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Recent progress of chemical methods for lysine site-selective modification of peptides and proteins

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ABSTRACT

Chemical modification of native peptides and proteins is a versatile strategy to facilitate late-stage diversification for functional studies. Among the proteogenic amino acids, lysine is extensively involved in post-translational modifications and the binding of ligands to target proteins, making its selective modification attractive. However, lysine's high natural abundance and solvent accessibility, as well as its relatively low reactivity to cysteine, necessitate addressing chemoselectivity and regioselectivity for the Lys modification of native proteins. Although Lys chemoselective modification methods have been well developed, achieving site-selective modification of a specific Lys residue remains a great challenge. In this review, we discussed the challenges of Lys selective modification, presented recent examples of Lys chemoselective modification, and summarized the currently known methods and strategies for Lys site-selective modification. We also included an outlook on potential solutions for Lys site-selective labeling and its potential applications in chemical biology and drug development.

1. Introduction

The selective modification of peptides and proteins plays a vital role in disclosing post-translational modifications (PTMs), studying protein-protein interactions (PPIs), monitoring cellular physiological and pathological processes, and developing therapeutic drugs [1–8]. Compared to *de novo* synthetic and chemical biology methods, chemical modification of native peptides and proteins provides a more versatile approach to achieve late-stage diversification for functional studies [9–14]. Among the 20 proteogenic amino acids, cysteine (Cys) represents an attractive target for peptide/protein modification due to the high reactivity of its thiol group in physiological conditions and its low natural abundance, and a variety of modification reagents or methods have been well developed [15–19].

Lysine (Lys), another amino acid with high nucleophilicity, is extensively involved in PTMs and the binding of ligands to target proteins, rendering its selective modification meaningful [20–23]. In addition, numerous proteins lack free Cys residue due to its low natural abundance, with most Cys being buried inside through the formation of disulfide bonds. Consequently, disulfide bond reduction is required for Cys modification, and the operation may perturb the natural structure and function of proteins, making direct modification of Lys residue a preferable choice [24]. However, given the high natural abundance and solvent accessibility of Lys, as well as its relatively low reactivity compared to Cys, dozens of active Lys residues can exist in a single protein, making achieving both chemoselectivity and regioselectivity simultaneously the primary consideration for the Lys selective modification of native proteins (Fig. 1) [25].

Despite significant advancements in research related to Lys chemoselective modification, site-selectivity, another aspect of Lys selective modification, is much less resolved, and modifying a single Lys residue site-selectively and effectively remains a considerable challenge, yet it is meaningful [26–28]. Such as antibody-drug conjugates (ADCs), the majority of which in clinical use rely on random conjugation to Cys or Lys residues, resulting in a heterogeneous mixture of multiple ADCs types, each containing different numbers of drug molecules conjugated at diverse amino acid sites and possessing distinct pharmacokinetic, efficacy, and toxicity properties [29,30]. Consequently, there is a pressing pursuit for the development of novel chemical strategies or reagents to

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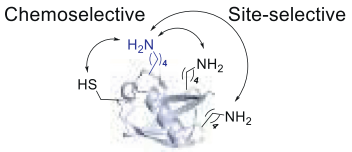

Fig. 1. Challenges in the lysine modification of native proteins.

achieve site-selective modifications of Lys in native peptides and proteins.

To provide an overview of the Lys site-selective modification, this review comprehensively categorized and analyzed reported Lys site-selective chemical modification methods based on the differences in modification reagents or strategies. At first, we covered some examples of Lys chemoselective modification methods. Subsequently, we provided an overview of the research advancements in Lys site-selective modification categorized into kinetically controlled site-selective labeling, proximity-directed modification, modified group transfer, and cooperative stapling of Lys with another amino acid. Finally, we discussed potential solutions for Lys site-selective modification and its application potential in chemical biology research and drug development. Overall, this review emphasized the application of modification reagents and strategies for Lys site-selective bioconjugation.

2. Chemoselective modification

The selectivity of protein modification initially depends on the factors that enable modification reagents to differentiate the reactivity of various reactive groups. Typically, deprotonated primary amines are the most nucleophilic in a native protein, while protonation drastically reduces their reactivity. In proteins, the α-amino group of N-terminus and ε-amino group of Lys residues are present, which possess pK_a values of approximately 8 (α-amino group) and 10 (ε-amino group) (Fig. S1 in Supporting information) [31]. Therefore, the ε-amino group of Lys requires a higher pH value for deprotonation and differentiation from α-amino groups. A pH range of 8.5–9.5 is generally suitable for modifying Lys residues. To date, a variety of reagents or strategies can accomplish Lys chemoselective modification, including electrophiles, photochemistry, and transition-metal catalysis [32,33].

2.1. Electrophiles

2.1.1. Activated esters

Activated esters represent a class of ideal reagents for Lys bioconjugation due to their good reactivity and acceptable hydrolysis stability, and including *N*-hydroxysuccinimide (NHS) esters, sulfotetrafluorophenyl (STP) esters, HOBt esters, and thioesters, *etc.* have been used in Lys-targeted covalent drug, protein proofing, *etc.* NHS activated esters were developed in forming the peptide bond by Anderson *et al.* in 1964 and are widely used in bioconjugation today (Fig. 2A) [34]. NHS esters conjugate with proteins *via* a very stable aliphatic amide bond and are considered among one of the most powerful protein modification reagents, and widely used in the elucidation of protein-protein or protein-drug interactions, protein structural analysis, and preparation of peptide/protein conjugates with drugs or other molecules [35]. STP esters are another class of Lys selective active esters and they are more stable than NHS esters in aqueous media. STP esters have been successfully applied to the preparation of antibody-drug conjugates and quantitative profiling of global reactive Lys in human cell proteomes (Fig. 2A) [36]. More recently, Li *et al.* developed pyridinium-based activated esters for selective Lys labeling, and this approach was applied to activity-based protein profiling in MCF-7 cells [37].

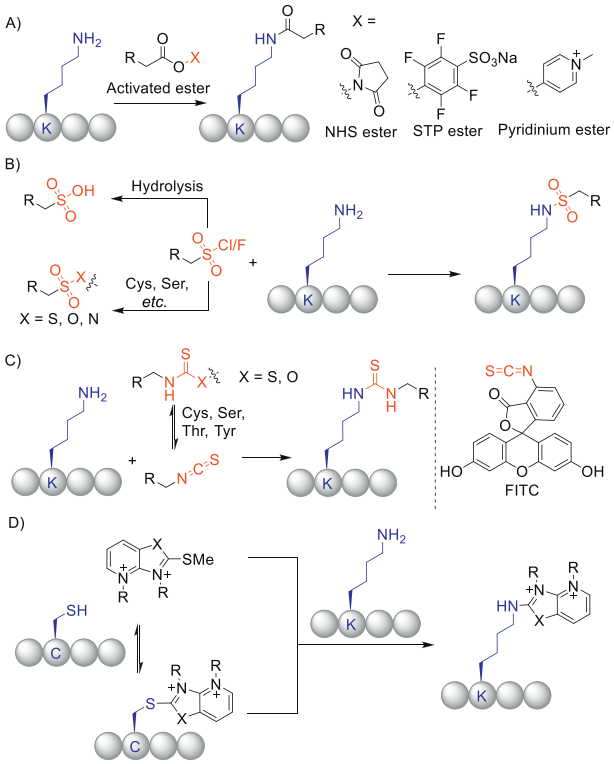

Fig. 2. Lys modification of proteins by electrophiles.

2.1.2. Sulfonyl halides and sulfonates

Dansyl chloride is the first widely used sulfonyl chloride in the modification of proteins and shows incontestable popularity for the study of protein structure and functions (Fig. 2B) [38]. Sulfonyl fluoride electrophiles are powerful privileged covalent warheads that can probe the binding sites of enzymes or drug targets and have found significant utility in medicinal chemistry and chemical biology as reactive probes [39]. Despite sulfonyl halides being highly reactive, they are very unstable and easily hydrolyzed to sulfonic acid in aqueous media, especially at a high pH required for Lys deprotonation. Besides, sulfonyl halides are less selective than NHS esters due to their high reactivity towards nucleophiles, and they can also conjugate with cysteine, serine, threonine, tyrosine, and histidine in proteins (Fig. 2B).

2.1.3. Isocyanates and isothiocyanates

Isocyanates can react with amines to form stable urea derivatives, however, they are difficult to manipulate due to the instability to deterioration during storage. Isothiocyanates, which are commercially accessible, possess moderate reactivity and good stability in most solvents, making they are much more popular in Lys bioconjugation. Despite Cys and Tyr could react with isothiocyanates to give dithiocarbamates and *O*-thiocarbamates, respectively, the addition processes are reversible (Fig. 2C) [40]. Fluorescein isothiocyanate (FITC) is one of the most commonly used fluorescent reagents for protein labeling and has proven to be effective in diverse applications. However, conjugates prepared by isothiocyanates are less hydrolytically stable compared to those by NHS esters, and NHS esters labeling proceeds at lower pH, resulting in NHS esters being preferable to isothiocyanates for Lys bioconjugation in many respects (Fig. 2C).

2.1.4. Tunable amine reactive electrophiles

In 2022, Raj group discovered a series of tunable amine reactive electrophiles (TAREs) with good hydrolytic stability and high activity for Lys selective modification of proteins (Fig. 2D) [41]. This

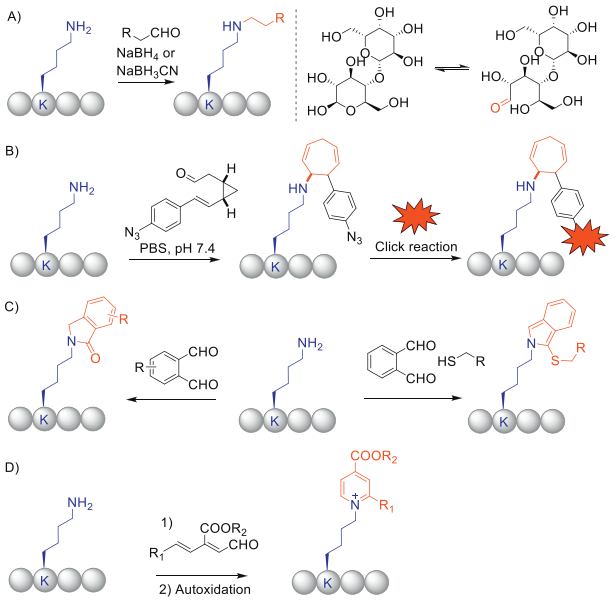

Fig. 3. Aldehyde derivatives for Lys modification of proteins.

type of TAREs reagent could react with Cys reversibly to generate another Lys-reactive electrophilic thioether and both the two thioether species could be nucleophilic attacked by Lys to generate stable modified products. Besides, The TAREs are highly tunable due to their reactivity and selectivity could be varied between Cys and Lys through the addition of different heteroatoms and their charged methylation.

2.2. Aldehyde and ketone derivatives

Amines can react with aldehyde or ketone derivative to form an imine intermediate, which can further be selectively reduced by sodium cyanoborohydride *et al.* to give a stable alkyl amine (Fig. 3A) [42]. Reductive amination of proteins generally proceeds at a pH range of 6.5–8.5, has low kinetics of conjugation, and the sodium cyanoborohydride may reduce disulfide bonds within proteins, so that this method is not used in Lys conjugation as frequently as the activated ester or isothiocyanate method. Francis group discovered Cp*Ir bipyridyl complex could be an alternative of typical reducing agents to the reductive alkylation of proteins *via* iridium-catalyzed transfer hydrogenation [43]. This method has played a significant role in the preparation of protein-carbohydrate conjugates due to the semiacetal structure of carbohydrates (Fig. 3A).

In recent years, several novel aldehyde and ketone derivatives were developed for the Lys selective modification of proteins. Christmann *et al.* developed a class of Lys-reactive 2-(2-styrylcyclopropyl)ethanal aldehyde derivatives for the functionalization of proteins (Fig. 3B) [44]. The reagent exploits a divinylcyclopropane-cycloheptadiene rearrangement to render the initial imine condensation process irreversible (Fig. 3B). Li and coworkers reported that *ortho*-phthalaldehyde (OPA) derivatives can chemoselectively react with amino groups on peptides and proteins to form phthalimidines under physiological conditions. (Fig. 3C) [45]. These kinds of reagents were also applied to chemoselective peptide stapling and bicyclization by reacting with Cys and Lys in a three-component reaction manner (Fig. 3C) [46]. Besides, the OPA was also used by Chen and coworkers for chemoselective bioconjugation *via* one-pot clamping of two different amine nucleophiles [47]. Tanaka group reported that $\alpha,\beta,\gamma,\delta$-unsaturated aldehyde derivatives could condensate with

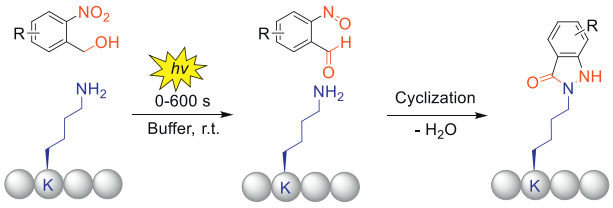

Fig. 4. Light-induced PANAC conjugation for Lys selective modification.

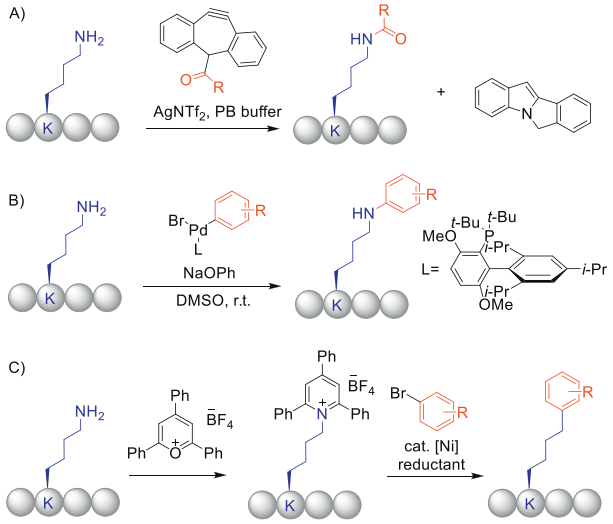

Fig. 5. Lys modification by transition-metal catalysis.

Lys selectively *via* a rapid 6π-aza-electrocyclization reaction, followed by autoxidation to generate stable pyridinium salt products (Fig. 3D) [48]. Besides, 2-carbonylbenzeneboronic acid derivatives and *o*-ester substituted diazonium terephthalates were also used in Lys bioconjugation (Fig. S2 in Supporting information) [49–51].

2.3. Visible-light-driven chemistry

Visible-light-driven chemistry has been widely used in labeling and cross-linking of biomacromolecules [2]. Chen group developed a light-induced primary amines and *o*-nitrobenzyl alcohols cyclization (PANAC) as a photoclick reaction to achieve the functionalization of small molecules and native biomolecules (Fig. 4) [52]. This method proceeded *via* addition/cyclization/dehydration/tautomerization pathway, and has been successfully applied to direct diversification of pharmaceuticals and biorelevant molecules, Lys-specific peptide modification and cyclization, protein labeling *in vitro* and in live cells, the capture of protein-protein interactions and protein-DNA interactions, and organelle-targeted temporal labeling in living systems (Fig. 4) [53,54].

2.4. Transition-metal catalysis

In 2020, Yan and Huang groups realized rapid Lys modification of peptides and proteins by switching the click reactivity of dibenzoazacyclooctynes (DBCO) from azide click component towards amine-reactive caged acylation reagent (Fig. 5A) [55]. The rearrangement of DBCOs precluded the click reactivity of DBCOs and provided a switched acylation reagent to amine substrate. This method has shown practical applications in the modification and fluorescent labeling of peptides, proteins, therapeutic antibody trastuzumab, and living bacteriophage.

Buchwald and coworkers developed a Lys arylation method for peptides using preformed or *in situ* generated [LPd(Ar)X] complexes (Fig. 5B) [56]. More recently, Watson *et al.* reported a Ni-catalyzed deaminative reductive coupling method of amino acid pyridinium salts with aryl bromides by leveraging high-throughput experimentation, enabling efficient synthesis of noncanonical aryl amino acids and late-diversification of peptides (Fig. 5C) [57]. However, both the two protocols proceeded under bioincompatible conditions and cannot apply to protein modification, limiting their application to some extent.

2.5. Others

Besides, water-soluble isatoic anhydride-based derivatives (Fig. S3A in Supporting information) [58], pyrocinchonimide, (Fig. S3B in Supporting information) [59], azaphilones (Fig. S4 in Supporting information) [60], 3-acyl-4-pyranone derivatives [61], imidoesters, squaric acid diester, and dichlorotriazine derivatives, *etc.*, were also used in Lys bioconjugation [16]. These above chemoselective methods provide valuable solutions to Lys bioconjugation of proteins, however, these methods make it difficult to distinguish multiple Lys existing in one protein. Achieving the site-selective modification of a specific Lys in proteins is of great significance in disclosing PTMs and drug development, and there is a great purpose for novel Lys site-selective modification methods.

3. Site-selective modification

Site-selective modification of proteins is vital for modulating their function and preparing homogeneous therapeutic agents. In nature, this process occurs at a specific residue within a protein sequence, is rigorously controlled by enzymes, and is generally involved in protein-protein interactions [62,63]. Despite the use of unnatural amino acids provides solutions to site-selective modification with good selectivity and reactivity, these methods often require tedious procedures to obtain pre-functionalization proteins and involve metal catalysis [64–66]. Currently, several strategies can effectively realize site-selective modification of a specific Lys in native peptides or proteins, including kinetically controlled site-selective labeling, proximity-induced modification, modified group transfer, and cooperative stapling of Lys with another amino acid (Cys, Arg, Tyr, *etc.*) [67,68].

3.1. Kinetically controlled site-selective labeling

Individual Lys residues in a protein have certain different reactivity towards modification reagents due to the local microenvironment surrounding each Lys, solvent accessibility, and higher-order structural features, thereby providing an opportunity for site-selective modification of the most active Lys residue directly. Bernardes group reported a computer-assisted design of sulfonyl acrylate derivatives for modifying a single Lys residue on native protein (Fig. 6A) [69]. The Lys residue with the lowest pK_a would be deprotonated at a slightly basic pH and was the kinetically favored residue. Moreover, modified products allowed for further bioconjugation through an aza-Michael addition of a nucleophile to the acrylate electrophile that was generated by spontaneous elimination of methanesulfinic acid upon Lys modification [27].

Activated esters were widely used in Lys site-selective modification of proteins *via* a kinetically controlled labeling approach [70]. Weil group reported the site-selective modification of lysozyme C, RNase A, and the peptide hormone somatostatin using NHS ester reagents (Fig. 6B) [71]. The method could site-selectively modify a single Lys residue with high reactivity on the surface of proteins at physiological pH and room temperature in a yield of

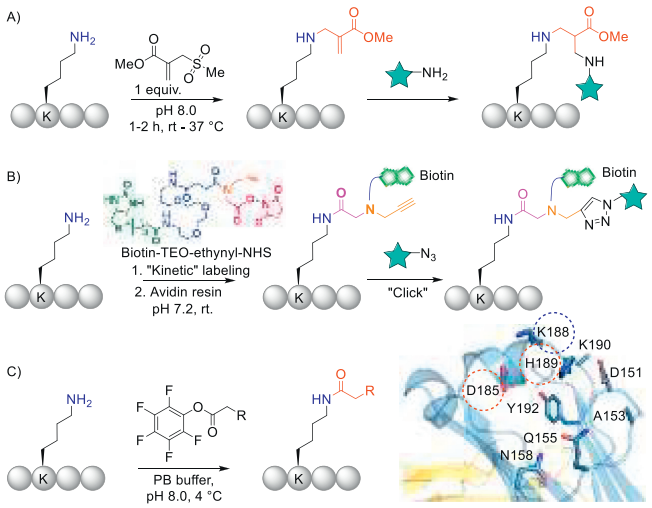

Fig. 6. Regioselective Lys modification by sulfonyl acrylate derivatives and activated esters. Reproduced with permission [72]. Copyright 2018, Wiley Publishing Group.

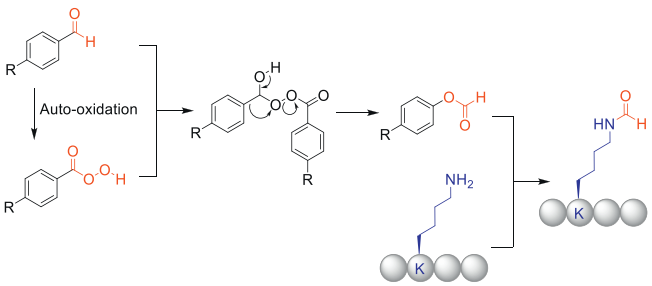

Fig. 7. Site-selective Lys modification by aromatic aldehyde auto-oxidation.

over 90%. Importantly, by employing multifunctional biotin-TEO-ethynyl-NHS (TEO: tetraethylene oxide) as a biorthogonal bioconjugation reagent, site-selective multi-functionalization of proteins was achieved and the products allowed for avidin resin purification and click chemistry (Fig. 6B). Besides, Cravatt group used STP ester for the investigation of global reactive Lys in the human proteome [36]. Fluorophenyl ester reagent was used by Cellitti *et al.* to label human kappa antibodies at a single Lys188 within the light-chain constant domain in a yield of 50%–70% [72]. The site-selectivity was attributed to the surrounding His189 and Asp151 which accelerated the labeling rate of Lys188 compared to other ~40 Lys sites (Fig. 6C). Besides, Rader group discovered β-lactam derivatives and heteroaryl methylsulfonyl-functionalized molecules could be applied to site-selective modification of h38C2, a humanized anti-hapten monoclonal antibody (mAb) that contains a unique reactive Lys99 (pK_a ~ 6) (Fig. S5 in Supporting information) [73,74].

In 2017, Rai *et al.* discovered a chemoselective and site-selective Lys formylation method for peptides and proteins enabled by aromatic aldehyde auto-oxidation (Fig. 7) [75]. Firstly, the aerobic oxidation of aromatic aldehyde can be initiated by a free radical chain reaction to form the corresponding peracid. Then, the nucleophilic addition of the peracid to another molecule of aldehyde generated a tetrahedral adduct, followed by the rearrangement of the tetrahedral adduct to afford the active formate reagent. Finally, the active formate ester was selectively trapped by the amine group to afford the formylated protein products. This protocol enabled the site-selective single-site formylation of Lys in a series of native proteins and offered late-stage modification. The site-selectivity was controlled by reversible protection of α-NH_2 of N-terminal by aldehyde, regulation of formate ester generation, and reduced effective concentration of Lys *via* imine formation.

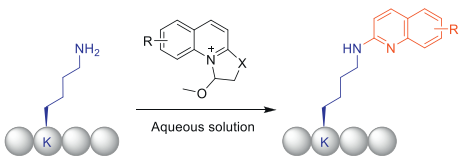

Fig. 8. Chemo- and site-selective Lys modification by water-soluble zolinium.

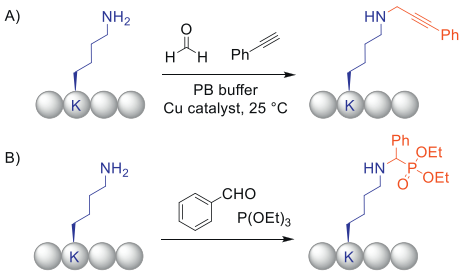

Fig. 9. Site-selective Lys modification by multicomponent approach.

In 2022, Zhu group reported a novel method for chemo- or site-selectively quinolylation of Lys residues in peptides, proteins, and living cells under mild conditions using their newly developed water-soluble zoliniums (Fig. 8) [76]. The site-selectivity of zoliniums was demonstrated by quinolylating Lys350 of bovine serum albumin (BSA), modifying and inactivating two highly conserved Lys residues (Lys5 and Lys61) of SARS-CoV-2 $3CL^{pro}$ protease that is essential for virus replication in host cells. Mechanism study indicated the Lys modification by zolinium mainly occurred in the hydrophobic or catalytically active pocket of proteins, where the negatively charged amino acid residues (such as glutamic acid) around the Lys may enhance the site-selectivity and feasibility of the modification.

In recent years, multicomponent approaches have been applied to site-selective labeling of native proteins. Rai group reported a multicomponent approach that involved protein, aldehyde, acetylene, and Cu-ligand complex to modify a single Lys residue in nine distinct proteins by constructing a C–C and C–N bond appending propargylamine motif (Fig. 9A) [77]. The site-selectivity was controlled by that aldehyde chemoselectively reacted with Lys in a fast and reversible manner and rendered latent stoichiometry acetylene reacted with imine intermediate in a rate-determining step, which served as a desired regulatory tool. However, this method showed limited modification efficiency, and a long reaction time was needed for a good conversion. This group also developed a phospha-Mannich protocol for chemoselective and site-selective modification of a Lys residue in native proteins without the use of a transition-metal catalyst by employing an aldehyde to form an imine with the amino group of Lys as an *in situ* electrophile, followed by nucleophilic alkylphosphite to site-selectively generate stable products (Fig. 9B) [78]. This method enabled the site-selective modification of mono-labeled Fab at Lys183 of the light chain.

Kinetically controlled Lys site-selective labeling has achieved certain success in the modification of a series of proteins. However, these methods that target the most reactive Lys in a protein often need strict control for the conjugation conditions (such as the pH of reaction buffer) and are generally unlikely to achieve completely selective modification as stoichiometric or substoichiometric modification reagents used. Besides, these methods have limited substrate tolerance and are generally limited to the modification of simple proteins.

3.2. Proximity-induced Lys site-selective modification

Proximity-induced site-selective modification chemistry links Lys selective reactive moiety with a nearby structural motif or functional group and *via* their binding with proteins to guide the site-selective labeling reaction of proximal Lys residue [79–81]. In recent years, several strategies have achieved proximity-induced Lys site-selective modification, including affinity labeling that uses small molecules, affinity peptide binders, or protein-protein interaction to guide the modification reagents towards the desirable Lys site, and covalent tethering that utilizes a temporary covalent attachment of a proximal amino acid site to enable the modification of the target Lys.

3.2.1. Affinity binding-directed site-selective modification

Lys site-selective modification directed by specifically binding of a small molecule ligand with a target protein was attractive and has gained widespread attention in Lys-targeted covalent drug discovery [21,82–84]. This strategy exploits a hybrid modification reagent that harbors a small molecule ligand with good affinity to a specific protein and an appropriately Lys reactive electrophilic warhead. During the modification process, the ligand reversibly binds with the protein *via* specific ligand-protein interaction to bring the warhead close to the desired Lys firstly, thereby facilitating covalent binding of the warhead with Lys residue inside or around the binding pocket (Fig. 10). Generally, the reactive group is relatively less reactive towards intermolecular Lys residues, and the reaction is accelerated by ligand-protein interaction to bring them together, thereby achieving the site-selective modification of Lys. Until now, a series of reactive moieties, including α,β-unsaturated sulfonamides (a), alkoxy-substituted *o*-nitrobenzoxadiazole (b), phenylbenzoate derivates (c), benzotriazole derivates (d), squarate derivates (e), *N*-acyl-*N*-aryl sulfonamide (f), *etc.*, have been developed for ligand-directed proximal Lys site-selective modification in proteins (Fig. 10) [85–91]. More recently, Li *et al.* reported that α-acyloxyenamide (g) prepared from ynamide through a one-step late-stage coupling reaction could engage conserved catalytic Lys residues in kinases with covalent inhibition [92]. Besides, the ynamide could also apply to amide bond formation [93–96].

Peptides generally possess higher specificity and affinity to protein targets compared with small molecules, and have exhibited great potential in affinity binding-directed proximal Lys site-selective modification. Xia *et al.* explored proximal Lys conjugation through fluorodinitrobenzene, isothiocyanate, and phenyl ester moieties guided by a specific protein-peptide interaction (Fig. 11) [97]. This strategy employed an affinity peptide with a Lys reactive motif and organized the Lys-reactive group to orient the ε-amino group of the proximal Lys in protein *via* the binding of peptide with protein. Site-selectivity of the protein-peptide interaction-guided Lys bioconjugation was examined on an SH3 domain and several ubiquitin-like proteins. Besides, Wang and coworkers developed a proximity-induced amino–yne reaction for Lys site-selective conjugation of MDM4 protein using propargylated-sulfonium tethered peptide that enables the reaction to occur at the selected Lys [98].

The affinity-directed conjugation strategy showed great potential in ADCs discovery [99–101]. In 2019, Yamada *et al.* reported an affinity peptide-mediated regiodivergent functionalization (AJICAP^{TM}) method to enable the site-selective modification of native IgG antibodies for preparing ADCs (Fig. 12A) [102]. The selectivity was controlled by specifically binding of affinity peptide with the Fc region of an antibody, thereby delivering the NHS ester moiety to the desired Lys residue *via* a linker with appropriate length. The method achieved site-selective modification of different amino acid sites in a monoclonal antibody with a yield of over

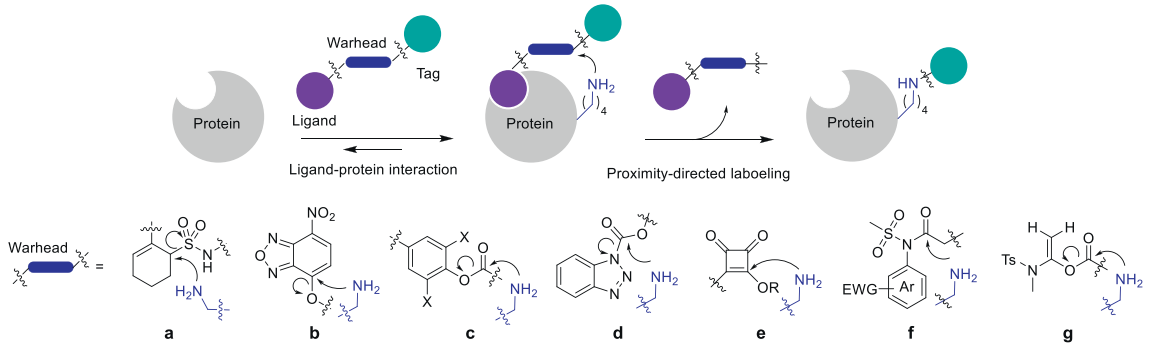

Fig. 10. Ligand-protein interaction directed Lys site-selective modification.

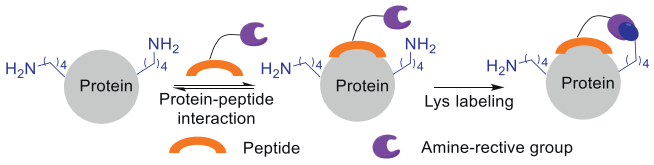

Fig. 11. Peptide-protein interaction directed site-selective Lys modification.

90% by simply changing the Fc affinity peptide reagents. Moreover, the synthesis of an ADC was achieved through the disulfide bonds reduction and cytotoxic payload conjugation *via* thiol-maleimide reaction, and the resulting product showed improved anti-tumor activity compared to the native trastuzumab antibody (Fig. 12A).

Xiao group developed a simple affinity labeling-directed antibody conjugation method to enable the preparation of homogeneous antibody conjugates from native antibodies without additional antibody engineering and post-synthesis treatments (Fig. 12B) [103,104]. This method installed a Lys reactive moiety in a chemically synthesized affinity peptide bearing a bioorthogonal handle for further payload conjugation. Upon specifically binding to a certain region of antibody, the affinity peptide would covalently attach to the antibody *via* spontaneous reaction of Lys reactive moiety with proximal Lys in the antibody, and the product could apply to bioorthogonal conjugation with a cytotoxic agent or fluorescent probe. The utility of this method was demonstrated by preparing well-defined ADC and multifunctional antibody conjugates with a variety of enhanced capabilities. Besides, Lam group reported a similar method for Lys site-selective modification *via* affinity binding of indole-based 5-fluoro-2,4-dinitrobenzene (FDNB) derivatized peptides with the conserved nucleotide-binding pocket (NBP) between the light and heavy

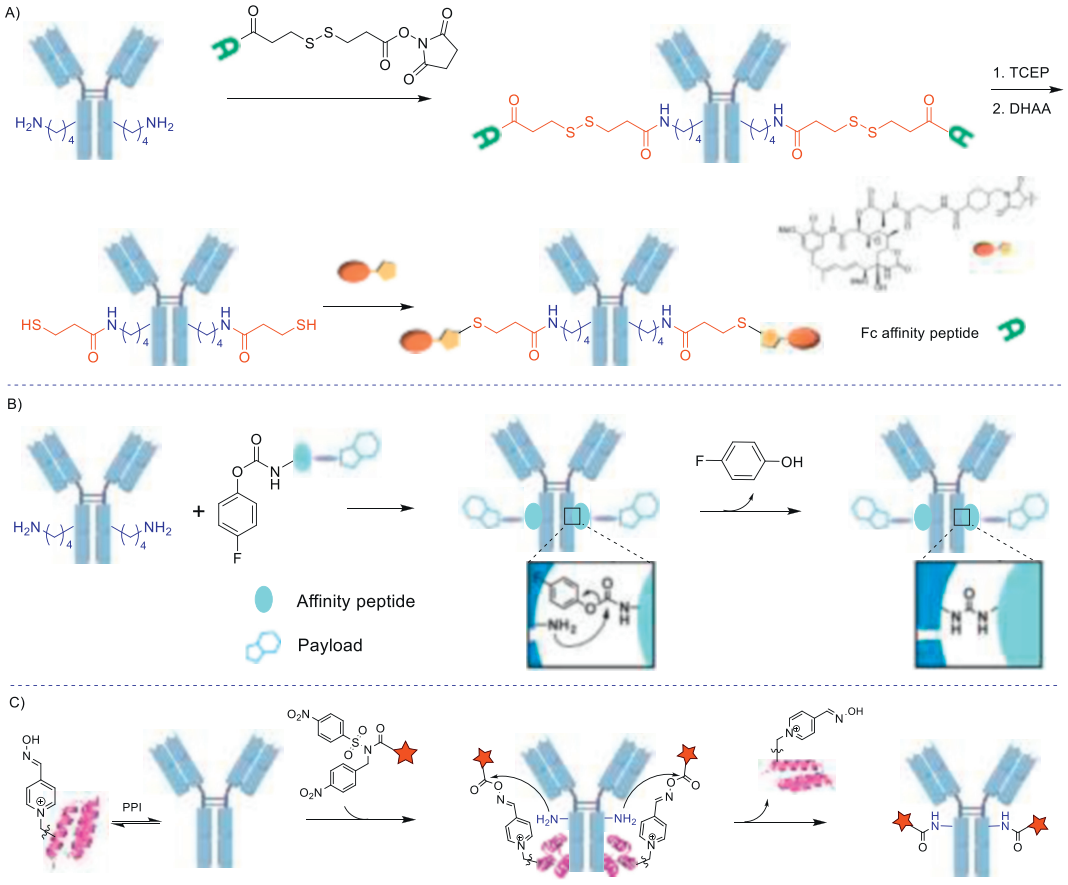

Fig. 12. Affinity peptide mediated Lys site-selective modification of antibodies.

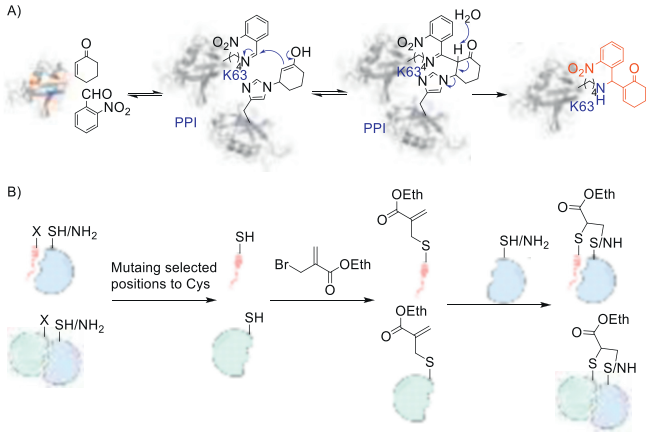

Fig. 13. Peptide-protein interaction directed site-selective Lys modification. Reproduced with permission [108]. Copyright 2023, American Chemical Society.

chains of the antibody [105]. Similar to these activated esters reagents, Chung group achieved site-selective modification of the Lys248 on trastuzumab using an IgG Fc-affinity peptide bearing a 5-norbornene-2-carboxylic acid thioester [106].

More recently, Lee and coworkers developed a novel approach to enable site-selective intact protein labeling by leveraging small Fc-binding protein and genetic code expansion technology (Fig. 12C) [107]. In this strategy, the Fc-binding protein acted as a mediator to efficiently and site-selectively transfer a chemical probe to Lys residue, thereby avoiding the operation to remove affinity peptides. The method was successfully applied to the site-selective labeling of Z-DM protein, Fc fragment, and trastuzumab with various tags, including fluorophore, biotin, and a drug. It is worth noting that only a catalytic amount of mediator was required of this method, in theory. Although these affinity peptide mediated methods provided invaluable solutions to homogeneous ADCs discovery, the introduction of extra affinity peptides may increase the difficulty of purification and result in immunogenic problems.

Compared with the small molecules and affinity peptides guided proximal Lys modification methods, achieving the site-selective modification of a specific Lys residue by protein-protein interaction was less disclosed. Rai group reported that protein-protein interaction could drive site-selective modification in a multicomponent aza-Morita-Baylis-Hillman (aza-MBH) reaction (Fig. 13A) [108]. The process involves simultaneously and reversibly generating of a pair complementary reactive intermediate, electrophilic imine and nucleophilic enolate that are generated with Lys and His in different proteins, respectively, followed by utilizing the specificity of protein-protein interaction to drive the subsequent site-selective irreversible step. More recently, London and coworkers mutated selected positions of peptides or proteins to Cys, then, the Cys residue was installed with methacrylate ester electrophiles, which further efficiently and selectively react with Cys or Lys residue on the target protein (Fig. 13B) [109]. By employing this strategy, a methacrylate-modified variant of colicin E9 immunity protein could irreversibly bind to E9 DNAse, resulting in higher thermal stability compared to the non-covalent complex.

The above methods that employ a small molecule, affinity peptide, or protein-protein interaction to specially bind target protein and enable the Lys reactive moiety to orient its proximal Lys residue, have achieved great success in the site-selective modification of a series of proteins. However, these methods showed limited substrate tolerance and cannot apply to other proteins due to the high specificity of affinity binding.

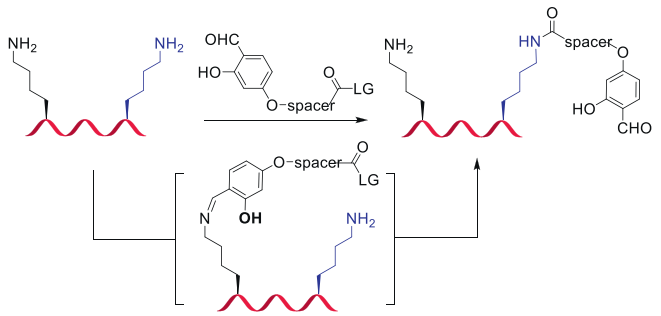

Fig. 14. Lys directed Lys site-selective modification.

3.2.2. Covalent tethering-directed Lys site-selective modification

Covalent tethering utilizes a modification reagent with two different reactive moieties and through a temporary covalent attachment of a proximal amino acid site to enable the site-selective modification of the desired Lys. In 2020, Rai group reported a linchpin-directed modification ($LDM_{K\text{-}K}$) technology by employing the *o*-hydroxyaldehyde derivative-spacer-activated ester reagent to enable the precise labeling of a single Lys residue (Fig. 14) [110]. This method enabled the site-selective installation (Lys169 in light chain and Lys395 in heavy chain) of fluorophore and payload on trastuzumab, and the synthesized ADC exhibited improved antiproliferative activity toward HER-2 positive SKBR-3 breast cancer cells compared to the native antibody.

Compared with the Lys-directed modification approach, Lys site-selective modification directed by another different amino acid would be more effective and Cys is ideal due to its inherent high reactivity and well-established labeling methods. In 2022, Li *et al.* developed a cleavable Cys labeling-directed Lys site-selective stapling and single-site modification approach for peptides and proteins (Fig. 15) [111]. They linked aryl thioether derivative that was developed by themselves for cleavable Cys modification and Lys selective *o*-nitrobenzyl alcohol moiety together [52,112]. Then, the cleavable and fast Cys labeling step was performed first to orient the most proximal Lys residue, followed by achieving the Lys site-selective single-site labeling with *o*-nitrobenzyl alcohol moiety under visible light irradiation, thereby avoiding multi-labeling or inaccurately labeling. The temporarily modified Cys residue could be either kept as a stapling anchor or recovered to its native state to achieve Lys site-selective single-site modification. Moreover, this method could achieve Lys site-selective single-site modification and multi-functionalization in a one-pot procedure.

Rai group reported a similar method for the site-selective modification of Lys in native proteins by employing a multifunctional reagent that contains a nitroolefin moiety for rapid, chemoselective and cleavable conjugation with Cys, and an activated ester moiety for relatively slow, stable, and chemoselective conjugation with Lys (Fig. S6 in Supporting information) [113]. The Lys site-selective modification protocol proceeded with C-S bond formation, C–N bond formation, C-S bond dissociation, aldehyde generation, and oxime formation sequentially in a one-pot procedure. However, multiple pH adjustments of buffer and extra additive addition were needed during the modification process. Besides, Lys conjugation step may proceed before Cys and afford multiple Lys residues modified byproducts.

3.3. Modified group transfer for Lys site-selective modification

The modified group transfer strategy employs a fast and reversible modification of another proximal amino acid (generally Cys) to deliver the reactive modified groups to Lys. Native chemical ligation reaction (NCL) that involves the ligation of an N-

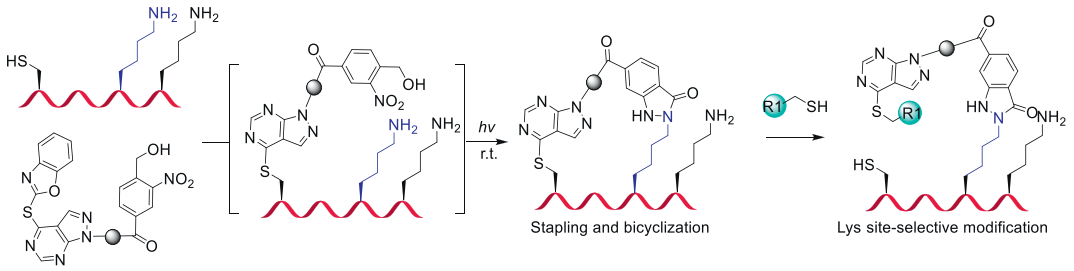

Fig. 15. Cleavable Cys labeling directed Lys site-selective modification.

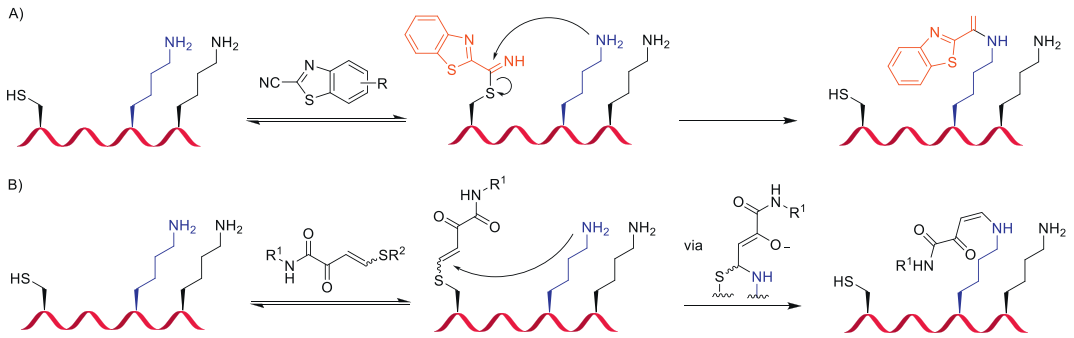

Fig. 16. Lys site-selective modification *via* modified group transfer.

terminal Cys with a C-terminal thioester-containing peptide fragment, has been widely used in protein synthesis [114]. Considering the high reactivity of Cys and the leaving ability of modified groups in some Cys-modified products, Lys site-selective modification can be effectively realized *via* the modification group transferring from Cys to its proximal Lys residue. Bertozzi *et al.* described the 2-cyanobenzothiazole (CBT) derivatives that are typically used for N-terminal Cys labeling could be applied to the Lys-selective modification of a Cys and Lys-containing 11-residue peptide motif (Fig. 16A) [115]. The Cys first reacted with the nitrile group to form a thioimidate intermediate, which is then attacked by the proximal Lys in peptide to form an irreversible covalent ligation.

In 2019, Poulsen and coworkers developed a class of semi-oxamide vinylogous thioesters (STEFs) for rapid Lys site-selective modification *via* a vinylic substitution reaction (Fig. 16B) [116]. During the transfer process of the modified group from Cys to Lys, the STEFs first reversibly conjugated with Cys residue, and subsequently a proximal Lys reacted with Cys-modified intermediate irreversibly to afford the desired Lys site-selective modification product *via* an addition-elimination mechanism. The STEFs were feasible to install onto an analog of the EGFR-inhibitor afatinib. It worth noting that the STEFs have some reactivity to amine nucleophiles, and the reagents were not only could modify Lys proximal to Cys but also label more reactive Lys residues without a proximal Cys, which resulted in inaccurate and multiple modifications of Lys. Besides, Baker and coworkers reported a Cys-to-Lys transfer methodology for the Lys site-selective bioconjugation of antibody fragments using thioester reagents (Fig. S7 in Supporting information) [117]. Liu group developed a Lys site-selective modification method that employed a Cys residue to covalently tether the Lys-reactive moiety to the target, followed by an intramolecular O–N acyl transfer process to yield desired proximal Lys-acylated products (Fig. S8 in Supporting information) [118].

3.4. Site-selective cooperative stapling of Lys with another amino acid

Cooperative stapling of Lys with another amino acid is an effective approach to achieve the site-selective modification, despite

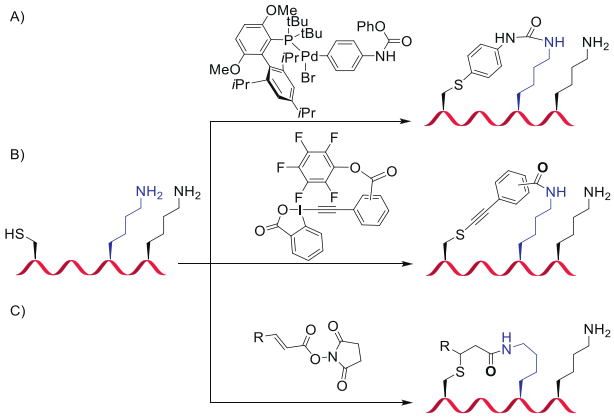

Fig. 17. Representative Cys-Lys site-selective cooperative stapling methods.

only stapled products could be obtained. Considering the high reaction efficiency of Cys residue and numerous modification methods, Lys cooperative stapling with Cys is the most attractive. Buchwald and coworkers reported a method for Cys-Lys intramolecular stapling and intermolecular cross-linking of peptides and proteins by employing palladium oxidative addition complexes (Fig. 17A) [119]. This method was applied to site-selective stapling of sortase A and cross-linking between a peptide and MDM2 protein based on the p53-MDM2 interaction.

Waser group achieved site-selective Cys-Lys stapling to yield structurally diverse thioalkyne linkers in multiple Lys-containing peptides using hypervalent iodine reagents bearing an activated ester group (Fig. 17B) [120]. The stapling efficiency and helicity of stapled products can be effectively controlled by adjusting the geometry and length of the linker in the stapling reagents. Besides, the stapled peptides could be applied to further functionalization through Ru-catalyzed cycloaddition of the formed thioalkynes linker with azides. This method was applied to the synthesis of a stapled peptide with enhanced α-helicity and binding affinity to the MDM2 protein.

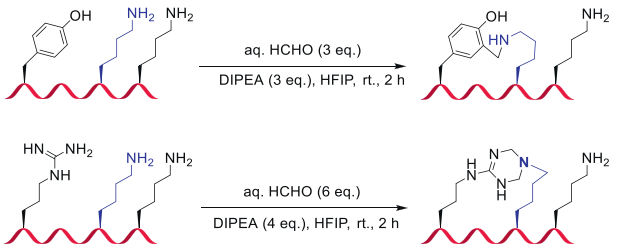

Fig. 18. Representative site-selective cooperative stapling methods of Lys with other amino acid.

Gois group reported an efficient amino-sulfhydryl stapling approach using bifunctional NHS-activated acrylamides (Fig. 17C) [121]. This method allowed for a fast functionalization of N-terminal Cys and site-selective stapling of an in-chain or C-terminal Cys with a proximal Lys residue. The method was successfully applied to the site-selective Cys-Lys stapling of BSA, which contains a free Cys and 59 Lys residues, and trypsin digestion and molecular dynamics (MD) simulations of stapled BSA demonstrated the amino-sulfhydryl stapling occurred at Cys34 and nearby Lys136.

While achieving Lys site-selective stapling *via* Cys is versatile, the stapling of Lys with other amino acid residues is attractive. Chen *et al.* developed a cooperative stapling approach of Lys with a nearby Tyr or Arg residue in native peptides using formaldehyde (Fig. 18) [122]. The stapling reactions proceed stepwise that the peptide first reacts with formaldehyde reversibly to generate an imine intermediate, followed by an intramolecular nucleophilic attack of the nearby Tyr or Arg with imine to form a stable linkage irreversible. The method enabled unique control of site-selectivity for modifying peptides bearing multiple Lys residues *via* reactivity built into the peptide sequence. However, this cooperative stapling method proceeds under bioincompatible conditions and is unsuitable for site-selective stapling of proteins. Based on the developed Lys-Tyr cooperative stapling method, Metanis group synthesized a stapled analog of salmon calcitonin, and the Lys11-Tyr22 stapled analog exhibited improved stability in both simulated gastric and intestinal fluid with retained α-helical structure and activity to the calcitonin receptor [123].

4. Conclusion

As a proteogenic amino acid with high nucleophilicity, Lys is extensively involved in post-translational modification and the binding of ligands to target proteins, prompting significant attention to its selective modification. In this review, various chemical modification reagents and strategies for Lys bioconjugation were summarized and organized to facilitate the understanding of their features, applications, and drawbacks. Although significant progress has been made in Lys modification, most of the methods still require further improvement, including enhancing selectivity, optimizing reaction kinetics, and simplifying modification conditions and operation. While many methods have accomplished chemoselective modification of Lys in peptides and proteins, site-selectivity, a crucial goal for protein bioconjugation, remains largely unfulfilled.

Kinetically controlled site-selective labeling and affinity binding-directed Lys site-selective modification methods showed good site-selectivity in some proteins, and have been employed in the preparation of ADCs and the development of Lys covalent drugs. However, these techniques exhibit limited substrate tolerance and are generally only suitable for specific protein modifications. Cooperative stapling and covalent tethering-directed modification approaches have facilitated the site-selective functionalization of a specific Lys residue in peptides or proteins by anchoring another proximal amino acid. However, these methods either yield only stapled products or involve tedious procedures for achieving Lys site-selective modification, thereby limiting their practical application. The modified group transfer strategy offers a potential solution to achieve Lys site-selective labeling *via* a rapid and transient modification of another amino acid (typically Cys), followed by intramolecular nucleophilic substitution of the proximal Lys residue. Compared with modifying Cys directly, the modified product prepared by this strategy generally possesses improved stability. Furthermore, the Cys for direction could be applied to further bioconjugation, which may be used in multiple labeling and dual-drug ADCs discovery. The site-selectivity control of this strategy is similar to covalent tethering-directed approaches and merits further exploration for its potential to deliver effective and general Lys site-selective modification, despite the need for addressing issues such as insufficient modification efficiency and the requirement for reagents with low reactivity towards intermolecular Lys to prevent directly random labeling bypassing the transfer process.

With the advancement of newly designed Lys site-selective bioconjugation reagents or strategies in the modification efficiency and the ability to control which Lys residue to modify, accurate control of the physicochemical properties of the conjugates may be achieved. Such an approach will be crucial for disclosing PTMs, protein-protein interaction research, and Lys-targeted covalent drug development. In a word, Lys site-selective modification methods need to be further explored, and the advancement could facilitate the development of bioconjugates with accurate structure and function, which will have a significant impact on academic research and drug discovery.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Jian Li: Writing – review & editing, Writing – original draft, Project administration, Investigation. **Jinjin Chen:** Writing – original draft. **Qi-Long Hu:** Writing – original draft. **Zhen Wang:** Supervision, Project administration. **Xiao-Feng Xiong:** Supervision, Project administration.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cclet.2024.110126.

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