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Review

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Recent progress in selective bioconjugation

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ABSTRACT

Selective installation of proteins using chemical reagents is important for the development of potential biomaterials for the treatment of human diseases. However, modification in a chemo- and regioselective manner under physiological conditions is a great challenge due to the presence of multiple reactive centers in the protein. Currently, the majority of conjugations are limited to lysine (Lys)- and cysteine (Cys)-selective reagents. Thus, they have been extensively studied. Apart from Lys and Cys, widespread site selectivity has been recently achieved through most of the 20 naturally occurring amino acid-bearing reactive functional groups. Consequently, this review focused on several recent achievements in site-selective modification of the rarest amino acid backbones (e.g., methionine, serine, glutamic acid, and tyrosine).

Key Word: bioconjugation, antibody, chemoselective, site-selective

Introduction

The development of a new methodology for covalent bond derivatization between chemical reagent (exogenous moiety) and selective amino acids of peptides, proteins, and antibodies while preserving the native function is important for the construction of potential biomaterials and antibody-drug conjugates (1). Generally, the on-demand site-selective homogeneous product formation helps accomplish the required application. In this context, a large number of site-selective late-stage diversification of proteins exist. However, the development of a new methodology has increased biomolecule integrity via chemo- and regioselective modifications by maintaining a stable bond under physiological conditions (2,3).

Homogenous selective construction has been achieved via bioorthogonal chemistry and genetic engineering through the incorporation of reactive functional groups (e.g., alkyne, azide, or ketone containing noncanonical amino acids) (4,5). However, the direct modification of the native protein would be a preferable option as there is no need for a genetic manipulation step. Researchers have majorly used lysine (Lys) and cysteine (Cys) conjugation in the last few decades using various electrophiles (e.g., maleimides, N-hydroxysuccinimide (NHS) ester, and α-halo carbonyls). Generally, heterogeneous population of conjugates is formed on the surface because most of the peptides that contain highly distributed Lys. However, the Cys moiety does not exist in the free form on the protein surface because of the oxidation and formation

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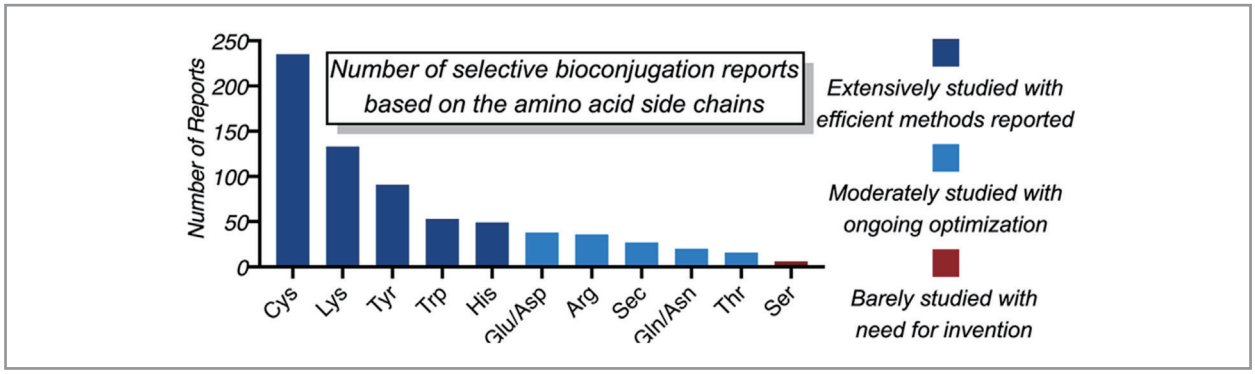


Figure 1. Selective bioconjugation reported based on amino acid sidearms.

of disulfide bonds. These disulfide bonds are very important for stabilizing the three-dimensional structure of the protein. Reductive pretreatment occurs at the initial step to generate the reactive thiol group from Cys for modification. However, this type of pretreatment damages protein structure and function (6). Recently, various site-selective conjugations have been reported using most of the naturally occurring amino acids (Figure 1). Typically, probe construction is preferred based on amino acid reactivity and the environment. This review covers the recent progress of site-selective modification of the rarest amino acids (e.g., methionine, serine, glutamic acid, and tyrosine).

Serine bioconjugation

Serine is one of the 20 naturally occurring amino acids holding various functional groups at their variable regions. Unfortunately, the functionalization of serine (in both chemo- and site-selective bioconjugations) has not been extensively studied due to its poor nucleophilicity compared to other available nucleophilic residues (e.g., lysine, cysteine, and tyrosine, and so on) (7). No reports currently depict the site-selective bioconjugation of serine residues on the native biomolecule. A new chemical approach would be of great interest in addressing the unmet challenge of serine bioconjugation. Nevertheless, such an approach provides site-selective protein labeling, as well as chemoselective peptide modifications (8). Naturally, various amino acids undergo phosphorylation through kinases based on this process (9). In addition, Baran et al. developed a P(V)-based reagent that acts as an electrophile, which typically exhibits an innate reactivity toward alcohol-based nucleophiles (10). Further, these reagents (based on the P(V) oxidation state) show noticeable reactivity toward serine residues. Thus, this approach turned out to be the first general method for bioconjugation of serine residues by overcoming other competing amino acid residues (11). First, reaction conditions were optimized using the P(V)-1 reagent with serine. An unprecedented reactivity with serine was then reported even in the presence of various competing amino acids (e.g., lysine, cysteine, threonine, tyrosine, and selenocysteine). For serine bioconjugation, the most competing amino acid was threonine. Using this method for labeling resulted in almost seven-fold higher reactivity for serine compared to threonine (Figure 2). In all the conjugations, the labeling conversions were comparable with reported protein modifications (e.g., 4-methyl-3H-1,2,4-triazoline-3,5(4H)-dione for tyrosine/tryptophan, iodoacetamide for cysteine, and NHS ester for lysine). Furthermore, competing experiments were performed using tetrapeptides with P(V)-1, P(V)-2a, and P(V)-3 reagents. Moreover, applications were also explored using linear and cyclic peptides containing various nucleophiles. Overall, labeling was moderate (19%) to excellent (100%). Finally, the serine residue protein (proteasome

targeting) was successfully labeled with ubiquitin. Site selectively at the S65 residue was confirmed using liquid chromatography with tandem mass spectrometry (MS/MS). In addition, this conjugation method was applied to efficiently label the DNA-binding 434 protein. The mechanism was evaluated to distinguish serine selectivity over other amino acid residues using density functional theory studies. Overall, the current method delivered extraordinary labeling for the bioconjugation of serine residues in both peptides and proteins. However, a few limitations need to be addressed, such as minimizing the usage of higher reagent concentrations, the stability of P(V)-based reagents in an aqueous medium, optimization of reaction conditions in the pure aqueous medium, and low-efficiency labeling, especially for proteins.

Methionine modification

Cysteine and methionine are two of the essential sulfur-containing and naturally occurring amino acids among the 20 amino acids found in proteins. In the context of chemoselective bioconjugation of methionine, Chang et al. explored a new strategy, termed redox-activated chemical tagging, utilizing oxaziridine-based reagents. Two mechanistic approaches were discussed when oxaziridine was in aqueous conditions. Oxygen and nitrogen transfer products yielded sulfoxide and a stable sulfimide product, respectively (12). Furthermore, a copper-assisted azide-alkyne cycloaddition (CuAAC) reaction was demonstrated in model proteins for antibody-drug conjugates (ADCs) using sulfimide-intermediated methionine bioconjugates with alkyne-containing oxaziridine and substituted azides. In addition, the elaborative systematic approach showcases the stability of sulfimide with the help of various oxaziridine substrates in the development of methionine-selective bioconjugation (13). Similarly, Gaunt et al. described an eminent alternative strategy to show methionine bioconjugation in proteins and peptides via the formation of sulfonium conjugates with the help of hypervalent iodonium salts. In the same attempt, due to the electrophilic nature of diazo-containing sulfonium salts, single electron transfer via the photocatalytic reaction was investigated and the removal of the diazo group affording trialkyl sulfonium salt conjugate was achieved (14).

Recently, Allentoff et al., inspired by Chang et al. and Gaunt et al., further explored this chemoselective methionine bioconjugation and applied it to site-selective fluorine-18 labeling of model proteins and peptides (15). The azide-containing precursor reported by the same group was utilized for labeling (16). Initially, the viability of this approach demonstrated that N-acetyl methionine methyl ester conjugated with alkyne-containing oxaziridine was radiolabeled with 70% of the radiochemical yield within 10 min. Similarly, when alkyne-containing iodonium salts were used, CuAAC cycloaddition resulted in low radiochemical yield and unmodified starting methyl ester, as well as triazole-containing alcohol, formation as the major product due to the interaction of diazo sulfonium

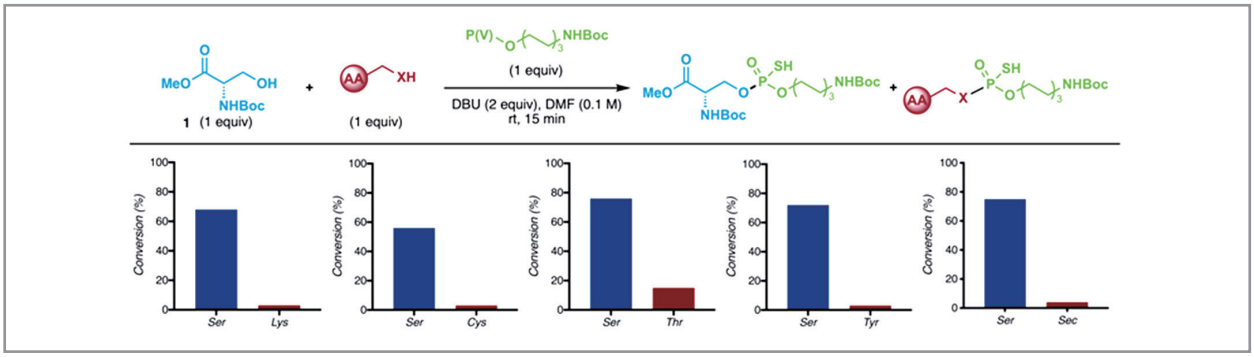


Figure 2. Competing experiments with various amino acids in the presence of serine.

salts with copper(I) catalyst. This issue was resolved by utilizing the photocatalytic reaction reported by Gaunt et al. In addition, the diazo-containing sulfonium salt was irradiated in the presence of the Hantzsch ester and iridium catalyst to reduce the diazo group, forming a stable trialkyl sulfonium salt, affording 75% radiochemical conversion after CuAAC with a fluorine-18-labeled azide precursor (Figure 3). Finally, 76% of labeling was achieved when applied to the methionine-containing peptide fragment of the human fibroblast growth factor 21. Following peptides, alkyne-modified bovine serum albumin (BSA) was radiolabeled with fluorine-18. Moreover, using a ligand-free copper catalyst resulted in a low yield (44%), whereas radiochemical conversion (81%) was observed by the inclusion of the copper ligand tris(3-hydroxypropyltriazolylmethyl) amine. Overall, labeling was recently explored for chemoselective bioconjugation focusing on methionine in proteins and peptides to the applicative site-selective fluorine-18.

Selective modification of aspartic and glutamic acids

Generally, free carboxylic acids are present in the C-terminus or sidearm of aspartic and glutamic acids in proteins. Carboxylic acid reactivity in the protein is poor because of the presence of both hydrogen bond donors and acceptors. Thus, the selective conjugation of this carboxylic acid is not easy (17). To date, the available method is limited to both carboxylic acid conjugations in proteins and living cells. Recently, Raines (2015) and Lei et al. (2018) separately demonstrated the esterification in the carboxylic acid group of a protein with a highly reactive diazo compound (18,19). In 2017, Waldmann et al. established the covalent modification of glutamic acid using Woodward's reagent K (20). However, these approaches are not utilized in live cells. In 2017, Li et al. reported the covalent modifications of Glu and Asp in living cells using highly reactive tetrazole-based probes under UV irradiation (21).

In 2020, the same group developed highly reactive azirines, probe-like aliphatic, di-aromatic substitution,

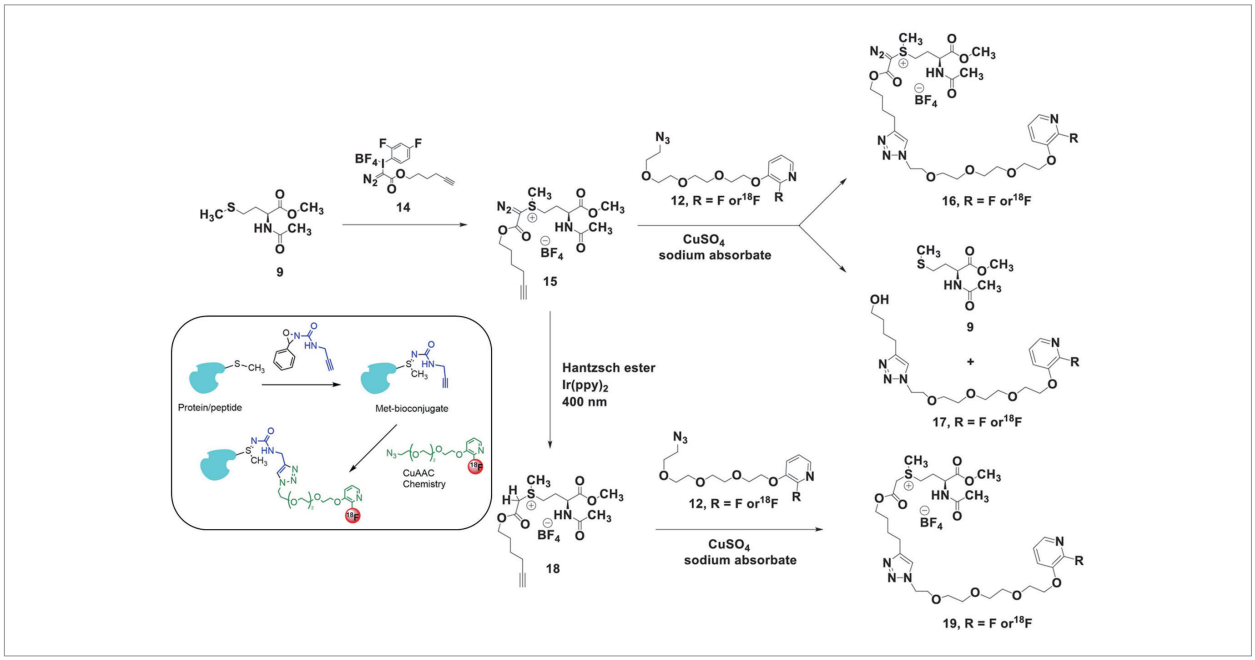


Figure 3. Site-selective fluorine-18 labeling via CuAAC reactions after the conjugation of iodonium salt and methionine methyl ester-forming alkyne-containing diazo sulfonium salt. The methionine-selective conjugation of a protein with an oxaziridine-bearing alkyne followed by a CuAAC reaction with fluorine-18-labeled azide (*inset*).

and monoaryl containing 2H-azirines, 2,3-diphenyl-2H-azirines, and 3-phenyl-2H-azirine for selective carboxylic acid functionalization (Figure 4). Consequently, phenyl 2H-azirine was observed to selectively react with a carboxylic acid under physiological conditions along with the stable adduct N-phenacylacetamide formation (22). However, an unstable ester was formed with the respective probe in previous bioconjugation methods. The ability of the synthesized probe to check for the easily available enzymes, BSA, ribonuclease A, myosin, β-amylase, and human serum albumin were used next. Under these conditions, 3-phenyl-2H-azirine showed that the modification of the Glu site was much easier than that of Asp due to the lower steric hindrance of the glutamic acid.

Further, selective glutamic acid labeling for ALDHI expression was carried out in vitro and in situ. The low concentration of the probe was sufficient for labeling in this condition. Moreover, the docking experiment explained that the 3-phenyl-2H-azirine probe was accommodated well in ALDHI expression. This experiment explained the selectivity of the amino acids in both in vitro and in situ conditions. The reactivity profile of the phenyl 2H-azirine probe in mammalian live cells (MCF-7, THP1, HeLa, and HEK293) showed high selectivity. The probe showed 98.5% selective labeling of Glu and Asp in the MCF-7 cell line. To quantitatively measure the reactivity of probe labeling on the carboxylic acid, the well-established isotope–isotopic tandem orthogonal proteolysis activity-based protein profiling method was explained. In this experiment, 1,749 binding sites from 583 proteins were identified, and 95.9% of these were proved to be Asp and Glu.

Tyrosine-selective modification

In 2010, Barbas et al. reported the Tyr-selective bioconjugation-type click-like reaction using 4-phenyl 3H-1,2,4-triazole-3,5(4H)-diones (PTAD). In this case, highly reactive PTAD was stable in acetonitrile, whereas it decomposed to phenyl isocyanate under aqueous (physiological) conditions (23,24). These isocyanide substrates reacted easily with Lys to generate urea as a byproduct (this issue was controlled using tris buffer-isocyanide scavenger). Additionally, various PTAD analogs have been used for protein modification with fluorescent marker attachment to proteins, PEGylation, ADCs, glycoconjugate vaccines, and protein-DNA conjugates. In addition, di-ortho functionalization of the tyrosine moiety and cross-reactivity with Cys were disadvantaged in this condition. Consequently, Guands et al. recently demonstrated the potential electrochemical tyrosine click (e-Y click) method to solve all the limitations (25). In this case, the applied potential controlled the

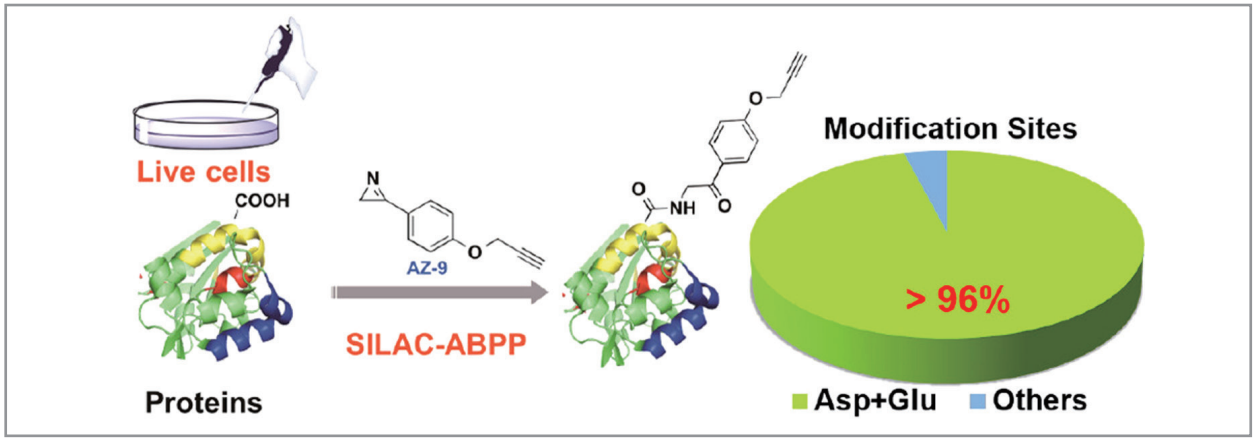
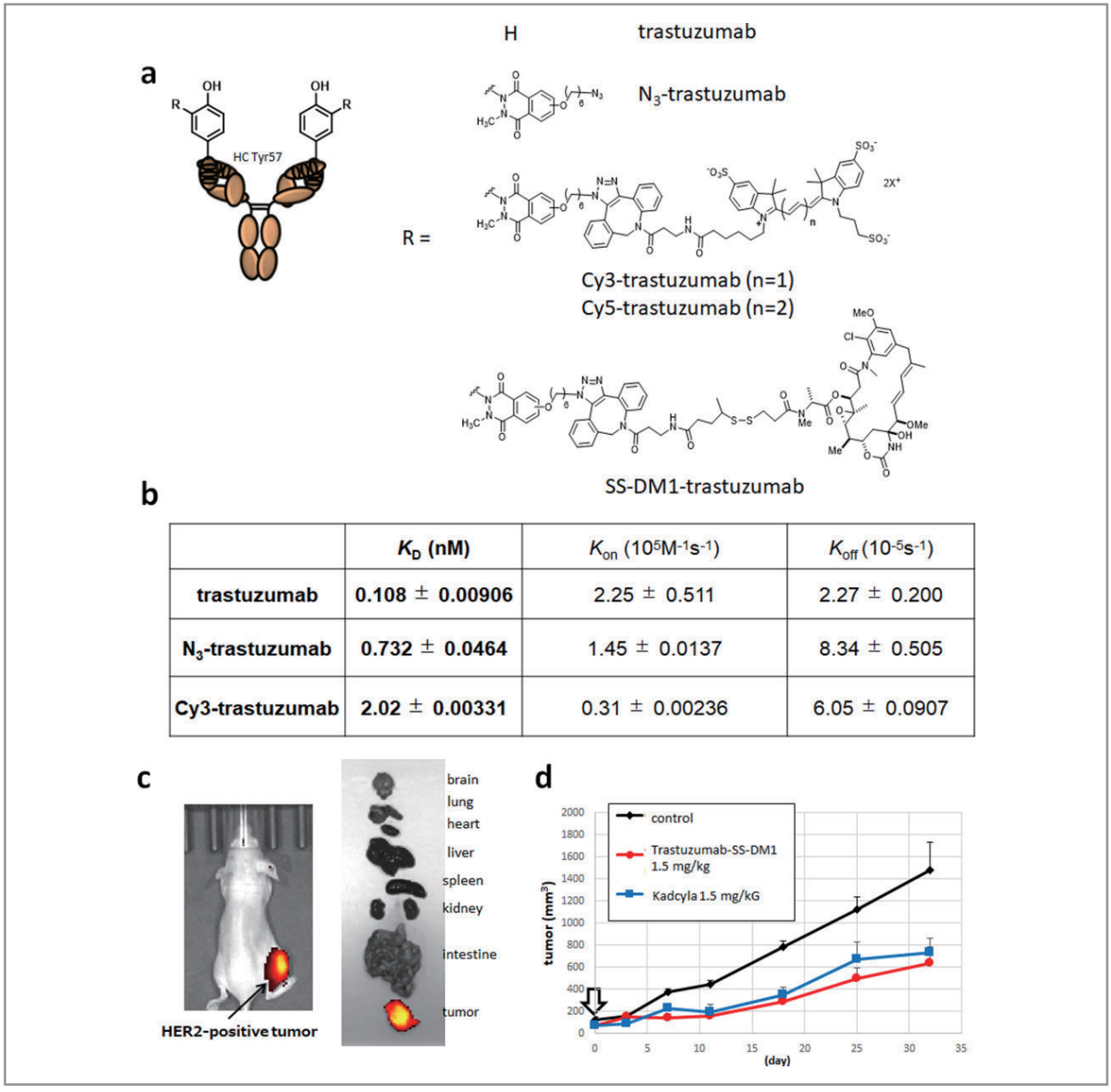


Figure 4. *2H*-Azirine-based reagents for chemoselective bioconjugation at carboxyl residues inside live cells.

oxidation of amino acids and conjugate products. More significantly, the PTAD decomposition and byproduct formation were reduced. This method was demonstrated to be more competent than chemical oxidants to synthesize PTADs. In addition, the conjugation ability with the Tyr moiety on BSA doubled the value compared with chemical oxidation (26,27). This groundbreaking result encouraged researchers to focus more on the electrochemical modification of Tyr-selective labeling.

Recently, Nakamura et al. utilized luminol for selective Tyr conjugation to avoid the side reaction of PTADs (28). Precisely 95% conjugation was achieved with angiotensin II using a hemin/H_2O_2 catalyst with N-methyl luminol. Interestingly, MS/MS data clearly showed that no electrophilic byproduct was formed, and selectivity was more predominant with the Tyr moiety. However,



	K_D (nM)	K_{on} ($10^5 M^{-1}s^{-1}$)	K_{off} ($10^{-5}s^{-1}$)
trastuzumab	**0.108 ± 0.00906**	2.25 ± 0.511	2.27 ± 0.200
N_3-trastuzumab	**0.732 ± 0.0464**	1.45 ± 0.0137	8.34 ± 0.505
Cy3-trastuzumab	**2.02 ± 0.00331**	0.31 ± 0.00236	6.05 ± 0.0907

Figure 5. Tumor imaging and antitumor activity. *a)* Modified antibody structures; *b)* HER2 binding affinities of antibodies using the biolayer interferometry; *c)* Cy5-modified trastuzumab, in vivo distribution; *d)* antitumor activities of anti-HER2 ADCs in the NCI-N87 xenograft tumor model (female BALB/cSlcnu/nu mice, n = 5).

the addition of excess H_2O_2 significantly damaged the Cys moiety, as confirmed by adding maleimide to the Tyr conjugation in the BSA protein. The result showed that almost 40% of the Cys moiety could not be modified with maleimide. This unwanted protein damage was minimized using horseradish peroxidase (HRP) catalyst with a lower quantity of H_2O_2 or nicotinamide adenine dinucleotide (29). Interestingly, laccase with atmospheric oxygen dissolved in the solution showed better results compared with the HRP/H_2O_2 or electrochemical PTADs (e-Y click) method. Still, this protocol showed a 38% Cys oxidation, whereas it was 49% using the HRP method. However, e-Y click showed only 15% oxidation. Based on this observation, the same group in 2020 demonstrated that the luminol substrate showed a more efficient substrate for the e-Y click method for Tyr conjugation (27).

Furthermore, this method was utilized for the selective modification of proteins containing a more solvent-accessible Tyr-containing substrate. This was confirmed by reinforcing the modification in this exact condition. For example, the modification of Y83 occurred in streptavidin. Similarly, Y57 occurred in trastuzumab. All four solvents were observed to access Tyr upon rituximab modification. The modification did not affect the antigen-binding affinity. The antibody Tyr moiety modified with a drug molecule demonstrated selective cytotoxicity. The drug-to-antibody ratio was <2 and exhibited comparable activity to Kadcyla in the NCI-N87 xenograft tumor model (Figure 5).

In summary, researchers have developed only a handful of highly reactive probes for site-specific conjugation based on the amino acid variable site for the advancement of ADCs. This development opened up various possibilities of modifications along with the traditional Cys and Lys modification methods. Still, the achievement of site-selective bioconjugation of Ser, Thr, and Gln/Asn is very limited. This review discussed the recent progress in the site-selective modification of the rarest amino acids (e.g., methionine, serine, glutamic acid/aspartic acid, and tyrosine). However, increasing the homogeneity and rate of the reaction under physiological conditions for the development of biomaterials and ADCs is still needed. Future studies are needed for additional progress in the potential synthetic protocol for site-selective modification of proteins and antibodies as very important areas for theranostic approaches and development of ADCs.

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