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Recent advances in site-specific modifications of peptides and proteins

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Modification at specific sites has long been a central goal in peptide and protein chemistry. Introducing functional groups at specific sites is an ideal state for protein function research and the modification of protein properties, as it can eliminate the interference caused by heterogeneous modifications. Recent downstream applications have placed even greater demands on site selectivity. This review examines the latest advances in the site-specific modification of peptides and proteins, organizing the reactions into four categories: exploiting disparities in chemical environments, tag-and-modify approaches, proximity-driven chemical modification, and enzymatic strategies. For each reaction, we detail the underlying design rationale and highlight downstream chemical and biological applications. Collectively, these methods have achieved remarkable gains in terms of specificity, efficiency, and scope, furnishing a versatile chemical toolbox for the site-selective functionalization of peptides and proteins. We conclude by summarizing the current state of the field and outlining prospective directions for future development.

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1 Introduction

Chemical modification is a powerful tool for engineering peptides and proteins, greatly expanding their functional diversity.[1–4] Site-specific modifications deliver precisely engineered products whose structures can be tailored on demand, enabling exact control over peptide and protein architecture and function and thereby deepening our understanding of structure–function relationships.[5,6] Introduced functional handles, such as bioorthogonal reaction groups, enable further modification of peptides and proteins, allowing their multifunctional engineering for diverse downstream applications, including cellular imaging, omics profiling, immunotherapy[7] and the design of antibody–drug conjugates.[8,9] From a different perspective, the rapid evolution of these applications continually raises the bar for site selectivity.[10] More precise modification translates into the more effective realization of the intended functions. Together, these factors underscore the vast potential of site-specific peptide and protein modifications.

In recent years, a wealth of new site-specific modification reactions have emerged, resulting in the assembly of a rich chemical toolbox.[11] Leveraging innate disparities in nucleophilicity and redox properties, researchers have developed reactions that target the N-terminus, C-terminus,[12] and even specific internal residues.[13] Exogenous tags can be appended to introduce new addressability, enabling direct and specific modification.[14] Residue-mediated or ligand-directed positioning proximity can trigger nearby functionalization. In addition to chemistry, enzymes that recognize specific sites offer another layer of selectivity (Fig. 1). In summary, the landscape of site-specific modifications is replete with advances that merit a comprehensive review.

This review provides an up-to-date, integrated survey of recent site-specific modification reactions for peptides and proteins, with a particular emphasis on their chemical and biological applications. We also speculate on future directions, aiming to guide and accelerate the next wave of discovery.

2 Exploiting disparities in chemical environments

The same functional groups do not always exhibit the same reaction characteristics because they are affected by the chemical environment. When a specific site of a protein is chemically modified, a natural approach is to exploit differences in the chemical environment within the protein. For instance, the pK_a of amines in different chemical environments varies, which in turn affects their nucleophilicity and reactivity.[15,16] The C-terminal carboxyl group also exhibits distinct oxidative reactivity compared to the carboxyl groups on amino acid side chains.[17] Researchers have used disparities in chemical environments to develop a variety of chemical modifications. We divide these methods into differences in nucleophilicity and oxidation–reduction properties.

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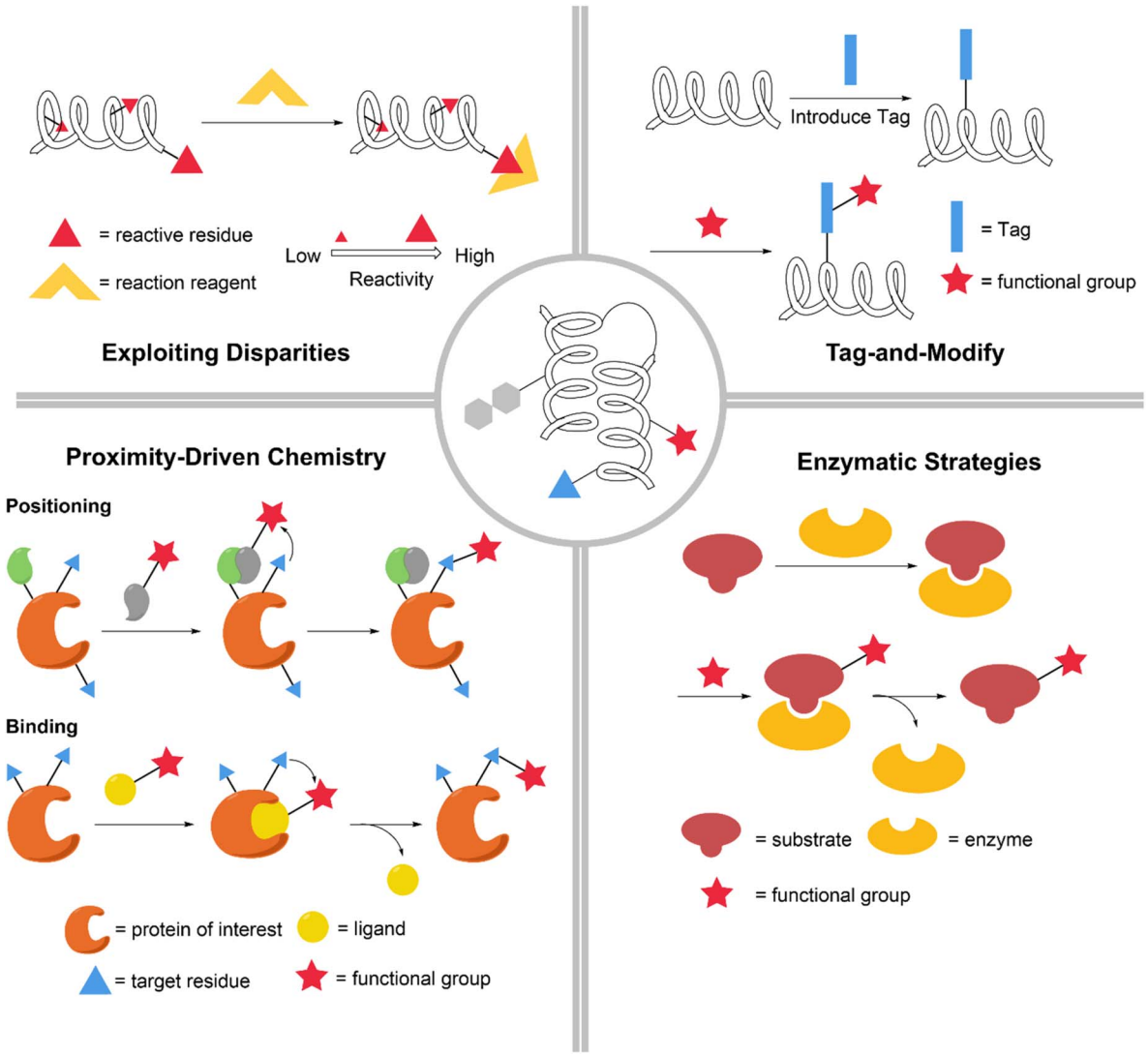

Fig. 1 Strategies for the site-specific modification of peptides and proteins.

2.1 Differences in nucleophilicity

2.1.1 Amino groups with distinguishable nucleophilicity. The N-terminal amino group resides in a chemical environment distinct from that of the lysine side-chain ε-amino groups, conferring increased nucleophilicity and a distinctive transition-metal coordination ability. Consequently, the N-terminal amine can undergo site-selective nucleophilic substitution or form transition metal complexes that enable cross-coupling, cycloaddition, and other transformations that are generally inaccessible to side-chain amino groups. Researchers have developed a variety of modifications specific to the terminal amino group. The cross-coupling reactions of amines under physiologically relevant aqueous conditions remain largely underdeveloped, and the issue of selectivity among multiple amino groups is far from resolved. In 2020, Miller *et al.* developed a method for selective N-terminal amine arylation that does not involve competition with lysine. Copper(II) salts mediate selective N-terminal reactions with arylboronic acid reagents under mild, aqueous-buffered conditions (Fig. 2A). The authors demonstrated that the reaction tolerates a broad range of N-terminal residues except proline, which bears a secondary amine. The authors exploited this method to install arylated products bearing sulfonamide, sulfone, and halogen functionalities. The charge and structural perturbations introduced by *N*-arylation did not compromise the enzymatic stability of the modified peptides.[18] In 2024, Hanaya *et al.* targeted the N-terminus of proteins for reaction with maleimides and 2-pyridinecarbaldehyde (2-PCA) under mild and aqueous conditions, generating derivatives *via* copper(II)-mediated [3 + 2] cycloaddition (Fig. 2B). Maleimide derivatives are widely employed in Michael addition reactions with cysteine residues. Given the scarcity of accessible free cysteines in native proteins, the N-terminal amino group represents an attractive alternative target for site-specific bioconjugation with maleimides. A copper(II) ion forms a stable ternary complex with the Schiff base of the N-terminal amino acid and 2-PCA. Subsequent α-proton deprotonation at neutral pH generates a Cu(II)-enolate *in situ*, which acts as a latent 1,3-dipole to react with the maleimide.

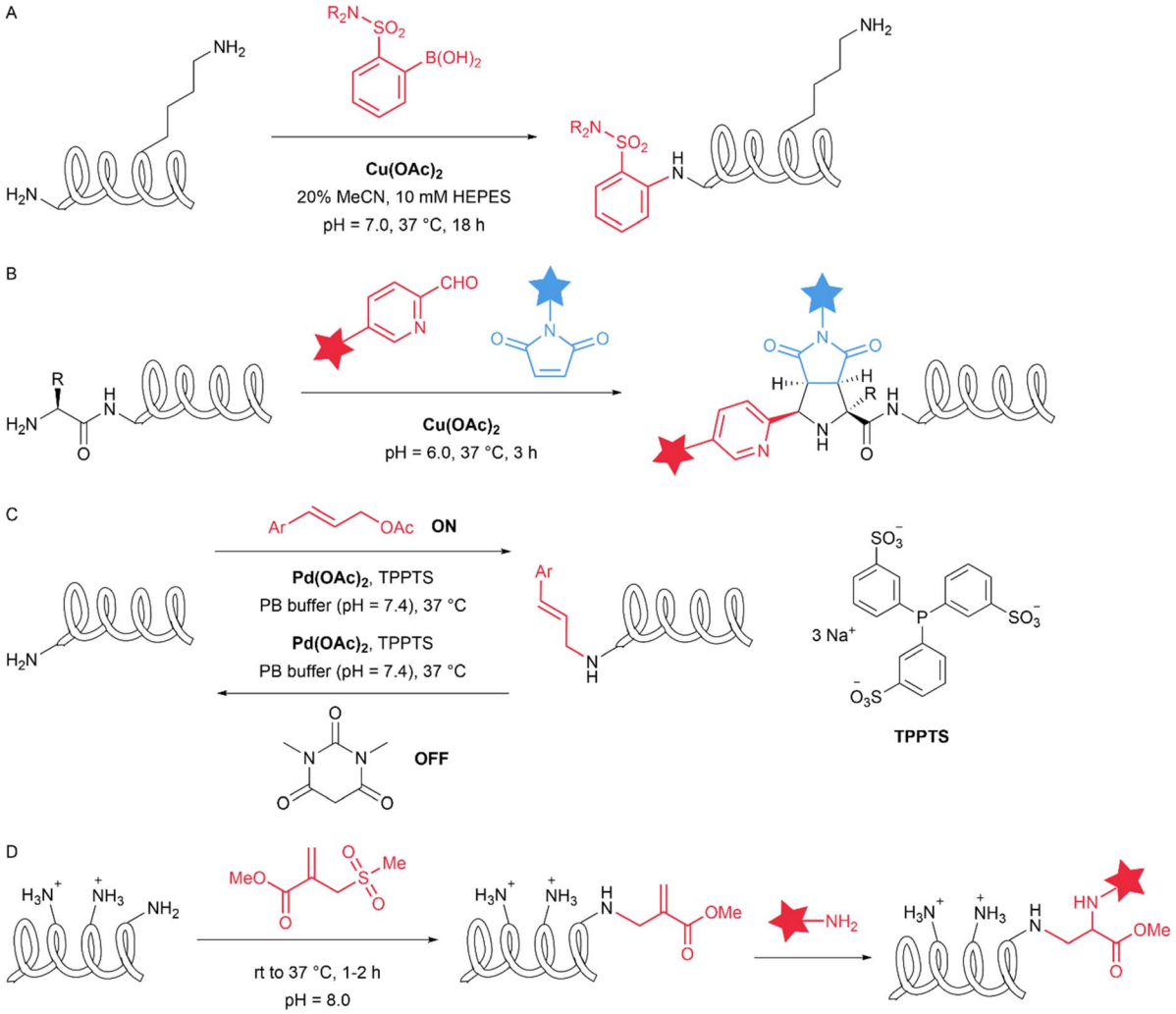

Fig. 2 Methods targeting the amino groups with distinguishable nucleophilicity. (A) Utilization of boronic acid reagents for copper-mediated amine arylation of the N-terminus. (B) Copper-mediated [3 + 2] cycloaddition enables dual functionalization of the N-terminus. (C) Controlled, reversible N-terminal modifications of peptides and proteins. (D) pK_a-guided, regioselective lysine modification.

With this method, a wide range of functional groups can be installed in high yield onto proteins bearing diverse N-terminal amino acids using simple, commercially available maleimide derivatives. Leveraging click-chemical handles, the authors demonstrated the preparation of trimeric protein complexes cross-linked at the N-terminus and dual-modified trastuzumab.[19] Reversible modification strategies enable on-demand control for studying protein function, yet site-selective and reversible modification at the N-terminus remains scarce. A universal approach compatible with diverse N-terminal residues is urgently needed. In 2024, Lin *et al.* developed a specific reversible strategy for modifying the N-terminal amino groups of proteins. Peptides and proteins were N-terminally cinnamoylated *via* allyl ester through palladium catalysis (Fig. 2C). The method is compatible with all twenty canonical amino acids at the N-terminus, thereby broadening the substrate scope for N-terminal protein modification. By introducing azido or biotin tags on the cinnamyl group, trastuzumab can be further modified with toxins or fluorophores for gain of function. In the presence of 1,3-dimethylbarbituric acid, Pd-catalysed decinnamoylation efficiently restored the native peptide or protein, operating in a switchable on/off manner. Leveraging this unique reversibility, the authors demonstrated the precise tuning of the binding affinity of trastuzumab and traceless enrichment of N-terminally free proteins from cell lysates.[20]

Beyond experimental strategies, computational and bioinformatic tools have emerged as valuable resources for predicting site-specific reactivity from protein sequences. Machine learning algorithms trained on structural and reactivity databases can now identify reactive hotspots and subtle electronic environment differences among identical amino acid residues. Cysteine exhibits strong nucleophilic reactivity and serves diverse functions within proteins. Predicting the reactivity of cysteine residues facilitates the annotation of protein functions. In 2012, Anandakrishnan *et al.* reported a tool that can predict the pK_a values of cysteine thiol groups, which in turn can be used to estimate their relative nucleophilic reactivity.[21] In 2015, Soylu and Marino developed an algorithm termed HAL-Cy and implemented this algorithm in a web service (Cy-preds). Cy-preds is capable of performing calculations for comprehensive

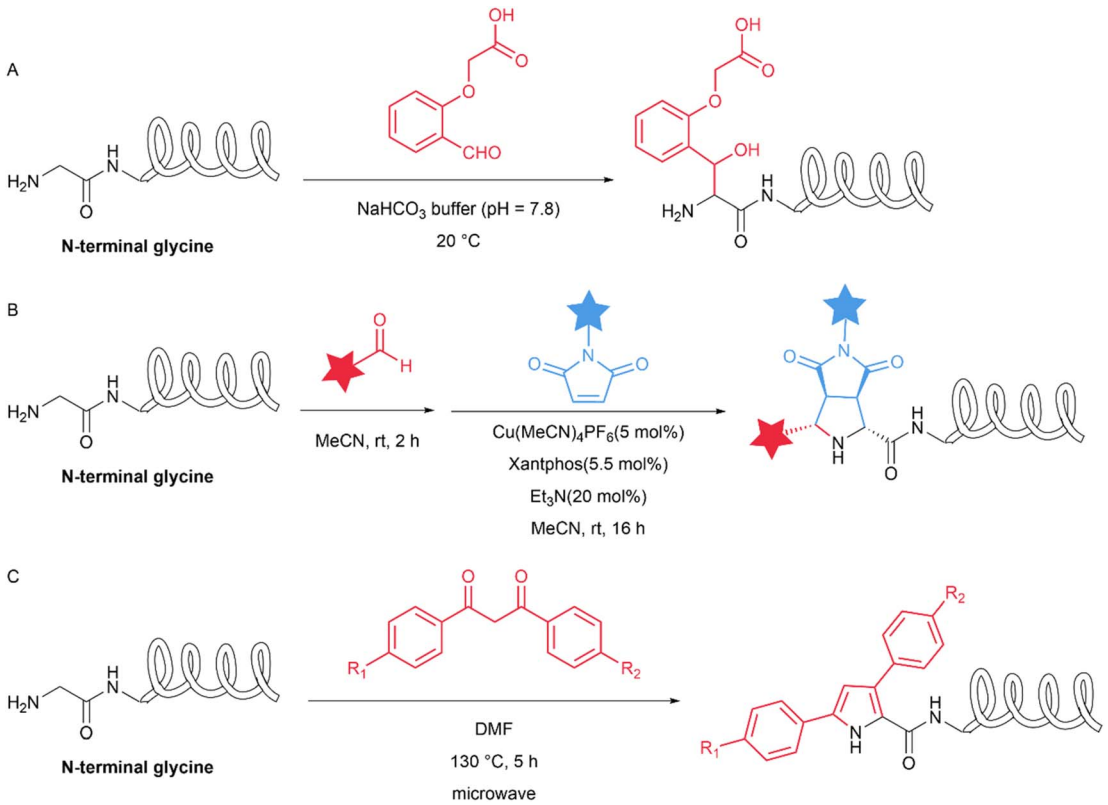

Fig. 3 Methods targeting N-terminal glycine residues. (A) An aldehyde bearing an appropriate hydrogen bond acceptor enables the selective modification of the N-terminal glycine. (B) N-terminal-glycine-specific dual modification through copper-catalysed [3 + 2] cycloaddition. (C) N-terminal glycine labelling *via* methylene "double activation".

analysis of Cys reactivity in proteins, encompassing reactivity predictions and functional characterization.[22] In 2017, Wang *et al.* reported the development of a sequence-based computational algorithm named sbPCR (sequence-based prediction of cysteine reactivity). This method enables to predict cysteines with hyper-reactivity based on only local sequence features and provides a large inventory of potential hyper-reactive cysteines in proteomes.[23] The ε-amino groups of different lysine residues on a protein exhibit subtle yet intrinsic differences in reactivity, thereby indicating the possibility of site-selective modification. The pK_a value of the lysine amino group can also be predicted for site-selective reactions. In 2023, Wilson *et al.* developed a theoretical computational method for predicting the pK_a values of lysine residues in proteins.[24] The computational approaches complement chemical intuition and experimental screening, potentially accelerating the rational design of site-specific modification strategies. In 2018, Matos *et al.* computationally predicted and designed sulfonyl acrylate reagents for the targeted modification of a single lysine residue. Under mildly alkaline conditions, the kinetically favoured reaction occurs at the lysine with the lowest pK_a, while exhibiting chemoselectivity in the presence of other nucleophilic residues (Fig. 2D). A transient hydrogen bond forms between the sulfonyl group of the reagent and the ε-amino group of the lysine side chain, enabling the key N–C bond to be forged *via* a low-energy, H-bond-assisted chair-like addition transition state. Subsequent elimination of the sulfonyl group then furnishes an additional thermodynamic driving force for the reaction. The authors achieved site-specific lysine modification in five different proteins. The resulting acrylate-bearing proteins were then conjugated in a chemoselective and regioselective manner with synthetic amine-containing cargoes, including PEG, fluorophores, and anticancer drugs, *via* a subsequent Michael addition.[15]

2.1.2 N-terminal glycine. The distinct chemical microenvironments created by the side chains of different N-terminal amino acids can also impart unique reactivity to the α-amino group. For example, the N-terminal glycine serves as a target that is distinct from all other proteinogenic amino acids. In 2019, Purushottam *et al.* engineered an aldehyde bearing a precisely positioned hydrogen bond acceptor that directs the exclusive modification of the N-terminal glycine. Leveraging the geometric constraint imposed by the aromatic ring of *ortho*-substituted benzaldehyde, the authors quantitatively converted the N-terminal glycine into an amino alcohol (Fig. 3A). After an imine formed between the aldehyde and the α-amine, equilibrium tautomerization to an enolate and subsequent aldol reaction with a second molecule of the aldehyde formed a robust C–C bond, resulting in irreversible modification specifically at the N-terminus of glycine. The scope of this strategy extends to proteins in crude cell lysates, and a symmetrical dialdehyde reagent allows one-step installation of diverse probes while the mild reaction conditions fully preserve protein structure and function.[25] In 2024, Machida and

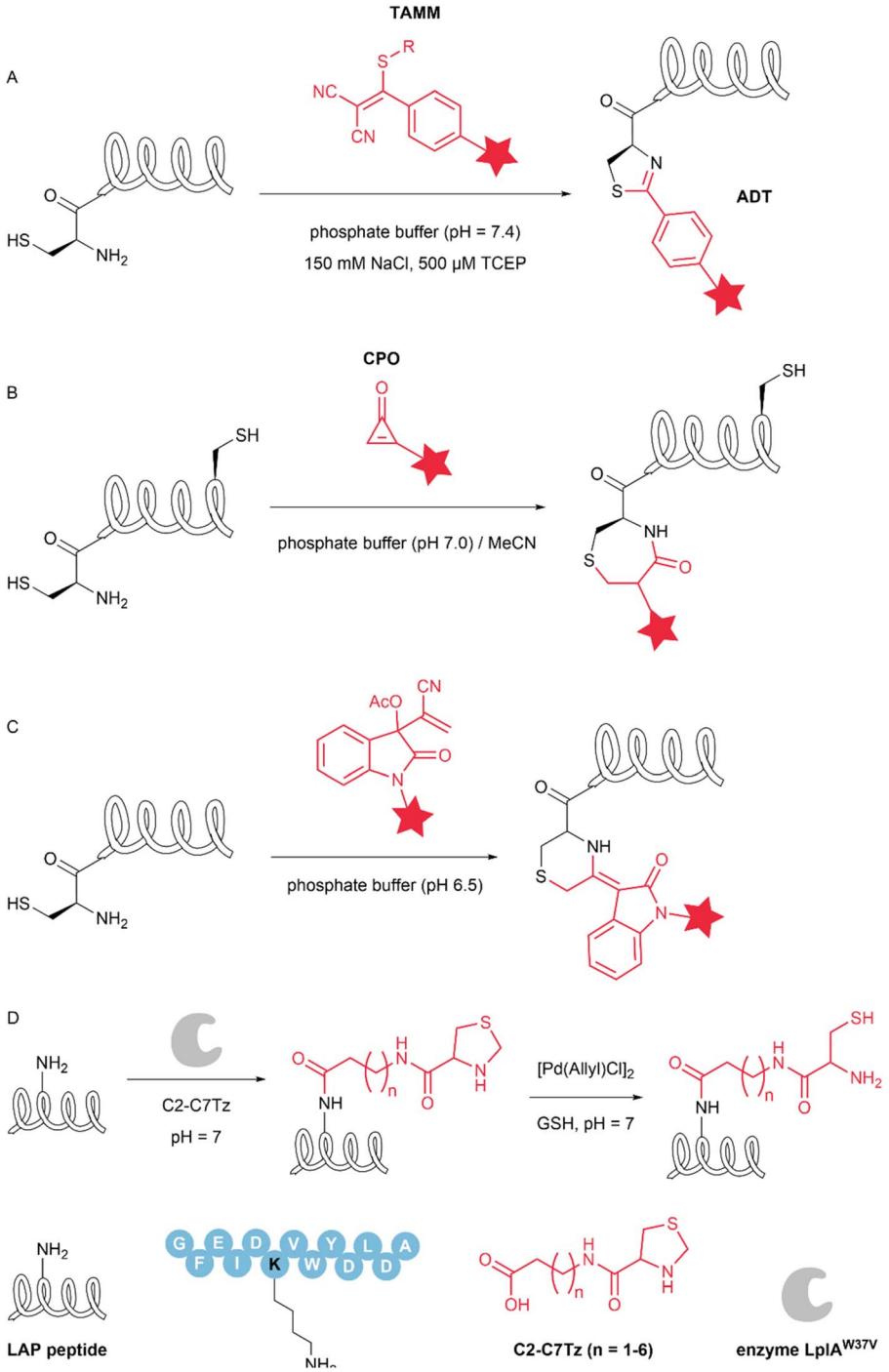

Fig. 4 Methods targeting the 1,2-aminothiol group. (A) TAMM-directed condensation with 1,2-aminothiol. (B) CPO targeting the 1,2-aminothiol group of N-terminal cysteine residues. (C) Baylis–Hillman-orchestrated protein aminothiol labelling (BHoPAL). (D) Lipoic acid ligase-based technique to install a 1,2-aminothiol group within proteins.

Kanemoto developed a method for the N-terminal glycine-specific dual modification of peptides through a three-component [3 + 2] cycloaddition with aldehydes and maleimides catalysed by copper (Fig. 3B). This approach capitalizes on the exclusive and efficient generation of metalated azomethine ylides. It enables the precise N-terminal modification of a broad spectrum of oligo- and polypeptides, including sequences containing up to 26 amino acid residues with multiple lysine moieties. Given the broad functional group compatibility of the aldehyde and maleimide modules, this method enables the simultaneous installation of two distinct functional molecules onto a single peptide.[26] In the same year, Liu *et al.* achieved dual activation of the α-methylene of the N-terminal glycine, thereby distinguishing it from all other amino acids. The authors exploited a condensation reaction between dibenzoylmethane and the N-terminal glycine to form

Table 1 Methods exploiting differences in nucleophilicity

Modified residue	Reagent	Highest yield (%)	Reference
N-terminal amino group	Arylboronic acid	97	18
	Maleimides/2-pyridinecarbaldehyde	99	19
	Allyl ester	98	20
Lysine	Sulfonyl acrylate	98	15
N-terminal glycine	*Ortho*-substituted benzaldehyde	71	25
	Aldehyde/maleimide	99	26
	Dibenzoylmethane	76	27
1,2-Aminothiol	2-((Alkylthio)(aryl)methylene) malononitrile	>90	28
	Cyclopropenone	88	29
	Isatin-derived Baylis–Hillman adducts	95	30

an imine, followed by enolate tautomerization and intramolecular cyclization; subsequent dehydration and a final tautomerization yielded a fluorescent pyrrole ring (Fig. 3C). This approach is compatible with amino acids possessing reactive side chains. Furthermore, the method allows for selective pull-down assays of N- terminal glycine peptides from mixtures without prior knowledge of the N-terminal peptide distribution.[27]

2.1.3 1,2-Aminothiol groups. The 1,2-aminothiol group exhibits a distinctive nucleophilicity and can be readily installed *via* an N-terminal cysteine, allowing site-specific targeting that distinguishes it from all other thiol groups. In 2020, Zheng *et al.* reported that 1,2-aminothiol can react rapidly, specifically and efficiently with 2-((alkylthio)(aryl)methylene) malononitrile (TAMM) under biocompatible conditions to form 2-aryl-4,5-dihydrothiazole (ADT) (Fig. 4A). This reaction enables *in vitro* site-specific labelling and the modification of proteins on the surfaces of live mammalian cells and bacteriophages without compromising their viability or infectivity. The authors employed a TAMM conjugate containing a chloroacetyl group to cyclize peptides containing both an N-terminal cysteine and an internal cysteine and applied this strategy to the phage display technique. Using this approach, the authors identified ADT-cyclic peptides with high affinity for diverse protein targets, providing valuable tools for biological research and potential therapeutics.[28] In 2022, Istrate *et al.* employed mono-substituted cyclopropenone (CPO) reagents to selectively react with the 1,2-aminothiol group of N-terminal cysteine residues under mild, biocompatible conditions (Fig. 4B). A wide range of functional groups can be readily appended to CPO *via* the precursor CPO-pentafluorophenol (CPO-PFP). The reaction retains its ability to target the N-terminal cysteine even in the presence of other solvent-exposed and reactive cysteine residues.[29] In 2024, Mir *et al.* employed isatin-derived Baylis–Hillman adducts to react with 1,2-aminothiols, generating *Z*-alkene-linked bicyclic heterocycles that enable robust protein bioconjugation both *in vitro* and under live-cell conditions. The authors refer to this technology as Baylis–Hillman orchestrated protein aminothiol labelling (BHoPAL) (Fig. 4C). The authors also developed a lipoic acid ligase-based technique to install a 1,2-aminothiol group at virtually any desired site within a protein, thereby extending BHoPAL beyond N-terminal labelling (Fig. 4D). By combining the two technologies, the authors generated dual-labelled protein bioconjugates bearing distinct tags at two precisely defined sites within a single protein molecule (Table 1).[30]

2.2 Differences in oxidation–reduction properties

2.2.1 Terminal group. Differences in redox properties can be exploited to discriminate the reactivity of specific sites. Even identical functional groups can exhibit distinct redox potentials due to their unique chemical environments.[31] For instance, the differential oxidation potential between internal and C-terminal carboxylates can be leveraged to achieve the selective functionalization of the C-terminus. Photoredox catalysis is a powerful strategy for protein functionalization. Visible-light-mediated single-electron transfer generates radicals under mild conditions, and the stability of these radical intermediates is intrinsically linked to the ground-state oxidation potential of the reacting group. In 2018, Bloom *et al.* developed a photoredox bioconjugation strategy that selectively targets C-terminal carboxylic acids, employing a less-reactive α,β-unsaturated carbonyl as a radical trap for the C-terminal decarboxylative radical (Fig. 5A). This method was successfully applied to site-selective functionalization of endogenous peptides and insulin.[17] In 2019, Garreau *et al.* developed a metal-free, decarboxylative C-terminal alkynylation of peptides that is broadly applicable for peptide modification. By tuning the oxidation potential of the organic dye, the carboxyl groups at the C-terminus can be distinguished from those on side chains, enabling site selectivity for the C-terminus (Fig. 5B). A wide array of functional groups was successfully installed by using this method. The reaction tolerates most side chains except for tryptophan, which requires protection.[32] *Ortho*-quinone cofactors of copper amine oxidases (CuAOs) can readily oxidize primary amines to aldehydes *via* a transamination pathway using molecular oxygen as the co-oxidant, thereby completing the catalytic cycle.[33,34] Inspired by this reaction, in 2021, Wang *et al.* reported the development of a strategy for protein modification through biomimetic quinone-mediated oxidation at the N-terminus. Leveraging the distinctive reactivity of an *ortho*-quinone reagent, the amine at the protein N-terminus is oxidized to generate an aldehyde or ketone handle for orthogonal conjugation (Fig. 5C). The authors applied this method to

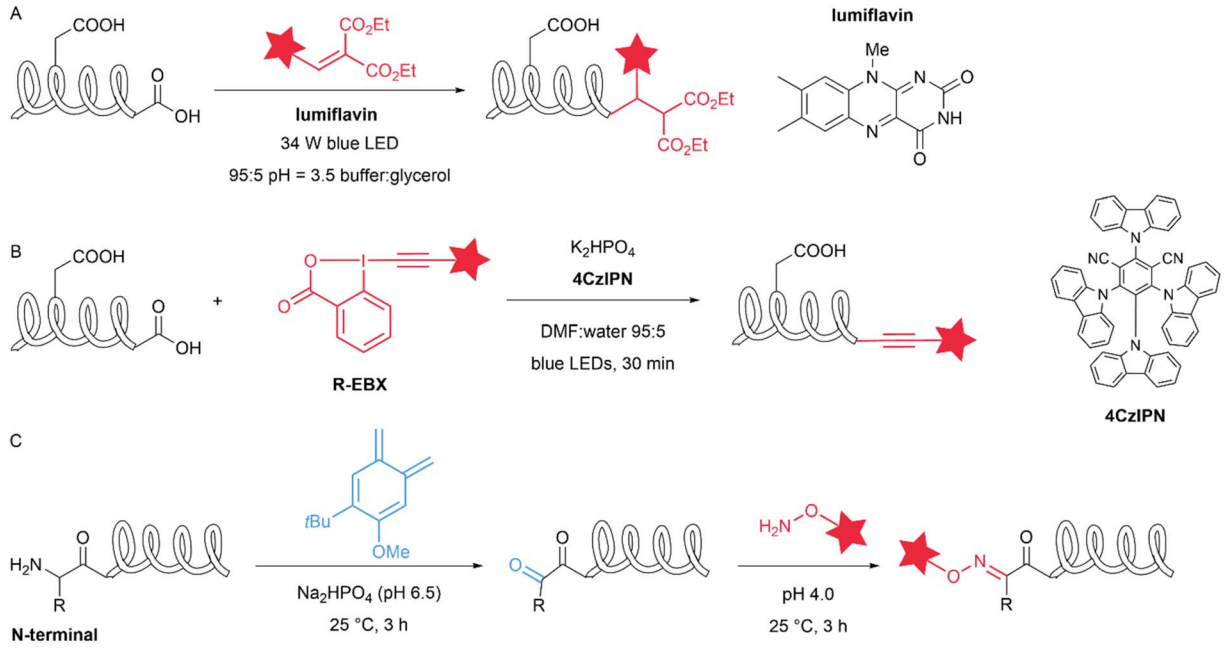

Fig. 5 Utilizing redox properties to target terminal groups. (A) Photoredox-catalysed decarboxylative alkylation. (B) Photoredox-catalysed decarboxylative alkynylation. (C) Modification of the N-terminal α-amine *via* biomimetic quinone-mediated transamination.

modify a series of proteins and construct a macrophage inflammatory protein-1β (MIP-1β) library.[35]

2.2.2 Side-chain group. Inherent differences in the redox potentials of natural amino acid residues can be exploited for site-selective modification reactions, and MacMillan's group has established several paradigms in this area. Methionine and tyrosine represent particularly challenging targets for conventional chemical modification. The thioether side chain of methionine is sterically shielded by alkyl groups, resulting in weak nucleophilicity. Its frequent burial in hydrophobic cores limits the accessibility of conventional reagents, rendering it recalcitrant to traditional nucleophilic substitution reactions that work well for cysteine or lysine. However, its inherent reducibility makes it readily addressable *via* photoredox catalysis. In 2020, Kim *et al.* reported a site-selective methionine bioconjugation strategy in which photoexcited lumiflavin generates reactive open-shell radical intermediates. The authors successfully modified diverse proteins, including ubiquitin and α-lactalbumin, and subsequently appended a variety of biologically relevant payloads (Fig. 6A).[36] Tyrosine residues are attractive targets for site-selective modification *via* photoredox catalysis. Due to their amphipathicity, tyrosines are commonly found in a wide range of hydrophilic and hydrophobic microenvironments. Consequently, different tyrosine residues can exhibit markedly varied surface accessibility and π-electronics, giving rise to a spectrum of reactivities. Multiple tyrosines in proteins vary in terms of solvent exposure and engagement in noncovalent interactions, making single-site discrimination challenging for selective modification. Tyrosines that are sterically hindered or engaged in cation–π interactions are rendered unreactive, whereas those that are surface exposed or capable of hydrogen bonding are readily reactive. In 2021, Li *et al.* devised a site-selective tyrosine bioconjugation strategy in which the water-soluble photocatalyst lumiflavin mediates oxidative coupling between a phenoxazine dialdehyde tag and a single tyrosine site, resulting in the formation of a covalent C–N bond (Fig. 6B). Even native proteins bearing multiple tyrosines can be efficiently labelled with tyrosine-specific single-site selectivity. Human lysozyme and ribonuclease A, each bearing six tyrosine residues, were efficiently labelled with complete selectivity for a single tyrosine residue (Y45 of human lysozyme and Y76 of ribonuclease A). This strategy involves the installation of an aldehyde handle directly onto the protein, enabling the subsequent attachment of diverse functional payloads.[37] Tryptophan (Trp) plays a variety of critical functional roles in protein biochemistry. However, due to its inherently low reactivity, developing a site-specific tryptophan modification strategy remains challenging.[38] In 2022, Hoopes *et al.* reported a strategy for achieving highly efficient tryptophan reactivity driven by photoinduced electron transfer (PET). The authors have engineered biaryl *N*-carbamoyl pyridinium salts that possess a donor–acceptor relationship enabling optical triggering with visible light. The probe efficiently reacts with surface-exposed Trp residues at micromolar concentrations upon irradiation with violet light for a short time (Fig. 6C). This method enables site-specific tryptophan modification in a wide range of proteins. β2-Microglobulin (B2M) contains two Trp residues and six surface-exposed Tyr residues. It was almost exclusively mono-labelled at the solvent-exposed Trp-60. The carbamate transfer group can be used to transfer useful functional groups to proteins, including affinity tags and click handles (Table 2).[39]

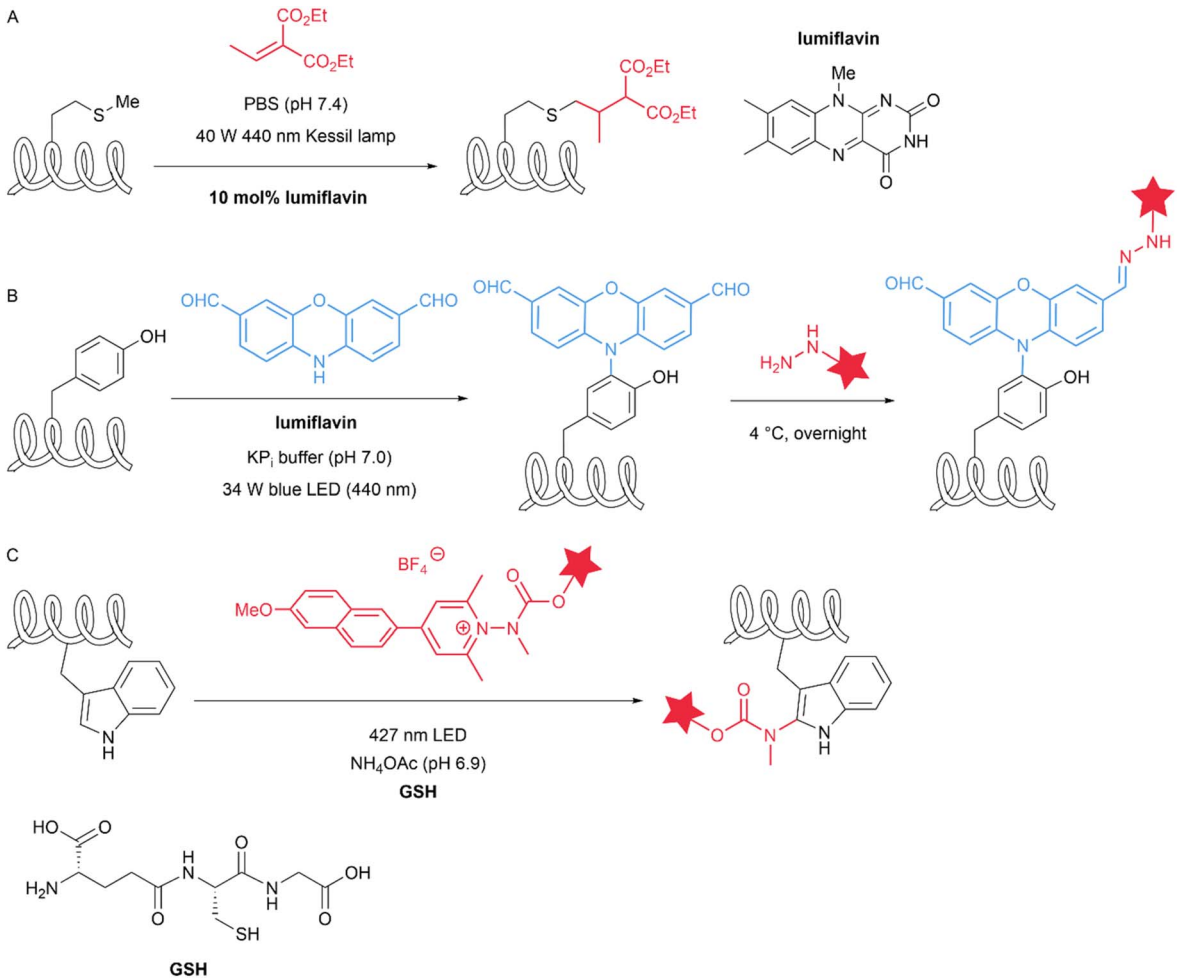

Fig. 6 Utilizing redox properties to target a single side chain group. (A) Photoredox-catalysed site-selective methionine functionalization. (B) Photoredox-catalysed site-selective tyrosine functionalization. (C) Photoinduced electron transfer-driven modification of tryptophan using visible light.

Table 2 Methods exploiting differences in oxidation–reduction properties

Modified residue	Reagent	Highest yield (%)	Reference
C-terminal carboxyl group	α,β-Unsaturated carbonyl	95	17
	Ethynylbenziodoxolone	>95	32
N-terminal amino group	*Ortho*-quinone	91	35
Methionine	α,β-Unsaturated carbonyl	96	36
Tyrosine	Phenoxazine dialdehyde	95	37
Tryptophan	Biaryl *N*-carbamoyl pyridinium salt	95	39

3. Tag-and-modify

In general, the inherent differences in the properties of peptide and protein substrates are limited. Relatively few examples of site-specific modification reactions relying on this difference are available. The artificial introduction of a positioning factor, which is referred to as a "Tag", is necessary to achieve more precise and controllable modifications.[1] This "Tag" can involve either altering a single amino acid with an unnatural amino acid or adding a new amino acid sequence. The introduction of unnatural amino acids allows for the precise targeting and modification of proteins at the desired site.[14] Special amino acid sequences can provide unique targeting properties. We divided this part into natural amino acid derivatives, the insertion of unnatural amino acids and the insertion of amino acid sequences.

3.1 Incorporation of natural amino acid derivatives

Natural amino acid derivatives exhibit only subtle differences in structure and reactivity compared with their native

counterparts, yet these distinctions are sufficient for the derivatives to serve as tags that mediate site-specific modification reactions. Dehydroalanine (Dha) can be easily introduced into proteins through chemical reactions. Cysteine can react with 2,5-dibromohexanediamide (DBHDA) *via* desulfurization to form Dha. Its structural feature of containing an α,β-unsaturated double bond enables a variety of transformations, including nucleophilic addition, radical addition and cycloaddition.[40] In 2020, Josephson *et al.* described the visible-light-driven installation of side chains at dehydroalanine residues in proteins through the formation of carbon-centred radicals that allow C–C bond formation in water. The authors used boronic acid catechol ester derivatives to generate $RH_2C^{\bullet}$ radicals and pyridylsulfonyl derivatives containing Fe(II) were used to generate $RF_2C^{\bullet}$ radicals. These radicals can react with dehydroalanine at specific sites to form β-CH_2–γ-CH_2 or β-CH_2–γ-CF_2 linkages, thereby incorporating a wide range of functional groups (more than 50 unique residues/side chains) into diverse protein scaffolds and sites (Fig. 7A).[41] Selenocysteine is an isostere of cysteine (Cys) with notable differences in properties, including a lower pK_a, high oxidation potential and nucleophilicity, relative to those of Cys. The differences in reactive properties make selenocysteine an ideal site for site-selective modification. In 2021, Zhao *et al.* introduced selenocysteine into peptides and proteins through solid-phase peptide synthesis (SPPS) methods, utilizing its reactions with alkyl/aryl radicals for the modification of peptides and proteins. The authors rapidly generated radicals *in situ* from hydrazine

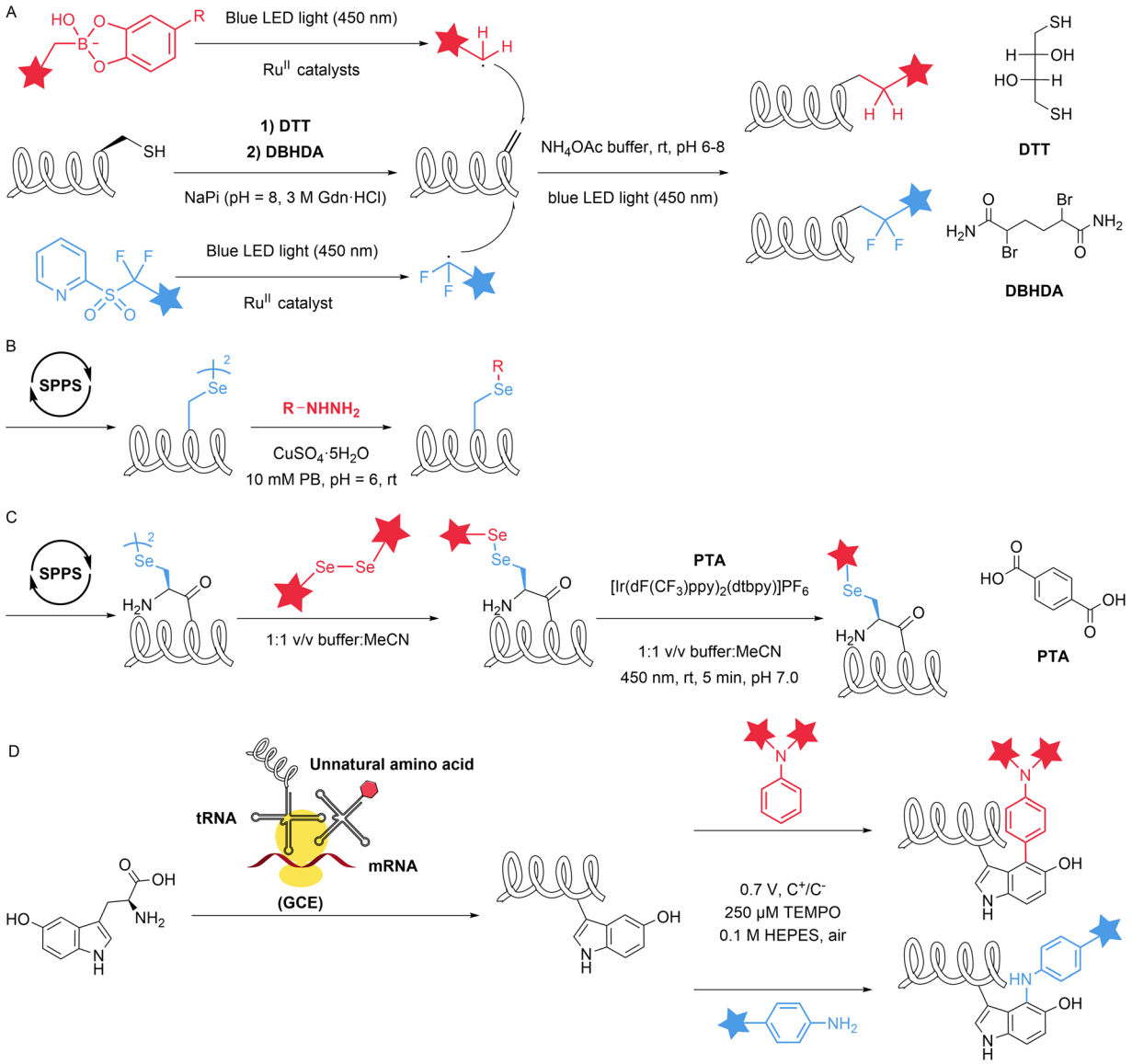

Fig. 7 Site-specific functionalization harnessing the properties of natural amino acid derivatives. (A) Light-driven radical addition to dehydroalanine enables posttranslational side chain installation on proteins. (B) Copper-mediated modification of selenocysteine and aryl/alkyl radicals. (C) Photocatalysed diselenide contraction for site-selective selenocysteine functionalization. (D) Site-specific electrochemical labelling of hydroxyindoles.

substrates and copper ions in an aqueous buffer at near-neutral pH values of 5–8 (Fig. 7B). This reaction is minimally affected by cysteine and methionine and involves the intramolecular cyclization of peptides and biotinylation of proteins.[42] Similarly, in 2022, Dowman *et al.* developed a photocatalytic method using selenocysteine called photocatalytic diselenide contraction (PDC). This reaction occurs upon the irradiation at 450 nm of an iridium photocatalyst and a phosphine and results in rapid and clean conversion of diselenides to reductively stable selenoethers (Fig. 7C). The authors achieved dimerization of selenopeptides, late-stage modification of calmodulin (K148U) diselenide and site-specific C-terminal functionalization of ubiquitin diselenide *via* a PDC reaction.[43]

Compared with canonical aromatic amino acids, the non-canonical amino acid 5-hydroxytryptophan (5HTP) possesses unique reactive properties and has substantially lower oxidation potential. Electrochemical methods, with their precise control over fundamental reaction parameters, mild reaction conditions, and ability to generate reactive intermediates *in situ*, have become attractive strategies for the chemoselective modification of proteins. In 2023, Loynd *et al.* reported an electrochemically promoted coupling reaction between 5-hydroxytryptophan and simple aromatic amines called electrochemical labelling of hydroxyindoles with chemoselectivity (eCLIC). This method selectively converts 5HTP into a reactive electrophile, enabling it to couple with nucleophilic aromatic amines that are inherently unreactive towards proteins (Fig. 7D). The authors used this method to achieve the site-specific modification of several different proteins, including full-length antibodies. The reaction is mutually compatible with the azide-alkyne cycloaddition, as well as the reaction between strained alkenes and tetrazines. The authors utilized this feature to express a protein containing 5HTP and azide-ncAA residues, successfully generating a homogeneous dual-labelled conjugate.[44]

3.2 Insertion of unnatural amino acids

The development of the genetic code extension (GCE) technique has made the insertion of unnatural amino acids more convenient. Their incorporation creates subtle differences in reactivity. This property is discussed in Section 2.2.2. Unnatural amino acids can possess latent bioreactivity and react with target natural amino acids. This aspect will be discussed in Section 4.3. Unnatural amino acids can carry bioorthogonal handles such as alkynyl or azide groups. These groups have enabled the development of a variety of site-specific modification reactions and will be the focus of the following discussion. Introducing an unpaired cysteine thiol into proteins that already contain internal disulfide bonds without perturbing the existing disulfide network is extremely challenging. In 2020, Ling *et al.* used the genetic code expansion technique to introduce a surface-exposed self-paired di-thiol functional group into proteins, which can be selectively reduced to generate active thiols without disturbing the internal disulfide bond. The introduction of the dithiol-containing amino acid has no influence on protein activity or the disulfide network. The two thiol groups generated can be specifically modified by iodoacetamide (IAM) (Fig. 8A).[45]

Quinone methides (QM) are excellent Michael acceptors for nucleophiles and have a high reaction rate. In 2020, Liu *et al.* introduced the amino acids FnbY and FmnbY, which contain photocaged quinone methides, into proteins using the genetic code expansion technique. Upon light-induced activation, these amino acids generate QM that enables rapid and selective conjugation with amine reagents (Fig. 8B). The authors used this method to install various functional groups onto proteins, offering advantages such as compatibility with low temperature and small linkages.[46] Introducing two mutually orthogonal groups into a protein enables specific modifications of two different types. In 2023, Wang *et al.* designed the encodable noncanonical amino acids pTAF and mTAF that simultaneously contain azide and tetrazine reaction handles. TAFs are double bioorthogonal handles that can react with commercially available fluorophores, radioisotopes, PEGs, and drugs in one-pot (Fig. 8C). The authors can use protein dual conjugates to assess combinations of tumour diagnosis, image-guided surgery, and targeted therapy in mouse models. Furthermore, simultaneously incorporating mTAF and a ketone-containing noncanonical amino acid into one protein allows the preparation of a site-specific protein triconjugate.[47] For the same type of unnatural amino acids, diverse functionalizations can be achieved by modulating their reactivity. In 2023, Cao *et al.* developed a supramolecular control system to reversibly regulate tetrazine reactivity through host caging. The authors synthesized naphthotubes and phenyltetrazine derivatives with high affinity and specificity as host–guest pairs, enabling the precise control of the unnatural amino acid-mediated reactions of tetrazine on demand (Fig. 8D). With respect to the tetrazine residues introduced at different sites in the proteins, the exposed residues could be reversibly caged, whereas the semi-buried residues were resistant to caging and retained their reactivity. This approach allows for the preparation of dual site-specific protein conjugates and sequential labelling of dual proteins on living cells using the inverse electron demand Diels–Alder (IEDDA) reaction. This system can also be used to control IEDDA reactions in living animals.[48]

3.3 Insertion of amino acid sequences

Natural proteins precisely control selective reactions and interactions through the construction of large three-dimensional structures. For example, enzymes have catalytic activity at specific sites, which is usually generated by their unique structures, resulting in the formation of a lock-and-key pairing pattern. In enzymes, particular amino acids are placed in a specialized active site environment. Similarly, researchers have designed a series of peptide sequences to adjust the reactivity of specific amino acids within the peptide sequences, thereby enabling modification reactions at specific sites. In 2016, Zhang *et al.* designed a four-amino-acid sequence (FCPF), which the authors call the 'π-clamp'. The authors hypothesize that the aromatic side chains of Phe activate the cysteine thiol and enhance the interaction with the perfluoroaryl reagents

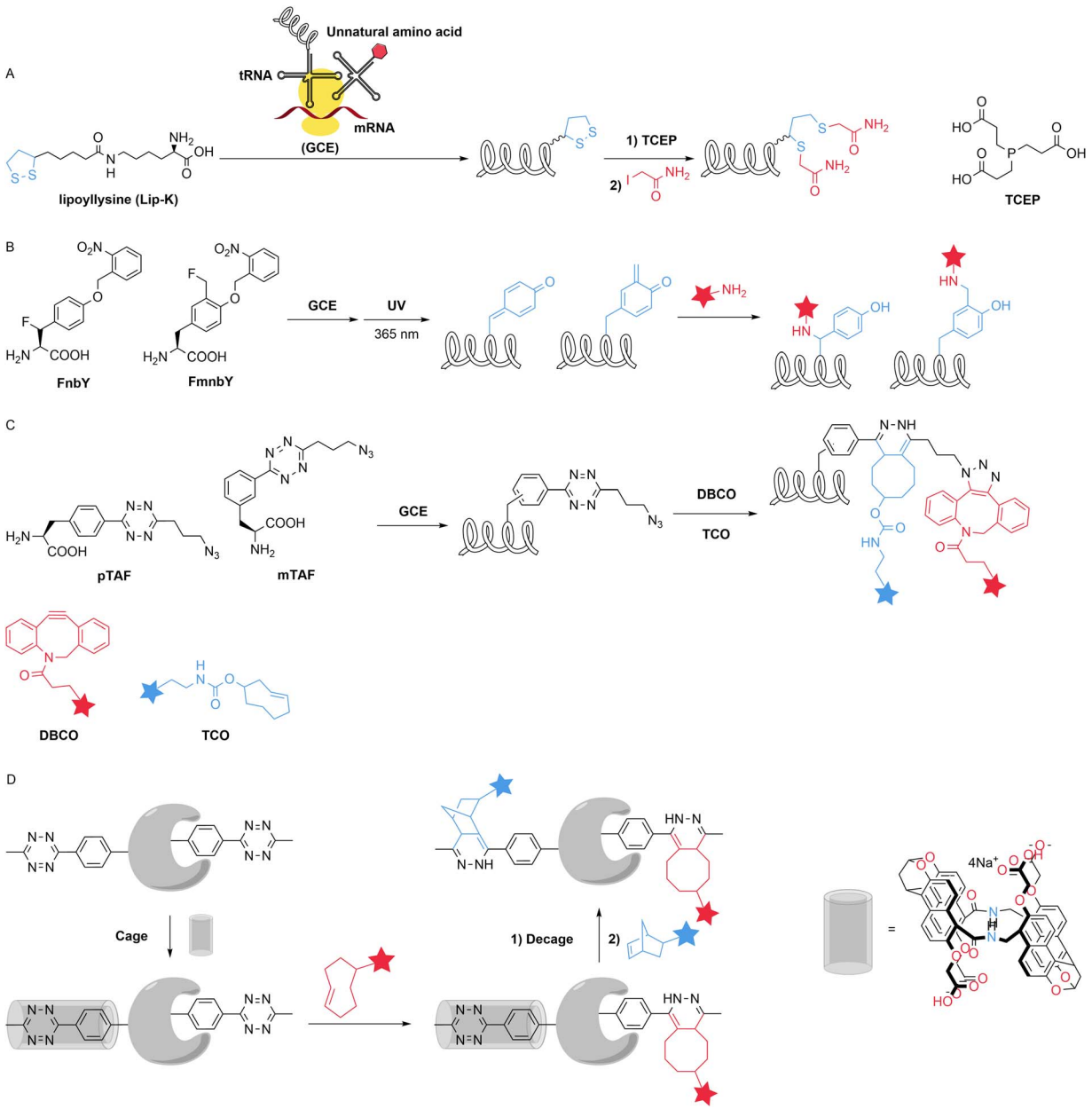

Fig. 8 Genetically encoded and orthogonally functionalized unnatural amino acids for site-specific protein modification. (A) Site-specific protein modification by disulfide-compatible thiols. (B) The incorporation of amino acids bearing photocaged quinone methides enables site-specific modification. (C) Noncanonical amino acids as double bioorthogonal handles for the one-pot preparation of protein multiconjugates. (D) Reversible control of tetrazine bioorthogonal reactivity by naphthotube-mediated host–guest recognition.

used in the reaction (Fig. 9A). Moreover, Pro serves to position the Cys and Phe residues into a conformation that promotes the reaction. The authors use the π-clamp to selectively modify the cysteine in the π-clamp sequence within proteins containing multiple endogenous cysteine residues. The authors applied this method to modify antibodies and enzymes where traditional cysteine modification methods cannot readily be used. Antibodies modified using the π-clamp retained binding affinity to their targets, enabling the synthesis of site-specific antibody–drug conjugates for the selective killing of HER2-positive breast cancer cells.[49] The π-clamp has been repeatedly exploited for protein modification and functionalization and has demonstrated good application prospects. In 2021, Gama-Brambila *et al.* inserted the π-clamp into Cas proteins (Cas9, dCas9, Cas12, and Cas13). FCPF-tagged proteins can be labelled with perfluoroarene carrying fluorescein in living cells and degraded by a perfluoroaromatic-functionalized PROTAC (PROTAC-FCPF). This strategy sufficiently suppresses the activity of Cas proteins, suggesting a wide range of applications of perfluoroaromatic-induced proximity in the regulation of stability, activity, and functionality of any FCPF-tagged protein.[50] The generation of protein–protein conjugates plays

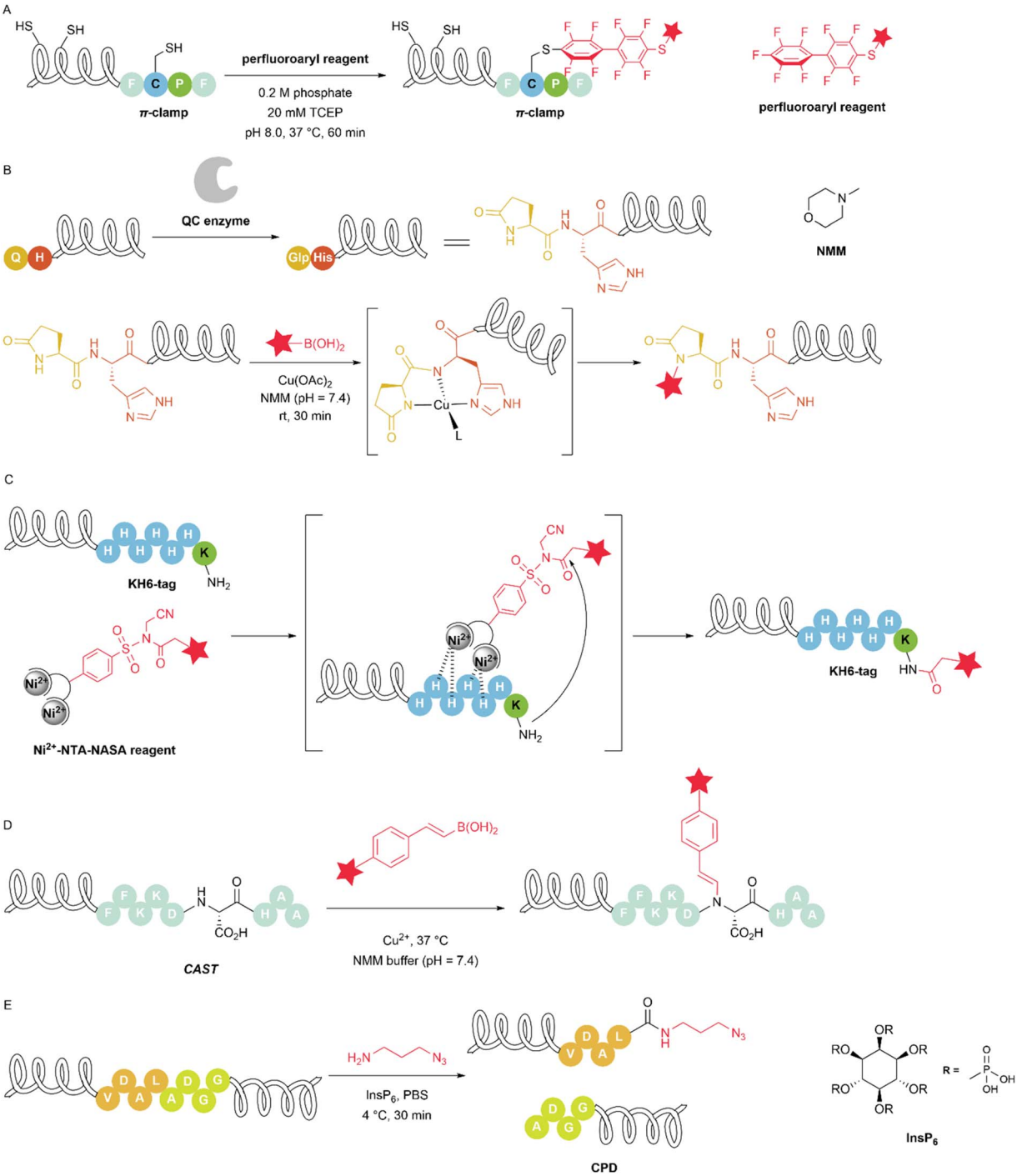

Fig. 9 Tuning site reactivity *via* unique amino acid sequences to achieve site-specific modification. (A) π-Clamp-mediated cysteine conjugation. (B) Pyroglutamate–histidine dipeptide-directed, copper-catalysed Chan–Lam coupling. (C) Peptide sequence-mediated, site-specific lysine modification by NASA reagents. (D) The copper-assisted sequence-specific conjugation tag (CAST) method was used to achieve the site-specific chemical modification of the protein backbone with pinpoint accuracy. (E) C-terminal functionalization *via* self-cleavage of the cysteine protease domain (CPD).

an important role in advancing the fields of biotechnology and biopharmaceutical research. Posttranslational chemical modification is a powerful approach for obtaining diverse protein–protein conjugates. In 2022, Taylor *et al.* developed a method for preparing protein–protein conjugates through π-clamp-mediated cysteine arylation with pentafluorophenyl sulfonamide functional groups. The authors used this method to achieve the homodimerization and heterodimerization of two antibodies that have π-clamps near their C-termini, resulting in a 10–60-fold increase in binding. Even in the presence of

Table 3 Methods by introducing the Tag

Tag type	Modified residue	Reagent	Reference
Natural amino acid derivative	Dehydroalanine	Boronic acid catechol ester/pyridylsulfonyl	41
	Selenocysteine	Hydrazine substrate	42
	Selenocysteine	Diselenides	43
	5-Hydroxytryptophan	Aromatic amines	44
Unnatural amino acid	Lipoyllysine	Iodoacetamide	45
	FnbY/FmnbY	Amine	46
	pTAF/mTAF	DBCO/TCO	47
	Phenyltetrazine	TCO	48
Peptide sequence	FCPF	Perfluoroaryl	49
	Glp–His	Boronic acid	52
	HHHHHHK	Ni^{2+}-NTA-NASA	53
	FFKKDDHAA	Styrylboronic acid	54
	VDALADGG	$InsP_6$	55

solvent-exposed cysteine residues in the protein, the cysteine in the π-clamp is still selectively modified. The other cysteines can participate in subsequent small-molecule conjugation, thereby constructing molecule–protein–protein conjugates with complete control over the modification sites.[51]

The limitations of traditional chemical modification methods in terms of site selectivity make fusion protein domains with unique reactivity widely valuable in protein conjugation. In addition to the π-clamp described above, researchers have developed a variety of amino acid sequences to achieve site-selective modification. In 2018, Ohata *et al.* discovered a dipeptide sequence, pyroglutamate–histidine (Glp–His), that can serve as the minimal tag for protein modification, featuring extremely rapid kinetics and excellent selectivity. The Glp–His sequence can be generated by the QH sequence using the glutaminyl cyclase (QC) enzyme. The use of the glutaminyl cyclase (QC) enzyme to generate specific Glp–His sequences *in vivo* is feasible and does not affect protein expression levels. The Glp–His dipeptide sequence exhibits remarkably fast and efficient copper-catalysed N–H coupling with boronic acids in 4-methylmorpholine (NMM) buffer, allowing specific and bioorthogonal functionalization at a natural posttranslational modification site (Fig. 9B).[52] A peptide motif could specifically bind a metal ion that catalyses a conjugation reaction with the bound peptide, thus simultaneously achieving chemoselectivity and site specificity. The His tag is a very common and effective example, as it can coordinate with Ni^{2+} and Co^{2+}. In 2021, Thimaradka *et al.* utilized a lysine in the short peptide sequence KH6 containing a His tag to react with Ni^{2+}-NTA-tethered *N*-acyl-*N*-alkyl sulfonamide (NASA) reagent for the modification of the amino group of the lysine side chain. Upon the complexation of Ni^{2+}-NTA with the H6 sequence, the lysine residue in the tag reacts with the NASA group to introduce a required group *via* the proximity effect (Fig. 9C). The authors characterized the properties of this reaction using model peptides and His-tagged enhanced green fluorescent protein (EGFP). The authors used this method to prepare a chemically functionalized nanobody and applied it to the imaging of membrane surface proteins in living cells.[53] Similar "metal-binding targeting" strategies have been developed to achieve site-specific protein conjugation. In 2023, Guo *et al.* developed a copper-assisted sequence-specific conjugation tag (CAST) method to achieve rapid, site-specific chemical modification of the protein amide backbone with pinpoint accuracy. The authors screened peptide sequences to optimize the reaction rates of His-containing peptides capable of binding metal ions. Using scanning point mutation and preferred residue combinations for sequence optimization, the authors ultimately generated the peptide FFKKDDHAA, which has the highest reactivity with styrylboronic acid. A screen of boronic acid substrates revealed that styrene boronic acid exhibited the best reactivity (Fig. 9D). The authors inserted the CAST peptide at different locations in proteins, including the N-terminus, internal loop, and C-terminus, to efficiently and quantitatively modify a diverse range of proteins. The authors employed this method to prepare stable antibody–drug conjugates (ADCs) with potent efficacy both *in vitro* and *in vivo*. CAST conjugation is orthogonal to all currently reported chemical or enzymatic conjugation methods, enabling the preparation of dual-payload antibody–drug conjugates.[54] Protease domains are frequently employed to increase the expression and purification of proteins of interest. Fusing the C-terminus of a protein with these unique domains facilitates site-specific modification by the corresponding enzymes. Unlike other tags, the cysteine protease domain (CPD) can be conveniently removed by a catalytic equivalent of inositol hexaphosphate ($InsP_6$). In 2023, Zeng *et al.* fused a cysteine protease domain on the C-terminus of the protein of interest and triggered cysteine protease domain self-cleavage with $InsP_6$ to achieve nonenzymatic C-terminal functionalization. CPD-induced C-terminal modification is compatible with diverse amine substrates and proteins (Fig. 9E). The authors leveraged this method to achieve the site-specific assembly of nanobody–drug conjugates and the construction of bifunctional antibodies (Table 3).[55]

4 Proximity-driven chemical modification

Tags enable site-specific modification but typically restrict modifications to the introduced tag rather than allowing

modification at native sites within the protein. By harnessing interactions near the protein's intrinsic modification site, the modifying agent can be confined to the vicinity of the target site, thereby increasing its local concentration. This approach exploits a local molarity effect to promote modification at a specific site, similar to the actions of enzymes. It can utilize either the amino acids adjacent to the modification site or the interactions with ligands to achieve site-specific modification. We refer to this strategy as the proximity-driven chemical modification approach. We divide these modifications into proximity-group-mediated modifications, ligand-directed modifications and affinity-peptide-directed modifications.

4.1 Proximity-group-mediated modification

4.1.1 Modification of side-chain residues. The reactivity of a target amino acid residue is usually insufficiently unique to enable single-site modification. The nearby amino acid residues or groups can serve as directing handles to preposition the reagent, increasing its effective local concentration. Rai's group developed the linchpin-directed modification (LDM), which enables site-controllable and site-specific modification through amino acid positioning. By positioning the linchpin at lysine residues, the researchers developed a variety of lysine-derived linchpin-directed modification reagents, resulting in the labelling of His (LDM_{K-H}), Lys (LDM_{K-K}), and Asp (LDM_{K-D}) (Fig. 10A).[56–58] In 2018, Adusumalli *et al.* reported the use of LDM_{K-H} technology to achieve the site-selective modification of histidine residues. The key to this method is the development of F_K-spacer-F_H reagents. The functional group F_K undergoes rapid and reversible chemoselective reactions with all accessible Lys residues, whereas F_H reacts slowly and irreversibly with the proximal His to control site selectivity. Tuning the reagent with the spacer could precisely match the relative orientation of F_K–F_H with the Lys and His residue pair to accelerate selective labelling (Fig. 10B). The authors applied this method to achieve the precise labelling of diverse proteins, including myoglobin, cytochrome C, aldolase, and lysozyme C, and allowed a single protein to be selectively tagged within complex mixtures. This method offers a convenient route for the late-stage, bioorthogonal attachment of NMR tags, affinity tags, and fluorophores, as well as for the preparation of antibody–drug conjugates.[56] In 2020, Adusumalli *et al.* reported LDM_{K-K} technology that allows the precise labelling of a single Lys. The design concept of the method is similar to that of the previous method. F_K^1 can preferentially react with one Lys residue of the Lys pair and rapidly reach equilibrium. Afterwards, F_K^2 undergoes an irreversible reaction with the second lysine to complete the modification. The coupling site can be regulated by the reactive sequence of Lys and by controlling the length of the spacer. This method is applicable to a variety of proteins with complex multiple lysine sites and allows the labelling of multiple domains on the protein surface. The authors used LDM_{K-K} to synthesize ADCs that exhibited highly specific antiproliferative activity against HER-2-positive SKBR-3 breast cancer cells.[57] In 2021, the authors continued to refine the linchpin-directed modification platform by employing nonconserved electrophiles. Using the linchpin to position and modulate the local concentration, the authors achieved the single-site-selective modification of Asp in ubiquitin and His in insulin, α-lactalbumin, and myoglobin. Examples of precisely labelled less-reactive residues are rare, indicating that the conserved chemoselectivity of electrophiles is not a necessary prerequisite for the precise single-site modification of native proteins.[58] The methods described above use high-frequency Lys residues to position the linchpin and directly modify other residues. However, when the number of proteins in a mixture increases, deriving the linchpin from low-frequency residues to modify high-frequency sites becomes highly valuable. In 2023, Reddy *et al.* reported a Cys-derived linchpin-directed single-site modification of lysine (LDM_{C-K}) to meet the technological demands. The authors developed nitroolefin (F_C) as an electrophilic reagent capable of undergoing a rapid, chemoselective reaction with cysteine. Through a retro-Michael/retro-Henry reaction, this reagent provided an aromatic aldehyde that enables probe capture, enrichment, purification, and late-stage installation (Fig. 10C). LDM_{C-K} enables the single-site-selective modification of proteins and demonstrates protein selectivity in protein mixtures or cell lysates. This method enables single-site installation of various probes *via* the generated aldehyde group and allows for the chemoselective installation of a second tag at the cysteine site to ensure precise dual labelling of the protein.[59] In the same year, Afonso *et al.* proposed a proximity-driven, cysteine-mediated, lysine-specific acetylation method to construct precisely acetylated histones. The authors engineered histone H3 by introducing a cysteine adjacent to the target lysine. First, the cysteine was conjugated with maleimide-dibenzocyclooctyne; then, an acetyl donor, *gem*-dithioacetate, was anchored *via* alkyne-azide cycloaddition. Finally, nucleophilic attack by the ε-amino group of the nearest lysine on the carbonyl of the acetyl donor triggers spontaneous acetylation, resulting in site-specific modification (Fig. 10D). Using this approach, the authors achieved single-site and dual-site precise acetylation of the external K9 residue and the internal K56 residue in recombinant histone H3. These acetylated histones interact with their relevant biological partners, even in the presence of the strain-promoted azide-alkyne cycloaddition (SPAAC) product at the nearby cysteine.[60] Supramolecular interactions with side-chain residues bring the reacting groups into close proximity, enabling single-site-specific modification. In 2021, Li *et al.* developed a new strategy in which cucurbit [8] uril (CB[8]) facilitated Michael addition to modify proteins and peptides. The cavity of CB[8] simultaneously accommodates both a tryptophan (Trp) and methyl viologen (MV) as guest molecules. Exploiting this property, a Trp residue on the target peptide and an MV on the reactive peptide are threaded into CB[8], drawing the cysteine and dehydroalanine into close proximity and thereby achieving single-site modification of the cysteine (Fig. 10E). This strategy overcomes the regioselective limitations of protein modification *via* Michael addition, enabling precise labelling within structurally complex targets. Notably, its successful application to KRAS highlights its potential for tackling intricate biomolecular systems.[61]

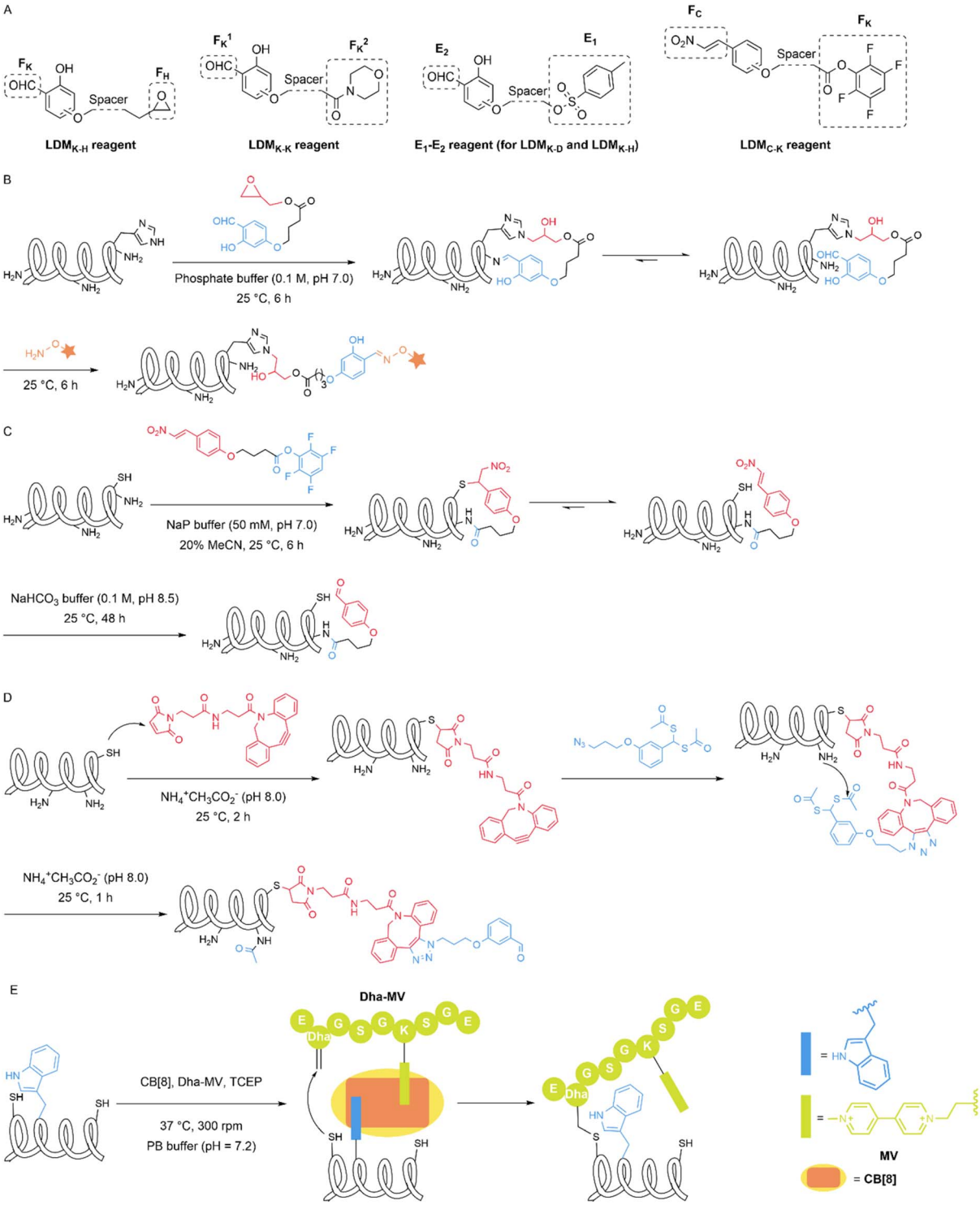

Fig. 10 Site-specific modifications mediated by proximity groups. (A) Design of reaction reagents for diverse LDM applications. (B) The sequential linchpin-directed His modification by Lys (LDM$_{K-H}$). The sequential targeting of Lys and His can regulate the site selectivity of the protein modification. (C) The sequential linchpin-directed Lys modification by Cys (LDM$_{C-K}$). (D) Cysteine-assisted click chemistry for the proximity-driven, site-specific acetylation of histones. (E) Cucurbit [8] uril facilitates Michael addition for regioselective cysteine modification.

4.1.2 C(sp^3)–H bond functionalization. Side chain residues can be modified through proximity-driven strategies, and C(sp^3)–H bonds in a peptide can be functionalized by directing the reagent to the target site *via* directing groups. Chen *et al.* developed a series of proximity-group-directed C(sp^3)–H functionalization reactions that enable the construction of macrocyclic peptides *via* intramolecular cyclization. In 2018, Zhang *et al.* reported a method for the synthesis of cyclophane-braced

peptide macrocycles from simple linear peptide precursors using the palladium-catalysed 8-aminoquinoline (AQ)-directed intramolecular arylation of sp^3 hybridized C–H bonds (Fig. 11A). This strategy provides a powerful tool to address the long-standing challenge of size and composition dependence in peptide macrocyclization and generates novel peptide macrocycles with uniquely buttressed backbones and distinct loop-type three-dimensional structures.[62] In 2019, Li *et al.* reported a generally applicable method for the synthesis of natural product-like cyclophane-braced peptide macrocycles *via* Pd-catalysed intramolecular C(sp^3)–H arylation with aryl iodides at the remote γ position of various N-terminal aliphatic amino acid units using a simple picolinamide (PA) directing group. Products with high structural and stereochemical complexity were quickly assembled from easily accessible peptide precursors prepared using standard solid-phase peptide synthesis (Fig. 11B). The cyclization of unprotected peptide substrates carrying various free polar side chains can proceed in aqueous medium. It offers valuable new orthogonal reactivity for peptide chemistry.[63]

Unmodified side chains of natural amino acids can be harnessed to assist the functionalization of C(sp^3)–H bonds in peptides. Asparagine, a natural amino acid bearing a primary amide side chain, can serve as a directing group. Its side chain and backbone can coordinate with palladium to form a bidentate Pd complex. In 2020, Weng *et al.* utilized the unmodified side chain of Asn combined with the backbone to form 5,6-fused bicyclic palladacycles that act as *N*,*N*-bidentate coordination sites, enabling the late-stage C(sp^3)–H arylation of peptides (Fig. 11C). This method avoids the need to install and later remove exogenous directing groups. The authors demonstrated the coordinating role of the Asn primary amide and calculated the reaction mechanism. Using this approach, the authors achieved the arylation of Asn-containing di-, tri-, and tetrapeptides.[64] In 2021, Chen *et al.* developed a late-stage, native Asp-directed C(sp^3)–H arylation and alkynylation of peptide sequences containing L- and D-alanine (Ala), yielding a series of modified products, including tyrosine (Tyr) analogues, phenylalanine (Phe) analogues, and alkynylated Ala analogues. When conducted at a mild temperature (50 °C), the reaction delivers a variety of modified peptides without the need for expensive ligands or exogenous additives (Fig. 11D). Using this protocol, the authors successfully constructed strongly fluorescent (BODIPY)-labelled peptides.[65] Transition metals tend to preferentially bind to peptide backbone amides rather than aliphatic side chains, leading to an inhibitory effect of the peptide backbone on side chain functionalization. The functionalization of aliphatic side chains at internal positions *via* amino acid coordination-assisted C(sp^3)–H activation remains underdeveloped. In 2024, Hou *et al.* developed a highly site-selective β-C(sp^3)–H arylation method for modifying the internal positions of both linear and cyclic peptides using *S*-alkyl cysteine as the directing group and 2-pyridone as the ligand for the Pd catalyst. The key to the success of the reaction lies in the formation of an *N*,*S*-bidentate directing group (Fig. 11E). The authors discovered that the protecting group on the sulfur atom is vital to preserve its coordinating ability and prevent side reactions. Based on this result, the authors developed a new protecting group, 4-methoxy-3-nitrobenzyl (PMNB), which promotes efficient C–H arylation and can be readily removed after peptide modification. The facile conversion of *S*-methyl cysteine to dehydroalanine (Dha) and the desulfurization of *S*-(*p*-nitrobenzyl)cysteine to alanine further expand the synthetic utility of the established C–H arylation strategy.[66]

4.2 Ligand-directed modification

The binding pockets of proteins can engage a cognate ligand. Applying a reactive warhead to that ligand converts it into a chemical probe. After protein–ligand interactions occur, the electrophilic group of the probe is positioned near the POI, thereby facilitating nucleophilic attack by residues near the ligand binding site and inducing covalent binding. Acrylamide has been widely employed as an electrophilic warhead for the covalent labelling of many cysteine-containing proteins. London's group developed a method based on covalent ligand-directed release (CoLDR) chemistry using α-substituted methacrylamide and performed a series of studies using this method. In 2021, Reddi *et al.* utilized this technology to achieve the site-specific labelling of endogenous proteins. Protein labelling *via* traditional affinity methods often inhibits protein activity because the targeting ligand permanently occupies the target binding pocket. The authors designed α-methacrylamide reagents with the targeting ligand as a leaving group, enabling the release of the targeting ligand while various functional tags were installed on the target protein (Fig. 12A). The authors documented a variety of applications of this method, including determining the half-life of BTK in its native environment and quantifying BTK degradation *via* proteolysis-targeting chimaeras (PROTACs). This method is a labelling strategy that maintains target protein activity and represents an important addition to the chemical biology toolbox.[67] In the same year, Reddi *et al.* found that hetero α-substituted methacrylamides have higher thiol reactivity and undergo a conjugate addition–elimination reaction, ultimately releasing the substituent (Fig. 12B). The authors altered the reactivity of α-methacrylamides by changing different α-substituents and used the BTK inhibitor ibrutinib as a model for modification, which exhibited low reactivity towards glutathione and high site selectivity. This method can be used to develop fluorescent turn-on probes. The authors generated a BTK CoLDR chemiluminescent probe that can be used for high-throughput screening of BTK inhibitors.[68] The tunability of electrophile reactivity can help determine the optimal balance between selectivity and potency. In 2023, Reddi *et al.* reported that sulfamate acetamide serves as a tuneable and low-reactivity electrophile. When the reagents react with cysteine, they release sulfamic acid. This "self-immolative" feature enables their use in covalent ligand-directed release chemistry. The authors achieved the generation of "turn-on" probes and traceless ligand-directed site-specific protein labelling (Fig. 12C).[69] In 2023, Wang *et al.* developed a strategy for the site-selective modification of the cysteine residues of target proteins using a sulfonium warhead based on a proximity-triggered approach. The authors used an

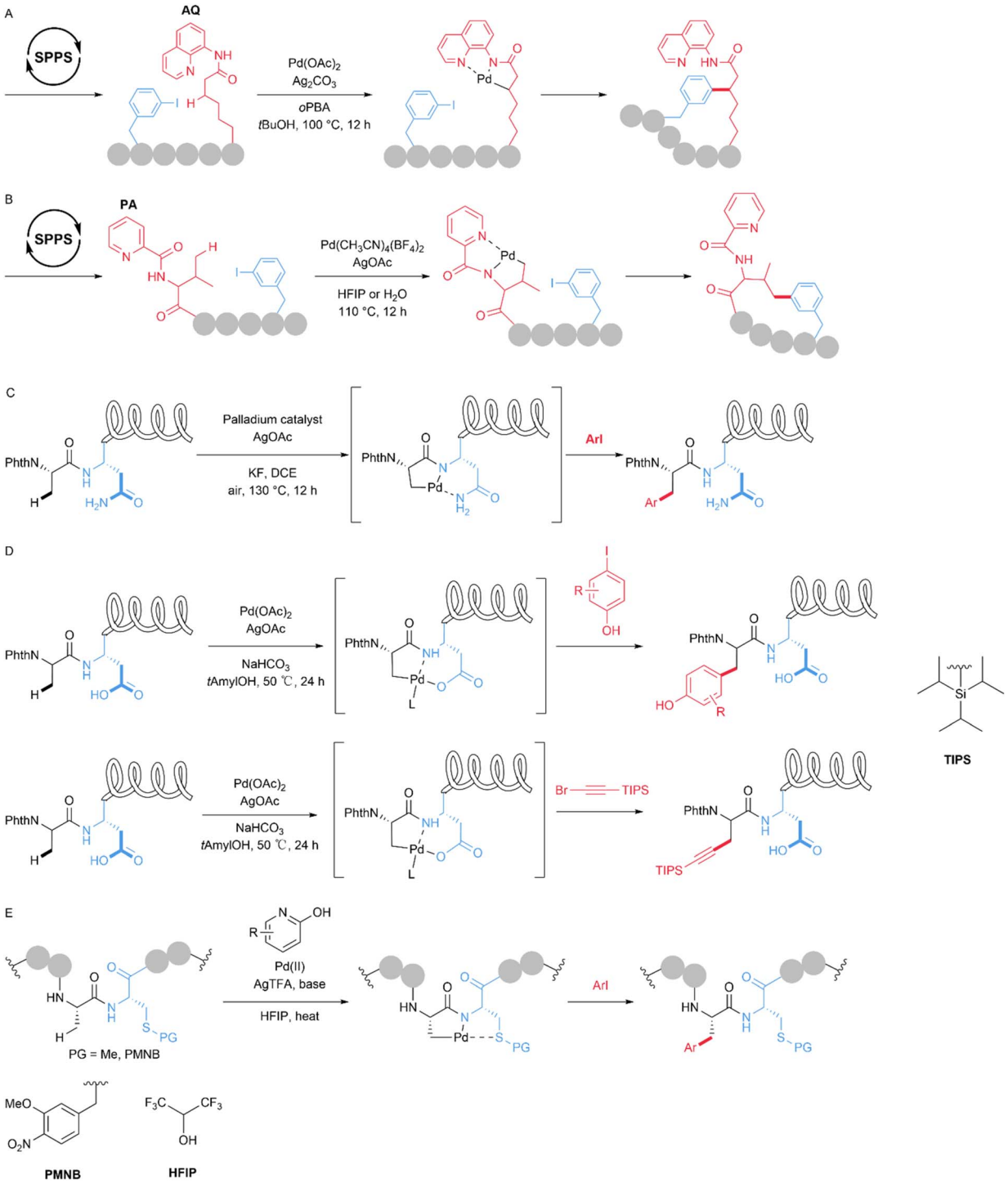

Fig. 11 Proximity-group-mediated C(sp^3)–H bond functionalization. (A) Palladium-catalysed aminoquinoline (AQ)-directed intramolecular arylation of the C(sp^3)–H bond. (B) Picolinamide group-directed intramolecular C(sp^3)–H arylation. (C) Site-selective arylations of C(sp^3)–H bonds at the N-terminus with the assistance of asparagine without exogenous directing groups. (D) Palladium-catalysed C(sp^3)–H arylation and alkynylation of peptides directed by aspartic acid. (E) Ligand-enabled, cysteine-directed β-C(sp^3)–H arylation of alanine.

inhibitor of epidermal growth factor receptor (EGFR), osimertinib, as the binding ligand and a model compound to develop a sulfonium-based reagent for EGFR modification. It can transfer different groups, such as bioorthogonal groups, dyes, and biotin, to EGFR (Fig. 12D). The authors used this method to achieve the transfer of covalent E3 ligase recruiter and hydrophobic tags to EGFR, achieving EGFR degradation based on the selectivity of the transferable PROTAC strategy and hydrophobic tagging strategy.[70]

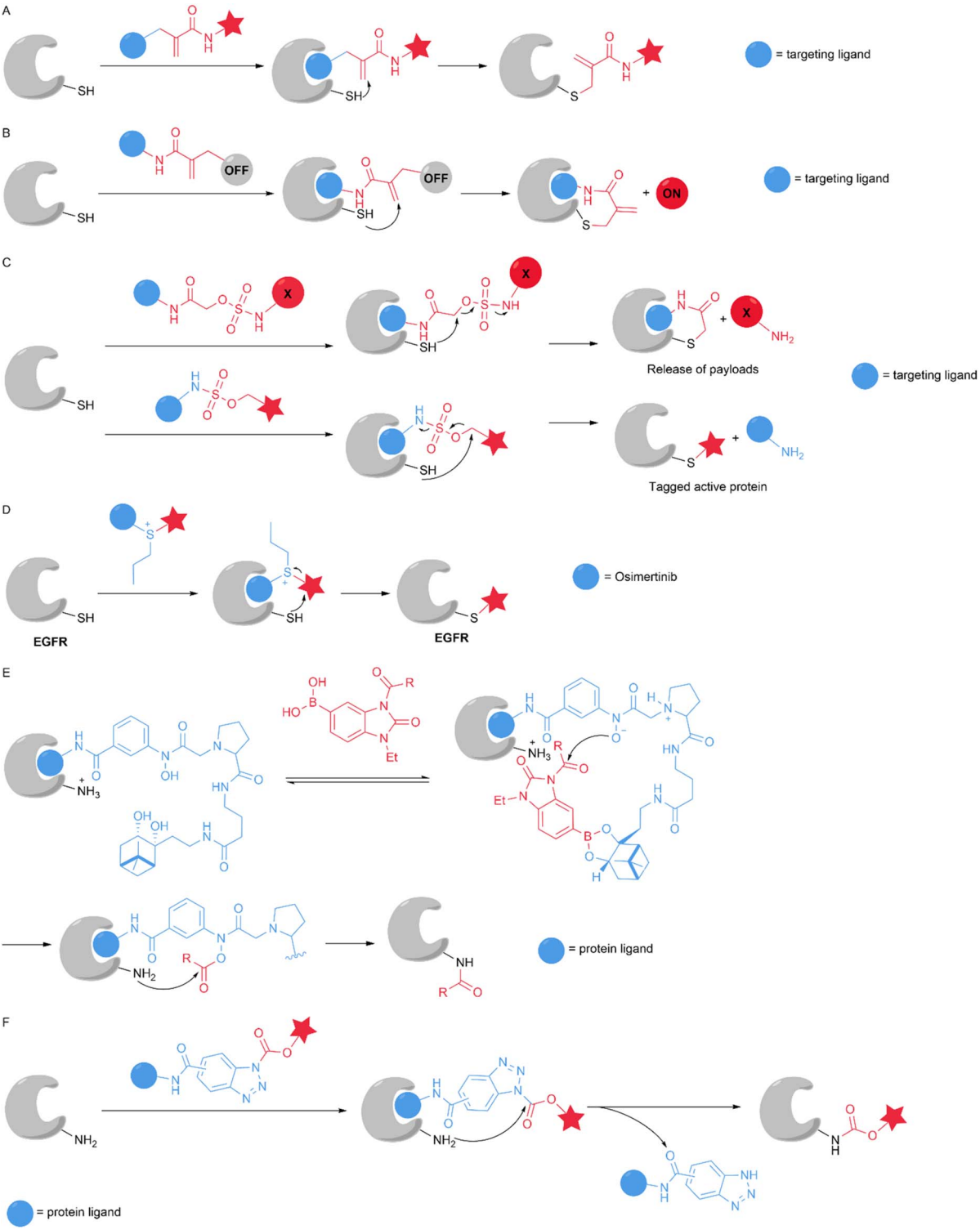

Fig. 12 Target-ligand-directed protein modification. (A) Site-specific labelling of endogenous proteins using covalent ligand-directed release (CoLDR) chemistry. (B) CoLDR chemistry for turn-on fluorescent probes. (C) Sulfamate acetamides as self-immolative electrophiles for CoLDR chemistry. (D) Proximity-triggered, sulfonium-based reagents for site-specific EGFR modification. (E) Boronate-assisted hydroxamic acid (BAHA) catalyst system using reversible boronate formation. (F) Ultrafast and selective labelling of proteins using affinity-based benzotriazole chemistry.

Selective methods for directly introducing post-translational modifications (PTMs) into proteins in living cells are highly important. In 2021, Adamson *et al.* targeted *E. coli* dihydrofolate reductase expressed within human cells as the substrate and utilized a boronate-assisted hydroxamic acid (BAHA) catalyst system to install a variety of acyl groups on the protein of

interest. The system comprises a protein ligand, a hydroxamate Lewis base, and a diol moiety. When used in conjunction with a boronic acid-containing acyl donor, the catalyst can facilitate acyl transfer to the target lysine residue by binding to the protein of interest (Fig. 12E). The system affords minimal off-target protein reactivity and provides resistance to glutathione. For proteins that have a suitable ligand, this approach enables both naturally occurring and abiotic lysine PTMs to be installed within live cells.[71] Most reactive groups currently used for protein affinity labelling have low reactivity towards nucleophilic residues, and the proximity effect accelerates the reaction to achieve selectivity. Great potential and an urgent need for the development of new reactive groups for affinity labelling that have fast reaction rates and good selectivity exist. In 2022, Xin *et al.* developed benzotriazole (BTA) chemistry that can be incorporated into affinity-labelled probes for the rapid and selective labelling of different cytosolic and membrane proteins both *in vitro* and in cells (Fig. 12F). Affinity-based BTA probes selectively and covalently label a lysine residue in the vicinity of the ligand binding site of a target protein with a reaction half-time of 28 s.[72]

4.3 Affinity biomacromolecule-directed modification

In enzymatic reactions, due to the affinity between the enzyme and the reaction substrate, the reaction substrate can move closer to the active centre of the enzyme. The affinity between substrate proteins, especially antibodies, and enzymes can also occur between the protein and affinity biomacromolecules. Similar to the proximity effect observed in natural enzymes, appending a reactive warhead to affinity biomacromolecules decreases the distance between the reaction reagent and the target residue, increasing the effective local concentration of the reagent. This strategy has been widely adopted by researchers for site-specific protein modification. Proteins can act as affinity biomacromolecules. Wang's group incorporated latent bioreactive unnatural amino acids (Uaas) into proteins to react with target natural amino acid residues through their proximity and reactivity. In 2018, Wang *et al.* reported the genetic encoding of fluorosulfate-L-tyrosine (FSY) as the first latent bioreactive Uaa that undergoes sulfur–fluorine exchange (SuFEx) on proteins *in vivo*. After FSY is incorporated into affinity proteins, it can selectively react with nearby lysine, histidine, and tyrosine residues on the protein of interest, directly generating covalent intraprotein bridges and interprotein cross-links between interacting proteins in living cells (Fig. 13A). FSY possesses proximity-activated reactivity, multitargeting capability, and excellent biocompatibility, making it highly important for *in vivo* protein covalent cross-links.[73] FSY has a relatively rigid and short side chain, which restricts the diversity of targetable proteins and the scope of applications. In 2021, Liu *et al.* designed and genetically encoded a new latent bioreactive Uaa, fluorosulfonyloxybenzoyl-L-lysine (FSK), in *E. coli* and mammalian cells. Compared with FSY, it features an aliphatic side chain, offering greater flexibility and a longer reaction distance. FSK is particularly useful for covalently connecting protein sites that are inaccessible to FSY (Fig. 13B). The authors utilized FSY and FSK to target different sites of the epidermal growth factor receptor to combat potential mutation-induced resistance. Applying these reagents to genetically encoded chemical crosslinking can capture enzyme–substrate–protein interactions in *E. coli*. FSK and FSY together provide a SuFEx system for generating covalent bonds in a variety of proteins both *in vitro* and *in vivo*.[74] Monoclonal antibodies (mAbs) contain a large number of reactive lysine residues, making site-specific modification with conventional chemistry challenging. Multisite reactivity typically leads to heterogeneous conjugates. Current approaches therefore rely largely on the genetic incorporation of noncanonical amino acids to introduce bioorthogonal handles. Peptides can also act as affinity biomacromolecules. Proximity-driven chemistry leverages the binding properties between affinity peptides and antibodies, eliminating the need for an additional antibody pretreatment. In 2019, Kishimoto *et al.* developed the method of chemical conjugation by affinity peptide (CCAP) for site-specific chemical modification using a peptide that binds with high affinity to human IgG-Fc. This method enables the rapid modification of specific residues (Lys248) in a single step under mild conditions, resulting in the formation of a stable amide bond between the peptide and the Fc (Fig. 13C). Antibody–drug conjugates and bispecific antibodies can be further developed by conjugating cytotoxic molecules or orthogonal reactive groups to affinity peptides, making it an effective conjugation method.[75] However, due to the presence of a noncleavable linker, the affinity peptide remains on the modified antibody. In the same year, Yamada *et al.* developed a site-specific lysine modification method for native IgG antibodies using similar peptides with special affinity for the Fc region of antibodies, enabling the creation of antibody–drug conjugates. This method is called affinity peptide-mediated regiodivergent functionalization (AJICAP). When a cleavable linker is used, the affinity peptide departs and allows the introduction of a free thiol group into IgG. The authors subsequently conjugated cytotoxic molecules *via* thiol-maleimide coupling (Fig. 13D). This modification method did not impair antigen binding. The results from the mouse model demonstrated that the resulting ADC selectively targeted and killed HER2-positive cells.[76] In 2021, Cao *et al.* reported a new proximity-induced site-specific antibody conjugation method (pClick) based on the proximity-induced reactivity between affinity peptide crosslinkers and a nearby antibody lysine residue. The authors designed and optimized a proximity-activated crosslinking peptide based on antibody-binding proteins (FB proteins) derived from *Staphylococcus aureus* protein A. The authors truncated the FB protein and modified it with 4-fluorophenyl carbamate lysine (FPheK) at position 25 (Fig. 13E). This modification resulted in excellent conjugation efficiency with the native trastuzumab antibody. The authors site-specifically conjugated the Tras antibody with monomethyl auristatin E to synthesize ADCs for the treatment of breast cancer and prepared multifunctional ADCs by coupling 5/6-carboxyfluorescein (FAM)-lysine to the N-terminus of the peptide. Using this technology, the authors prepared bispecific small molecule–antibody conjugates, which displayed excellent antitumour activity in mouse xenograft models.[77]

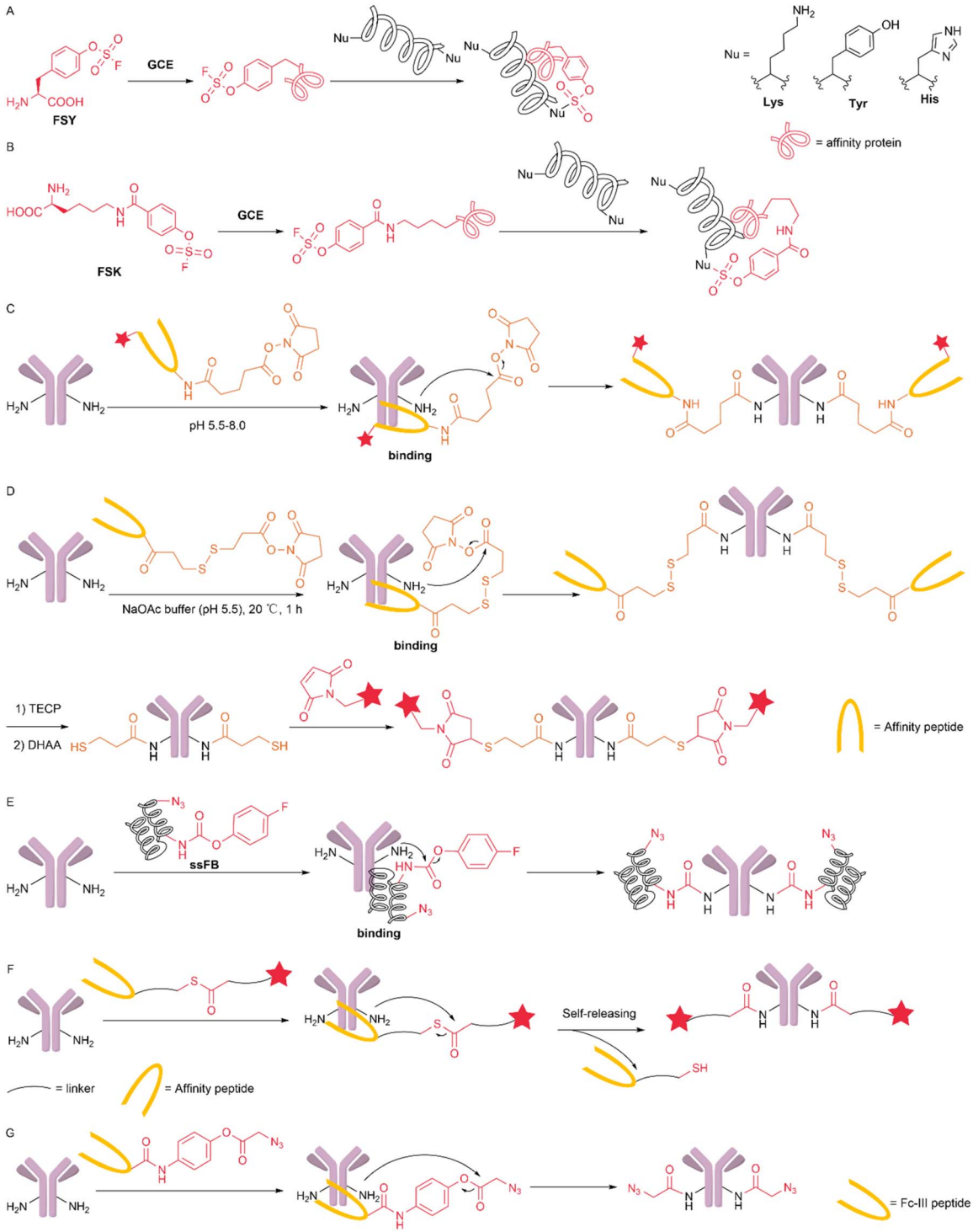

Fig. 13 Affinity biomacromolecule-directed protein functionalization. (A) Genetically encoded fluorosulfate-L-tyrosine (FSY) enables expanded protein modification *via* SuFEx chemistry. (B) Fluorosulfonyloxybenzoyl-L-lysine (FSK) offers greater flexibility and a longer reaction distance for protein modification. (C) The method of chemical conjugation by affinity peptide (CCAP) for site-specific modification. (D) The method of affinity peptide-mediated regiodivergent functionalization (AJICAP). (E) FB-protein-based cross-linked peptides for proximity-induced site-specific antibody conjugation (pClick). (F) A novel thioester-based acyl-transfer design enables ligand self-release during payload assembly. (G) Fc-III-peptide-directed site-specific lysine acetylation.

In the methods described above, after modification, the affinity peptides are either conjugated to the antibody or require subsequent cleavage. The self-release of the targeting peptide in a one-step reaction can be used to develop simple and efficient traceless modifications. In 2022, Zeng *et al.* developed a new traceless modification strategy using thioester-based acyl

transfer reagents, enabling the facile and efficient one-step synthesis of site-specific antibody–drug conjugates (ADCs). The authors designed the sequence of the Fc-binding peptide and optimized the linker. The optimized reagent can directly assemble the toxin precisely onto the K251 position and simultaneously self-release the affinity ligand in one step (Fig. 13F). Using this method, the authors synthesized a series of K251-linked ADCs, which exhibited excellent physicochemical and biological properties, including uniformity, stability, and *in vitro* and *in vivo* antitumour activity. This method enables the traceless, one-step synthesis of site-specific ADCs and provides a general model for the application of ligand-directed protein modification.[78] In the same year, Yuan *et al.* achieved the site-selective acetylation of single lysine residues in human immunoglobulin G. The authors designed and modified the Fc-III peptide identified from the bacteriophage library. Leveraging peptide–antibody affinity, the peptide positions a phenolic ester close to Lys248. Through a proximity-driven group transfer reaction, an acetyl group is transferred to Lys248. Using this method, functional groups such as azide, alkyne, fluorescent molecules, or biotin can also be selectively installed on Lys 248, allowing further derivatization of IgG (Fig. 13G). The authors constructed antibody-conjugated liposomes (immunoliposomes) targeting HER2-positive (HER2+) cancer cells and constructed a bispecific antibody complex (bsAbC) covalently linked to anti-HER2 antibodies and anti-CD3 antibodies.[79]

5 Enzymatic strategies

Enzymes possess an inherent ability to recognize specific sites within peptides and proteins, such as specific amino acid sequences or defined structural motifs. This natural selectivity makes them well suited for achieving site-specific modifications in a predictable and efficient manner. Current site-specific enzymatic modification strategies rely on the recognition of chemical or spatial structures. We divided this section into enzymatic modification strategies based on the recognition of native protein motifs, introduced tags, and certain spatial structures.

5.1 Engineered enzymes for the recognition of native protein motifs

Many natural enzymes achieve site-selective catalysis by recognizing specific peptide motifs within their substrates. Similarly, proteins possess unique amino acid sequences that can serve as intrinsic recognition elements for selective enzymatic modification. For example, *Staphylococcus aureus* sortase A (SaSrtA) recognizes the LPXTG motif and cleaves the peptide bond between threonine and glycine. This reaction generates a thioester-linked intermediate with the enzyme, which is then resolved by nucleophilic attack from an N-terminal glycine-containing acceptor, forming a new amide bond with high site specificity.[80] Thus, some strategies concentrate on engineering enzymes to recognize specific peptide sequences in natural proteins. For example, Whedon *et al.* engineered a sortase variant, cW11 (derived from sortase F40 reported by Schwarzer and colleagues[81]), which efficiently recognizes the APXTG motif, cleaves between threonine and glycine, and generates a threonine-linked product through nucleophilic attack at the threonine residue (Fig. 14A). This tool enables late-stage H3 functionalization with high precision and efficiency.[82] Similarly, based on sortase A, Podracky *et al.* employed a yeast display selection strategy to generate a sortase variant capable of the site-specific modification of Aβ peptides (Fig. 14B). The engineered enzyme selectively recognizes the natural LMVGG motif (residues 34–38 of Aβ) and cleaves between the two glycines, allowing the installation of diverse conjugates without the need for genetically introduced tags.[83]

5.2 Tag-guided enzymatic modification

In parallel with engineering enzymes for the recognition of native sequence motifs, an alternative strategy involves the introduction of short peptide tags that are specifically recognized by modifying enzymes. These tag-guided enzymatic approaches enable site-selective functionalization in a modular and predictable manner, expanding the toolkit for precise protein modification across diverse biological contexts. Compared with engineering enzymes for each individual target, this approach is more broadly applicable because, as long as a small tag can be introduced without disrupting the protein function, the same enzyme can be used to modify many different proteins without the need for further customization. However, the requirement to genetically introduce such tags presents limitations, particularly when working with native proteins or *in vivo* applications where tag insertion may be impractical or interfere with biological processes.

Using this tag-guided strategy, different types of modifications can be added. For example, in a 2020 study, Hofmann *et al.* described the use of the ubiquitin-conjugating enzyme Ubc9 to achieve site-specific lysine acylation on the tag in target proteins (Fig. 14C). Ubc9 selectively recognizes a minimal four-residue tag (IKXE, where K is lysine, X is any amino acid, and E is glutamic acid) and acylates the lysine within that tag, thereby directing the modification to a defined, tag-encoded site. This tag-guided strategy enables efficient and predictable lysine acylation under mild aqueous conditions and is compatible with a broad range of acyl donors, including those bearing biotin or biotin conjugated with PEG spacers.[84]

In earlier work, Ploegh, Durek and colleagues developed a C247A mutant of OaAEP1 ([C247A]OaAEP1). In this system, the NGL recognition motif is artificially introduced at the C-terminus of the protein. The enzyme cleaves the peptide bond between the asparagine and glycine residues and forms a thioester acyl-enzyme intermediate at its catalytic cysteine. This intermediate can subsequently be resolved by nucleophiles such as peptide N-termini or other primary amines, thereby producing different C-terminal ligation products (Fig. 14D).[85,86] In 2024, the authors subsequently expanded the application of OaAEP1 to peptide cyclization, employing it to catalyse head-to-tail and side chain-to-C-terminal cyclization (Fig. 14E). By extending the recognition motif with a His residue, the authors

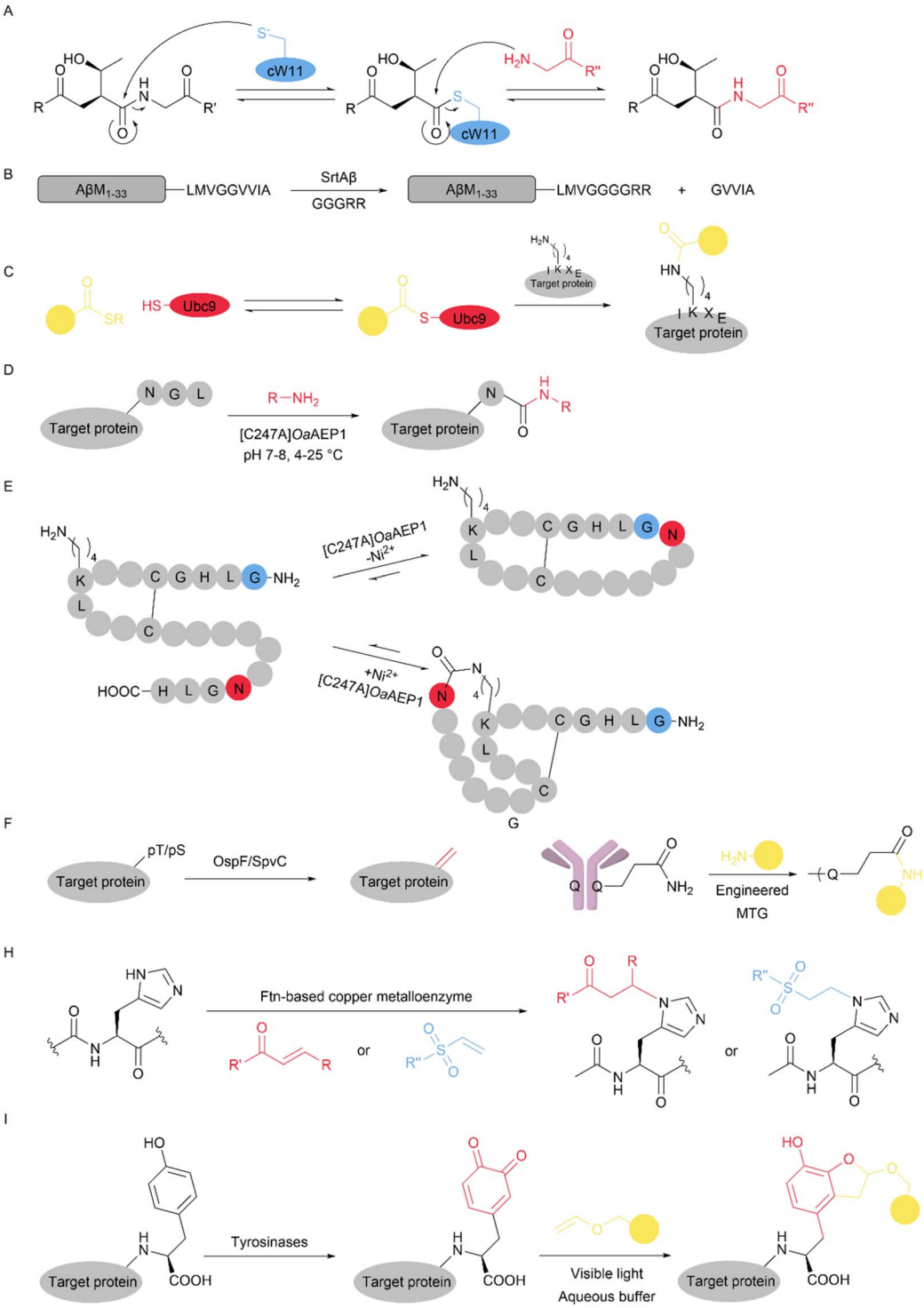

Fig. 14 Representative enzymatic strategies for site-specific protein modification. (A) Transpeptidation mechanism of cW11 sortase. (B) Transpeptidation reaction of Aβ catalysed by SrtAβ. (C) Strategy for isopeptide labelling using Ubc9 and peptide thioesters. (D) [C247A]OaAEP1-catalysed aminolysis. (E) Ni^{2+}-mediated control of the cyclization reaction catalysed by [C247A]OaAEP1. (F) Use of OspF and SpvC for the efficient enzymatic incorporation of Dha or Dhb. (G) Native antibodies were site-specifically conjugated using engineered MTG. (H) Site-specific histidine aza-Michael addition in proteins enabled by a ferritin-based metalloenzyme. (I) Tyr-specific protein modification *via* tyrosinase oxidation, *o*-quinone and visible light-induced photoaddition in aqueous solutions (pH 6.5 phosphate buffer).

achieved the modulation of cyclization. For instance, the peptide shown can be head-to-tail cyclized in the absence of Ni^{2+}. When Ni^{2+} is added, the N-terminal Gly-Leu-His sequence is quenched, and C-terminus-to-side chain cyclization occurs instead.[87] In addition, Veer *et al.* further expanded the substrate scope to include secondary amines such as proline and

hydroxyproline, enabling the formation of stable tertiary amide bonds and facilitating applications such as dual labelling.[88]

Dha is widely used for protein modification. The bis-alkylation-elimination strategy is one of the most powerful approaches for converting Cys to Dha in peptides or proteins.[89,90] However, this method may not differentiate different Cys residues. In 2022, Yang *et al.* reported an enzymatic approach to site-selectively introduce Dha/dehydrobutyrine (Dhb) into peptides and proteins using the bacterial phosphothreonine lyases OspF and SpvC (Fig. 14F). Using self-assembled monolayers for a matrix-assisted laser desorption/ionization mass spectrometry (SAMDI-MS)-based high-throughput screening platform, the authors identified optimized recognition motifs [F/Y/W]-pS or [F/Y/W]-pT that enable the conversion of single phosphorylated serine or threonine residues into Dha or Dhb with high efficiency. Using this method, the authors demonstrated the utility of this system in one-pot protein labelling and in modifying surface-displayed peptides on live *E. coli*, highlighting its potential for bioorthogonal functionalization under mild conditions.[91]

5.3 Structure-guided enzymatic protein modification

Another class of protein modification approaches achieves site selectivity by constructing local reaction environments that favour covalent attachment at a specific residue. Unlike sequence-guided enzymatic modification, which relies on the recognition of a defined peptide motif, structure-guided approaches instead exploit three-dimensional proximity and local surface features. Specifically, these methods typically involve engineering a catalytic pocket or reactive centre that confines reactivity to residues positioned within its spatial reach. Selectivity emerges from geometric constraints, such as coordination geometry, residue orientation, and steric accessibility. Such structure-guided strategies provide a powerful and orthogonal route to precision bioconjugation, particularly for residues traditionally considered difficult to modify selectively. For example, a microbial transglutaminase (MTG) was rationally engineered to selectively modify a native glutamine residue, Gln295, located in the hinge region of human IgG1 antibodies (Fig. 14G). The resulting MTG variant enables site-specific bioconjugation even on fully glycosylated antibodies, catalysing amide bond formation between the Gln295 side chain and amine-containing payloads, highlighting the utility of enzyme engineering in tag-free protein functionalization.[92] Although the authors did not explicitly delineate the molecular basis of this selectivity, they suggested that prior reports showed that efficient Q295 conjugation typically required Fc deglycosylation, which indicates that selectivity may be governed by conformational/structural accessibility rather than recognition of a certain sequence.

In 2024, Tsou *et al.* developed a ferritin (Ftn)-based artificial metalloenzyme platform capable of catalysing histidine-selective aza-Michael additions (Fig. 14H). By introducing a Cu(II)-binding noncanonical amino acid (such as 4-boronophenylalanine (BtA)) and fusing a substrate recognition peptide (αCT), the authors recruited insulin and stabilized a binding conformation that positioned His5 near the catalytic centre. This conformational restriction enables single-site histidine modification, even in the presence of more reactive residues, highlighting a general strategy for sequence-independent yet site-selective protein editing.[93]

Tyrosinases (EC 1.14.18.1) is another enzyme that catalyse the oxidation of tyrosine side chains to *o*-quinones. Certain tyrosine residues in peptides and proteins can also be oxidized by tyrosinases, enabling subsequent modification.[94] Using this enzyme, Chen *et al.* developed a tyrosinase-mediated strategy to achieve the site-selective modification of antibodies (Fig. 14I). By leveraging the structural accessibility of specific tyrosine residues (Fc-Y296), the authors selectively oxidized exposed Tyr sites on heavy chains, generating reactive *o*-quinones that could be further modified. This approach enables precise bioconjugation without disrupting antibody function, highlighting the potential of site-selective antibody modification strategies to enhance targeted immunotherapy for cancer.[95]

6 Conclusions

Site-specific modification is pivotal for constructing peptides and proteins with precisely defined structures and functionalities. Homogeneously modified products are indispensable for probing structure–function relationships. This review provides a comprehensive overview of recent advances in the site-specific modification of peptides and proteins, detailing both the design principles that underpin these transformations and their innovative subsequent applications.

Drawing on the intrinsic differences among protein sites, researchers have engineered site-selective reactions. By appending specifically modifiable tags or exploiting targeting moieties, they achieve precise positional control. Enzymatic strategies further extend this toolkit, offering exceptional site selectivity and broad application potential. In conclusion, site-specific modification offers innovative avenues for advancing traditional bioconjugation and bioorthogonal chemistry. Nevertheless, the field is still confronted with challenges. Some methods suffer from low conversion yields. Many efficient strategies additionally rely on the installation of exogenous tags or recognition motifs, restricting their use with native proteins in complex environments. Moreover, most current modifications are irreversible. The development of dynamically controllable labelling strategies remains a key direction for future research.

Moving forwards, the development of flexible yet precise modification strategies is crucial. Emerging computational tools, particularly machine learning algorithms trained on protein structure and reactivity databases, are increasingly enabling the prediction of subtle differences in the intrinsic properties of amino acids, potentially allowing tag-free discrimination among the same amino acid type in native proteins. Expanding the reaction toolkit through previously underexplored, orthogonal chemistries, organometallic catalysis, and photochemical and electrochemical transformations will broaden the scope of protein modifications. Notably, the rational design of novel electrophiles with tuned reactivity

profiles promises to unlock the site-selective modification of traditionally challenging, low-abundance residues such as histidine, aspartic acid, and tryptophan. Directed evolution and AI-assisted enzyme design will further allow the reprogramming of natural substrate-recognition motifs, delivering artificial enzymes with superior catalytic profiles. Finally, continued progress in supramolecular and stimulus-responsive chemistry should yield reversible, on-demand protein modifications that confer spatiotemporal control over protein function in real time. As ongoing research tackles these challenges, the prospects for the ongoing evolution and broader application of site-specific modifications in chemical biology and related fields are promising.

Author contributions

Xin-Rong Duan, Meng-Qian Zhang and Yan-Mei Li organize the overall narrative of the manuscript. Xin-Rong Duan, Geng-Hui Feng and Yan-Mei Li co-wrote the manuscript.

Conflicts of interest

There are no conflicts to declare.

Data availability

No primary research results, software or code have been included and no new data were generated or analysed as part of this review.

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