require a flanking consensus sequence for enzymatic specificity, only the structure of the sidechain phenol.

Chemical modification of natively encoded amino acids in a site-specific manner remains a challenge. By installing target amino acids that are not commonly found on protein surfaces, like cysteine, it is possible to engender proteins with sites that are preferentially modified. An alternative method to achieve selectivity is to harness the pre-evolved specificity of enzymes. Examples like FGE, TG, and sortase are able to discern canonical amino acids from their counterparts through surrounding sequence. To this growing list of tools, we would like to encourage others to explore the use of tyrosinase for site-specific bioconjugation. As with any method for protein conjugation, it is not perfect. The high reactivity of the *o*-quinone can lead to side products and fragmentation. But high reactivity is also a very useful tool that can be harnessed by protein chemists for efficient site-specific bioconjugation. The reactions are fast, selective, and mild. Some conjugates are stable against deconjugation, especially those from Diels-Alder cycloaddition, and methods to avoid oxidation-dependent deconjugation of thiol conjugates are under investigation.

MATERIALS

Distilled water (in-house facility generation) was deionized (18 MΩ). Tyrosinase from mushroom (T3824), 3-(4-Hydroxyphenyl)propionic acid NHS ester (H1256), tyramine (T90344), *N*-acetyl-cysteine (A7250), 4-(2-aminoethyl)aniline (123056) were purchased from Sigma-Aldrich and used as received. 3-(2-aminoethyl)aniline (51444) was purchased from AstaTech and used as received. The m-PEG_8-amine (BP-21111), DBCO-PEG_4-acid (BP-23760), endo-BCN-PEG_8-

acid (BP-23768), TCO-PEG_3-acid (BP-22420), m-PEG_8-thiol (BP-21118), m-PEG_8-NHS ester (BP-21103) were purchased from Broadpharm and used as received. Antibody fragments were generated in *E. coli* using a previously described expression and purification procedure.[27] Those incorporating site-specific cysteine mutations were prepared using "deblocking" procedures described for the production of THIOMAB antibodies.[24]

METHODS

RP-HPLC-MS—Reversed-phase high performance liquid chromatography mass spectrometry was performed on a 1290 Infinity II coupled inline to mass detection on a 6530 Q-TOF (Agilent). Sample separation was achieved on an AdvanceBio Diphenyl 2.1 x 75 mm column (Agilent) enclosed in the thermostat-controlled column compartment at 80 °C. UV and TIC integrations, and mass deconvolutions, were performed using MassHunter v7.1 (Agilent).

FPLC—Fast performance liquid chromatography was performed on a GE Healthcare Akta Protein Purification System. Antibody fragment conjugates were purified by affinity capture on KappaSelect (GE Healthcare).

Synthesis of Anilines

To a solution of m-PEG_8-NHS (5.0 mg, 0.0098 mmol, 1 equiv) in DMF (200 μL) in a 1.5 mL conical centrifuge tube was added either 4-(2-ethylamino)aniline or 3-(2-ethylamino)aniline (6.6 μL, 6.6 mg, 0.049 mmol, 5 equiv) as a neat liquid. The tube was briefly vortexed and allowed to react at room temperature. After 1h, RP-LCMS analysis on a C18 column showed complete conversion of the NHS PEG to the corresponding m-PEG_8-aniline derivative. The reaction was diluted to 2 mL with deionized water and purified directly by RP-HPLC on a Phenomenex Kinetex 5 μm XB-C18 250 x 10.0 mm (100 Å pore size) column using a 0.1% TFA in MeCN/water gradient elution (5% MeCN to 95% MeCN in 30 minutes at 3 mL/min). The fractions containing product were diluted 2x with pure water, frozen, and lyophilized in a pre-weighed vial. The purified material was dissolved directly in water to a concentration of 10 mM. Product $[M+H]^+$: 531.328 (calc'd); 531.331 (found).

Synthesis of m-PEG-phenol

To a solution of 3-(4-hydroyphenyl)propionic acid (56.7 mg, 0.25 M) in DCM (714 μL) in a glass scintillation vial charged with a Teflon stir-bar was added m-PEG8 amine (90.9 mg, 275 mM, 1.1 equiv. mol^{-1}) as a solid. The reaction was stirred until all components were dissolved at room temperature. After 20 min, TLC revealed complete consumption of the phenol starting material. The mixture was purified by normal phase chromatography with a gradient of 5-15% MeOH in DCM, giving 51 mg of m-PEG8-phenol, (97%). The purified material was evaporated and then dissolved directly in water to a concentration of 10 mM and analyzed by RP-LCMS for purity (98%) and identity: product $[M+H]^+$: 532.312 (calc'd); 532.356 (found).

Chemoenzymatic conjugation of nucleophiles to Fabs with engineered tyrosine residues (HOT conjugation)

Standard reactions conditions consisted of 50–100 μM tyrosyl Fab, 1–3 mol. equiv. of nucleophile mol^{-1} of tyrosine, pH 6, and 1% mol^{-1} tyrosinase. A specific example is as follows: to a solution of Fab–GGY (2 mg, 455 uL at 4.4 mg/mL in sodium acetate, 20 mM pH 5.0) in a 1.5 mL conical centrifuge tube were added buffer (MES, pH 6.0, 67 μL at 0.5 M), water (120 μL), m-PEG_8-thiol (8.5 μL at 10 mM), and tyrosinase (16.8 μL at 1.1 mg/mL), resulting in a reaction composition of Fab (3 mg/mL, 63.7 μM), 2 mol. equiv. thiol (127.5 uM), buffer (20 mM), and tyrosinase (640 nM, 1% mol^{-1}). The tube was sealed and incubated at 25 °C while vortexing at 500 RPM. After 1 h, the solution was purified by KappaSelect affinity chromatography, concentrated to 1.2 mL, and dialyzed against PBS, pH 7.4 (200 vol. equiv.). Fab [M]: starting material 47066.7 (calc'd); 47069.3 (found). Product: 47480.9 (calc'd); found 47483.7.

Chemoenzymatic conjugation of phenols to Fabs with engineered cysteine residues (HOP conjugation)

Standard reactions conditions consisted of 50–100 μM cysteine Fab, 1–3 mol. equiv. of phenol mol^{-1} of cysteine, pH 6, and 1% mol^{-1} tyrosinase. A specific example is as follows: to a solution of FabC (5 mg, 546 μL at 9.16 mg/mL, in sodium acetate, 20 mM pH 5.0) in a 1.5 mL conical centri-

fuge tube were added buffer (MES, pH 6.0, 250 μL at 0.5 M), water (404 μL), m-PEG_8-phenol (26.9 μL at 10 mM in water), and tyrosinase (23.4 μL at 5 mg/mL), resulting in a reaction composition of Fab (4 mg/mL, 86.1 μM), 2.5 mol. equiv. phenol (215.2 μM), buffer (100 mM), and tyrosinase (2.15 μM, 1% mol^{-1}). The tube was sealed and incubated at 25 °C while vortexing at 500 RPM. After 1.5 h, the solution was purified by KappaSelect chromatography, concentrated to 1.0 mL, and dialyzed against PBS, pH 7.4 (200 vol. equiv.). Fab [M]: starting material 47006.4 (calc'd); 47005.4 (found). Product: 47552.16 (calc'd); found 47548.8.

Chemoenzymatic conjugation of dienophiles to Fabs with engineered tyrosine residues (ABCD conjugation)

Standard reactions conditions consisted of 50–100 μM tyrosyl-Fab, 3–10 mol. equiv. of dienophile mol^{-1} of tyrosine, pH 6, and 1% mol^{-1} tyrosinase. A specific example is as follows: to a solution of Fab-GGY (5 mg, 1.14 mL at 4.4 mg/mL in sodium acetate, 20 mM, pH 5.0) in a 1.5 mL conical centrifuge tube were added buffer (MES, pH 6.0, 133 μL at 0.5 M), water (7 μL), TCO-PEG_4-acid (10.6 μL at 50 mM in water), and tyrosinase (46.2 μL at 5 mg/mL), resulting in a reaction composition of Fab (3.75 mg/mL, 79.7 μM), 5 mol. equiv. TCO (398 μM), buffer (50 mM), and tyrosinase (3.98 μM, 1% mol^{-1}). The tube was sealed and incubated at 25 °C while vortexing at 500 RPM. After 1.5 h, the solution was purified by KappaSelect chromatography, concentrated to 1.0 mL, and dialyzed against PBS, pH 7.4 (200 vol. equiv.). Fab [M]: starting material 47066.7 (calc'd); 47066.9 (found). Product: 47498.18 (calc'd); found 47498.24.

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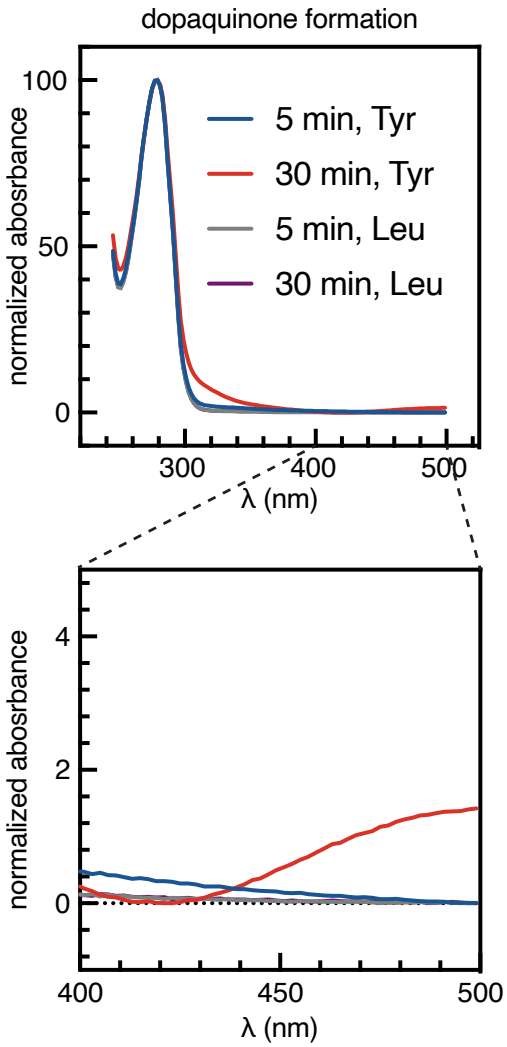

Figure S1. UV spectrum of transiently generated dopaquinone. The full UV/Vis absorption spectrum was extracted from the RP-LC peak corresponding to the Fab MS shown in Fig 1.

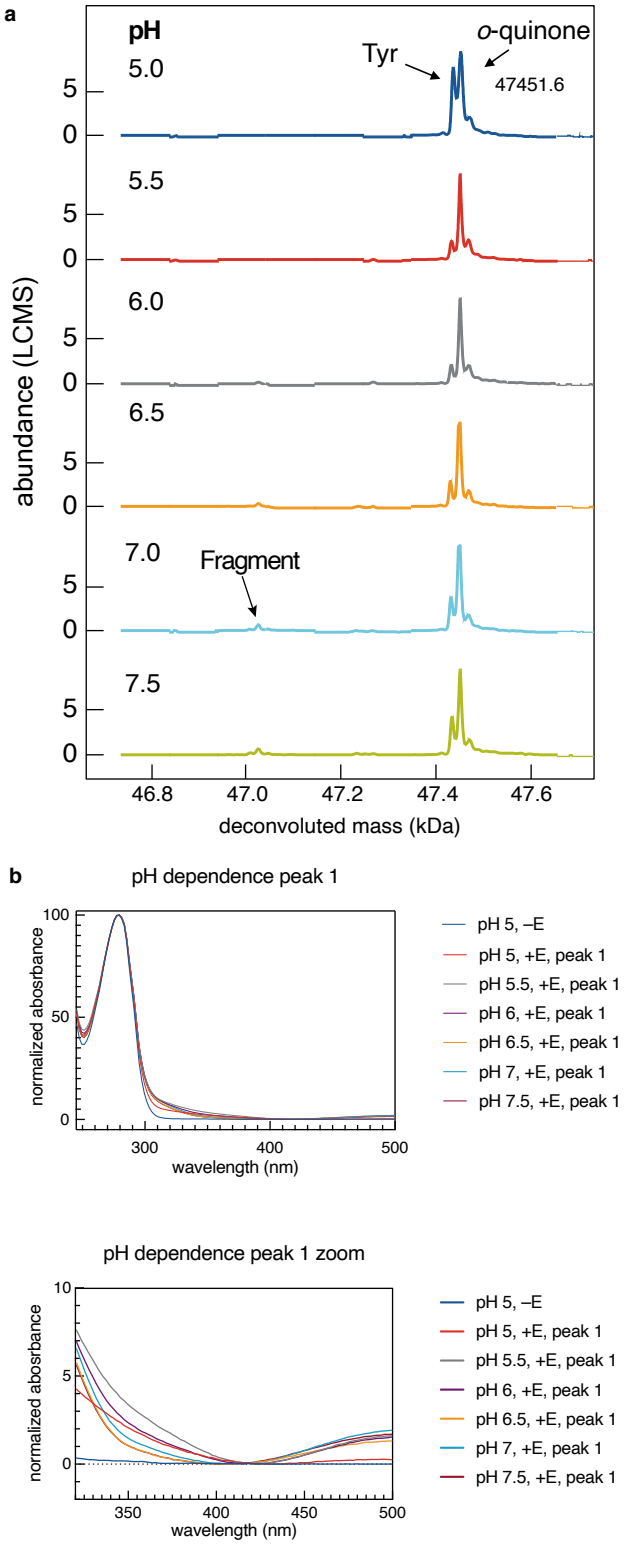

Figure S2. pH dependence of dopaquinone formation in situ. (a) MS spectra of a Fab containing an engineered Tyr residue upon treatment with tyrosinase across a range of pH values, after quenching with 1% TFA after 30 min of reaction. (b) UV/Vis absorption spectra of the peaks from (a).

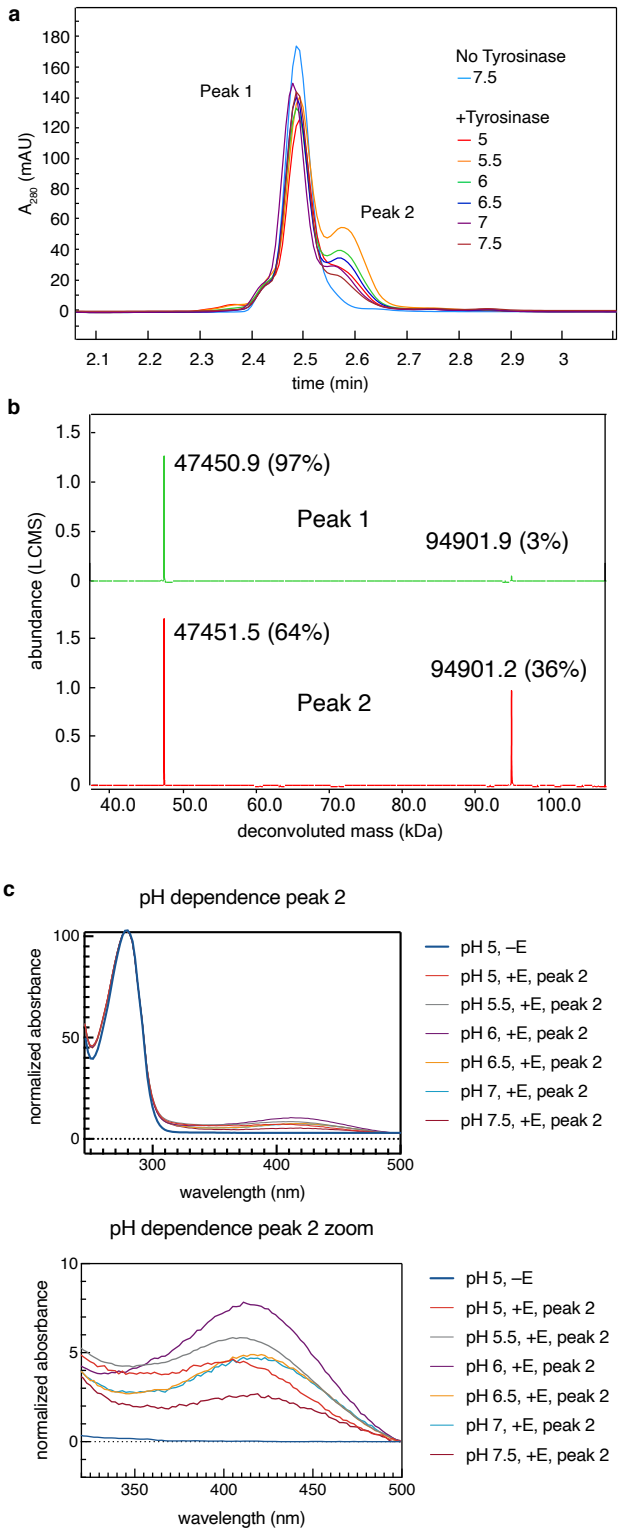

Figure S3. Formation of Fab dimer through intermolecular quinone crosslinking. (a) RP-LC chromatographic separation of two peaks across a range of reaction pH values. (b) Deconvoluted MS spectra of peak 1 (Fab M+14) and 2 (Fab M+14, 2x) for the pH 6 sample. Peak % are based on deconvoluted MS abundances, not UV spectrum integration. (c) UV/Vis absorption spectra of peak 2 across a range of pH values.

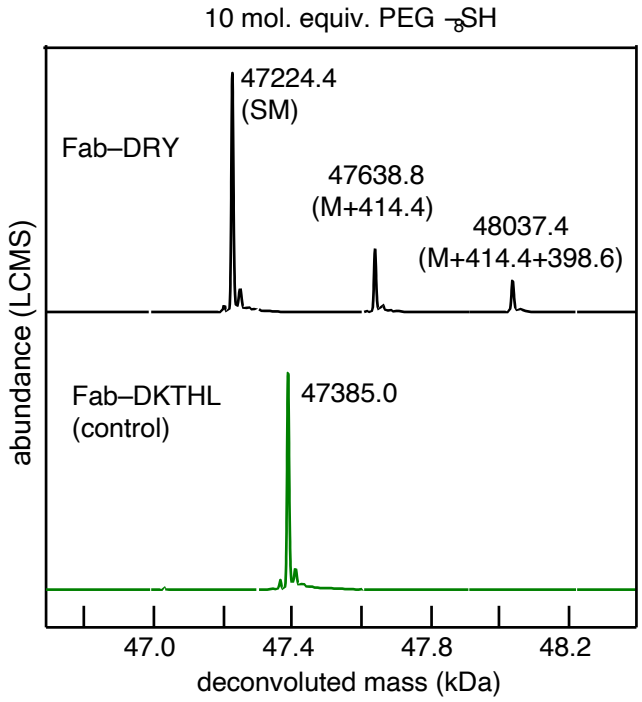

Figure S4. Formation of PEG_8-thiol HOT conjugate with excess nucleophile. Reactions consisted of 75 μM Fab–DRY or Fab–DKTHL, 750 μM (10 mol equiv.) PEG_8-thiol, 2 μM tyrosinase, pH 6.75. Shown are the deconvoluted mass spectra from samples quenched with 1:1 vol/vol 1% TFA at the times indicated.

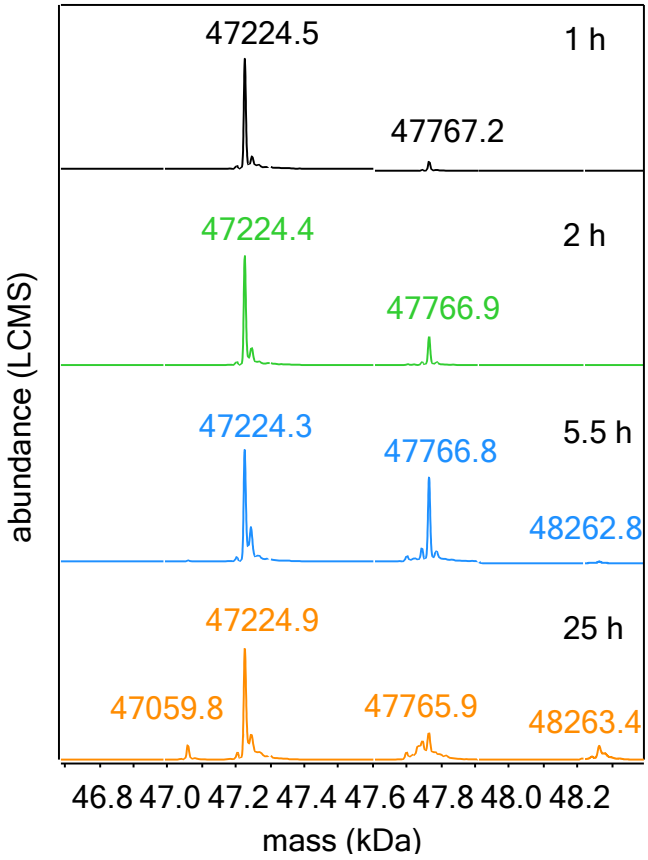

Figure S5. Timecourse of formation of PEG_8-*m*-aniline HOT conjugate. Reaction consisted of 75 μM Fab–DRY, 1 mM (13.3 mol equiv.) PEG_8-aniline, 2 μM tyrosinase, pH 6.75. Shown are the deconvoluted mass spectra from samples quenched with 1:1 vol/vol 1% TFA at the times indicated. Main peaks in each cluster are labeled with deconvoluted masses.

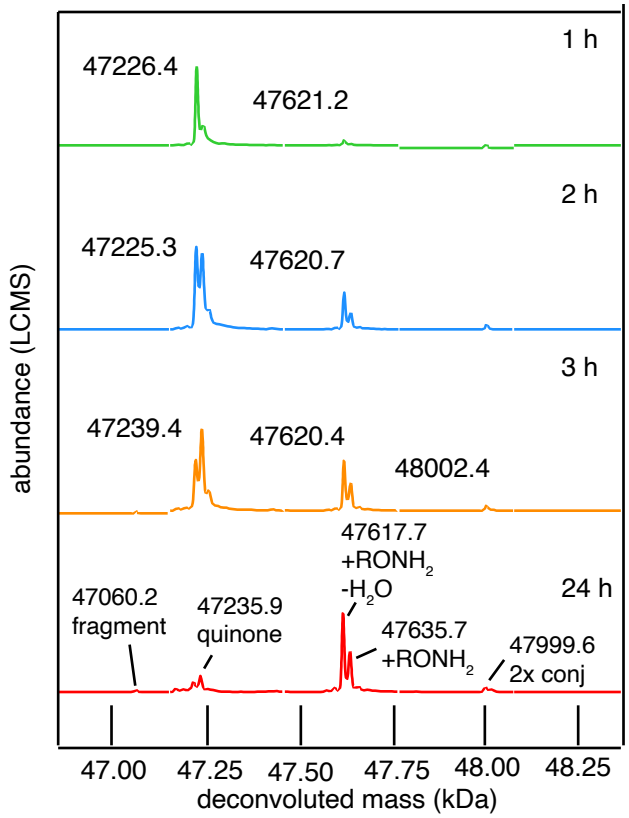

Figure S6. Timecourse of formation of PEG_8-alkoxyamine HOT conjugate. Reaction consisted of 75 μM Fab–DRY, 1 mM (13.3 mol equiv.) PEG_8-alkoxyamine, 2 μM tyrosinase, pH 6.75. Shown are the deconvoluted mass spectra from samples quenched with 1:1 vol/vol 1% TFA at the times indicated. In the 24 h spectrum, conjugate peaks and dehydrated byproducts are labeled with deconvoluted masses.

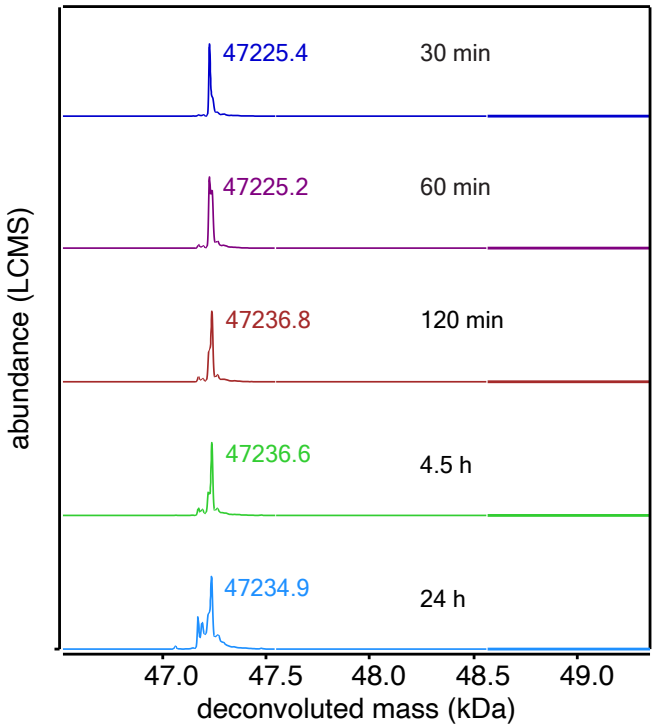

Figure S7. Timecourse of reaction of PEG_8-amine with dopaquinone formed in situ. Reaction consisted of 75 μM Fab–DRY, 375 μM (5 mol equiv.) PEG_8-amine, 2 μM tyrosinase, pH 8. Shown are the deconvoluted mass spectra from samples quenched with 1:1 vol/vol 1% TFA at the times indicated. The expected peak would appear at M+383.5 Da (47.5 kDa).

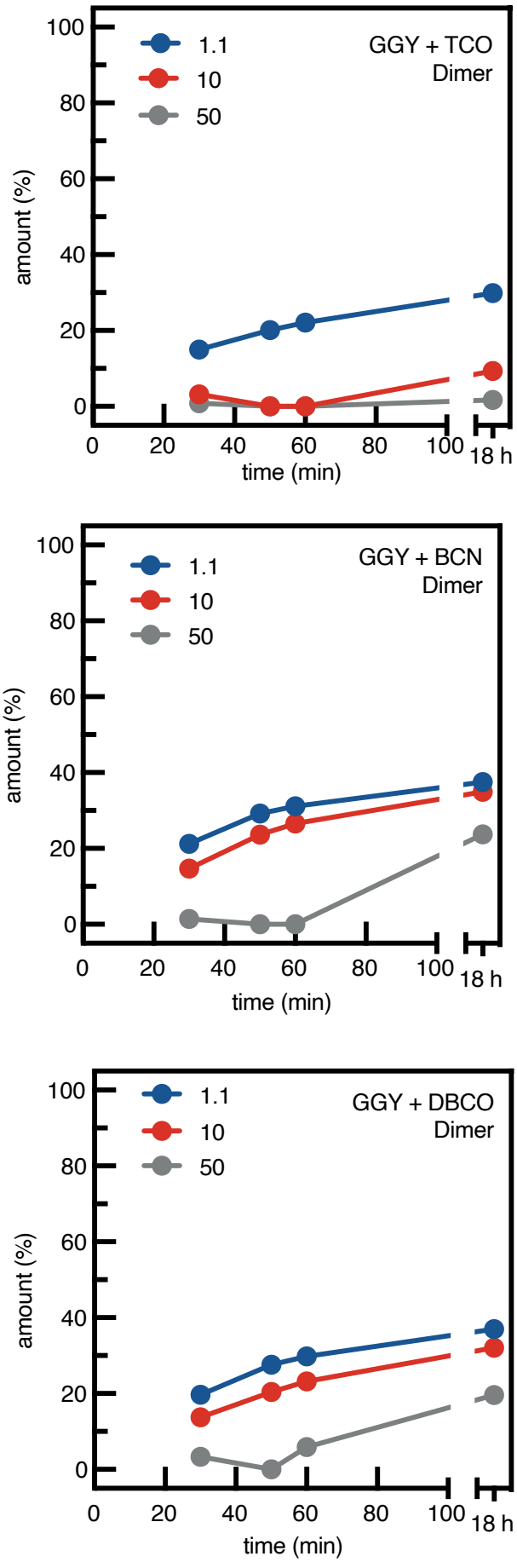

Figure S8. Timecourse of Fab dimer formation during ABCD conjugation of Diels-Alder reaction partners. Reaction conditions consisted of 50 μM Fab, 1 μM tyrosinase, pH 6.

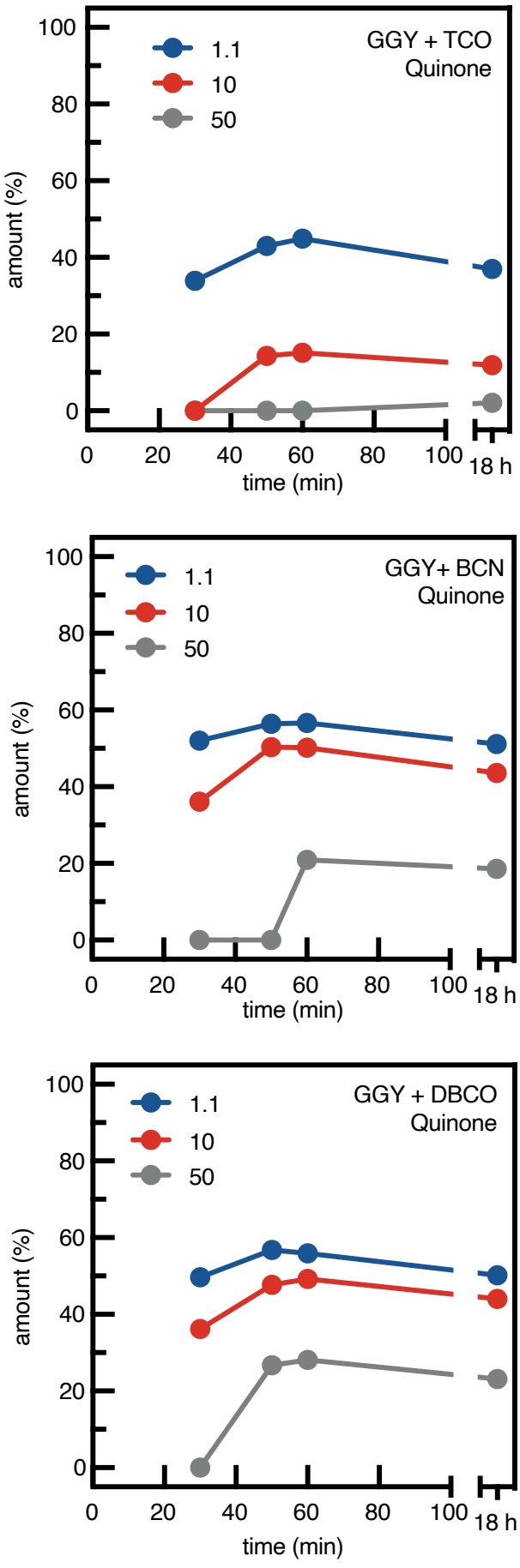

Figure S9. Timecourse of Fab dopaquinone formation during ABCD conjugation of Diels-Alder reaction partners. Reaction conditions consisted of 50 μM Fab, 1 μM tyrosinase, pH 6.

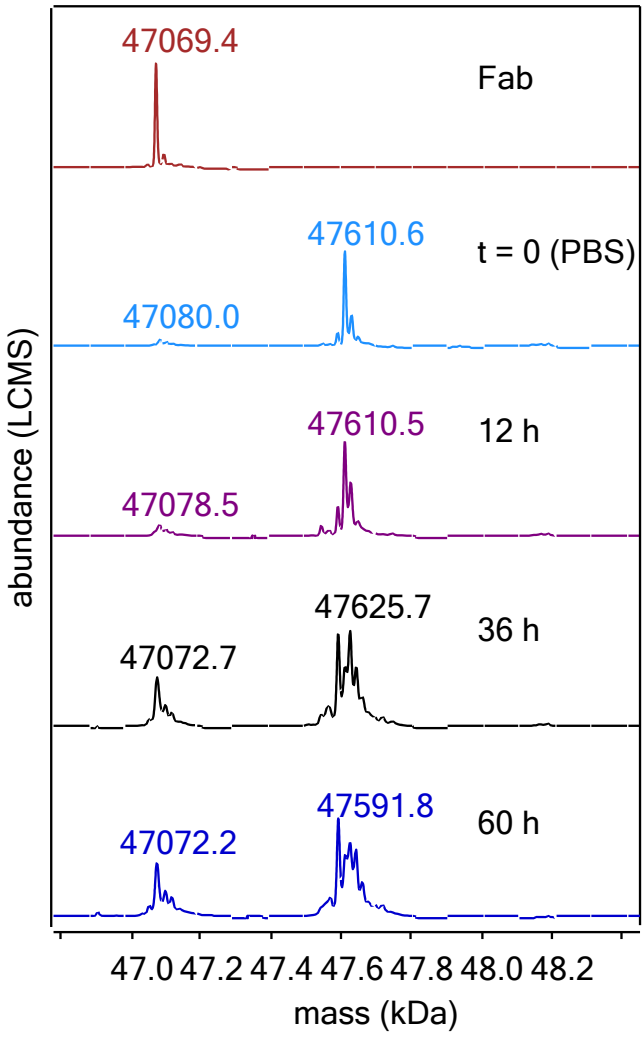

Figure S10. Stability of PEG_8-*p*-aniline HOT conjugate against PBS, pH 7.4, at 37 °C. Conjugate was purified using KappaSelect affinity chromatography, formulated in PBS by dialysis, and stored at 37 °C. Shown are the deconvoluted mass spectra from samples quenched with 1:1 vol/vol 1% TFA at the times indicated.

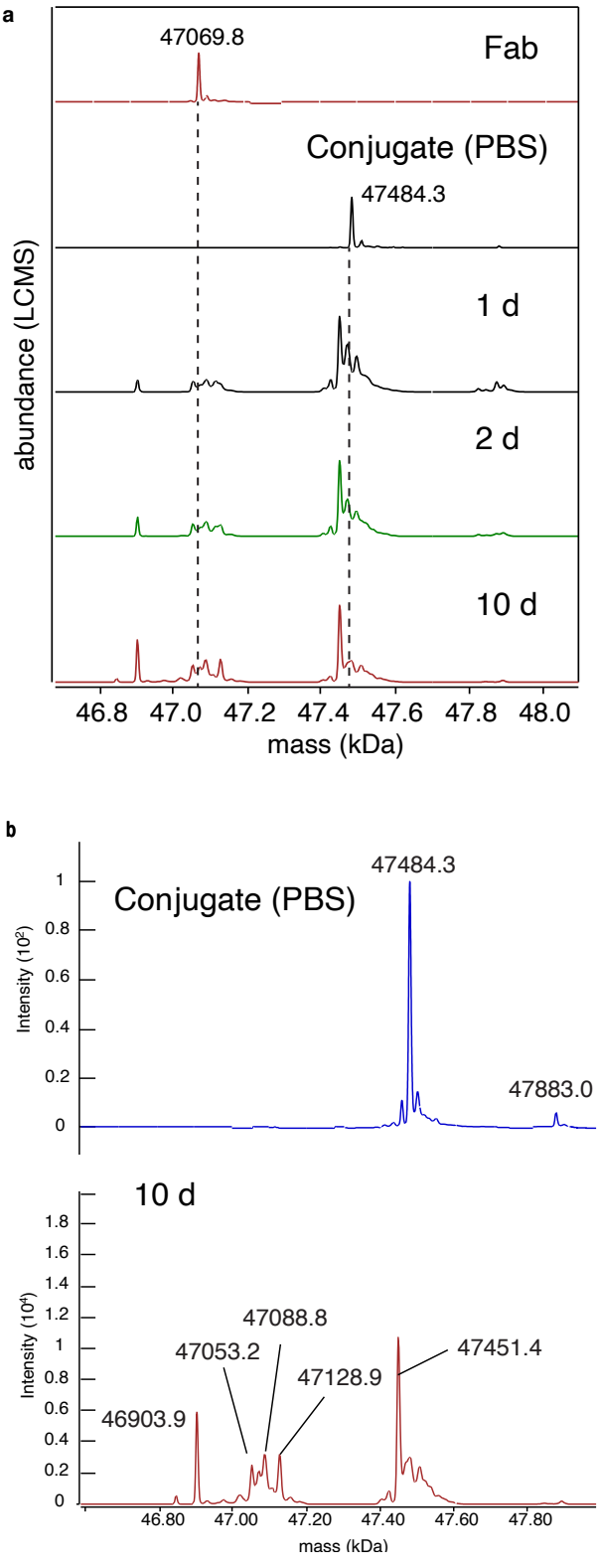

Figure S11. Stability of PEG_8-thiol HOT conjugate against PBS, pH 7.4, at 37 °C. Conjugate was purified using KappaSelect affinity chromatography, formulated in PBS by dialysis, and stored at 37 °C. Shown are the deconvoluted mass spectra from samples quenched with 1:1 vol/vol 1% TFA at the times indicated.

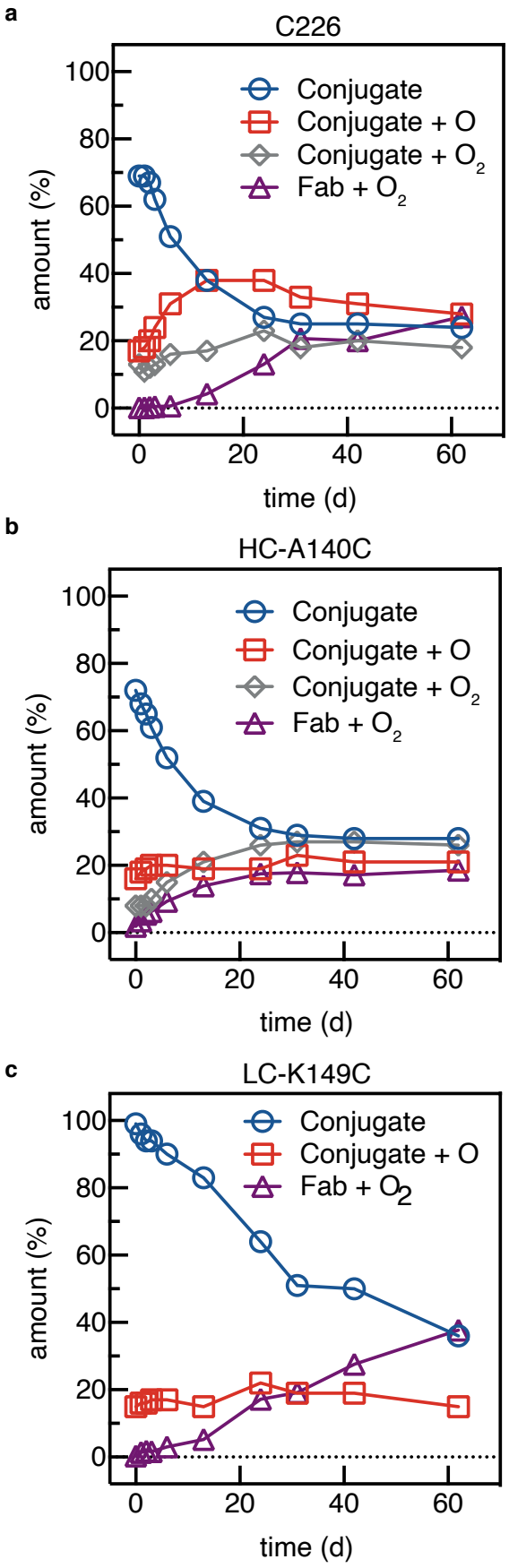

Figure S12. Stability of HOT thioether linkages is oxidation dependent. Species detected during the evaluation of stability of HOT linkages at (a) HC-C226 (FabC), (b) HC-A140C, and (c) LC-K149C.