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Recent advances in chemical protein synthesis: method developments and biological applications

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The central dogma of modern biology underscores the pivotal roles proteins play in diverse biological processes, the study of which necessitates advanced methods to produce proteins with precision and versatility. Chemical protein synthesis, a powerful approach utilizing chemical reactions for the *de novo* construction of structurally accurate proteins, has emerged as a transformative tool for studying proteins and generating protein derivatives/mimics inaccessible by natural biological machinery, including post-translationally modified proteins, proteins comprised of unnatural amino acids, as well as mirror-image proteins. This review summarizes recent strides in synthetic method developments for chemical protein synthesis, including innovative techniques in solid-phase peptide synthesis, the challenges presented by difficult sequences in either synthesis or folding and the exploration of novel ligation reactions using both chemical and enzymatic methods. Furthermore, the review also delves into newly developed protocols for site-selective protein modifications and the generation of stapled or macrocyclized peptides/mini-proteins, highlighting the power of chemical methods to make structurally diverse proteins. Recent applications of synthetic proteins in investigating post-translational modifications (phosphorylation, lipidation, glycosylation, ubiquitination, *etc.*),

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mirror-image biological processes and drug development are further discussed. Together, these topics provide a comprehensive overview of the current landscape of chemical protein synthesis.

chemical protein synthesis, solid-phase peptide synthesis, ligation reactions, post-translational modifications, mirror-image proteins, peptide drugs

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CONTENTS

1 Introduction

Proteins are essentially large-sized organic molecules that connect chemistry to life. They are intensively studied to understand how life works at the molecular level and are also increasingly being developed as diagnostics and therapeutics. In addition, proteins are receiving growing attention in the development of advanced materials. While advancements in biological techniques and technologies have enabled researchers to better study and understand the functions and structures of proteins, the complexity and diversity of the proteome require more powerful methods to produce proteins with increasingly structural precision and versatility. One such powerful approach is chemical protein synthesis [1–6], wherein synthetic chemistry is used to construct structurally defined proteins in a *de novo* fashion, and which has emerged as a transformative tool to study proteins, particularly those bearing posttranslational modifications (PTMs) [7–12]. As a complementary strategy to protein biosynthesis, chemical protein synthesis can lead to proteins of any chirality with exquisite control over sequence, structure, and functionality and enables the generation of proteins that would otherwise be difficult or impossible to obtain [13–17]. Beyond interrogating the structure-function relationships of naturally occurring proteins, chemical protein synthesis also enables the production of proteins incorporating non-canonical amino acids and

unnatural modifications and even mirror-image proteins entirely made of D-amino acids, opening doors to applications in fields from pharmaceuticals and materials to synthetic biology [18–27].

New chemistry (*i.e.*, new chemical reactions and new synthetic strategies) is the fundamental driver of chemical protein synthesis and vital for the development of the field. Compared with the pioneering synthetic studies on relatively small proteins such as insulin [28–30], ground-breaking new chemistry, such as solid-phase peptide synthesis (SPPS) [31], has revolutionized chemical protein synthesis, allowing many important and increasingly sophisticated proteins to be produced. However, there is still a strong need for innovative methods of chemical protein synthesis, especially new chemical reactions, and synthetic strategies, to obtain proteins with more complex structures and to continuously improve the efficiency of synthesis. This review describes recent advancements in chemical protein synthesis, including novel chemical and enzymatic ligation reactions and strategies, site-selective protein modification methods, and the application of chemically synthesized proteins in different areas of biomedical studies. These new advancements have greatly reshaped the field and supported the development of modern biomedicine, demonstrating the importance, power, and value of synthetic chemistry. By surveying these advancements, we will provide an overview of the current state of the field and illuminate potential avenues for future exploration.

2 Recent method developments for chemical protein synthesis

2.1 Peptide synthesis

SPPS technology [31,32] greatly simplifies the preparation of peptides by enabling the iterative coupling of protected amino acids onto insoluble polymer support to give peptide chains without the need for complex purification procedures at the end of every step (Figure 1). The initial Boc (*t*-butyloxycarbonyl) based protocol has now been largely supplanted by the Fmoc (9-fluorenylmethyloxycarbonyl) based method [33], which is preferred due to its milder reaction conditions, simpler reaction operations and greater amenability to automation. More recently, new Fmoc-based SPPS methods [34–36] have been developed, including the 4,6-dithiocyano-5-nitropyrimidine (NDTP, Figure 1b) coupling reagent [37] and the 2,4-dinitro-6-phenyl-benzene sulfenyl (DNPBS, Figure 1c) α-amino protecting group to suppress α-carbon racemization in peptide synthesis [38].

Other recent SPPS innovations include improvements in coupling reagents, such as the ynamide [39] and allenone [40] reagents developed by Zhao *et al.* (Figure 1c), which accomplish highly efficient amino acid coupling under mild conditions. These coupling reagents, as well as their derived

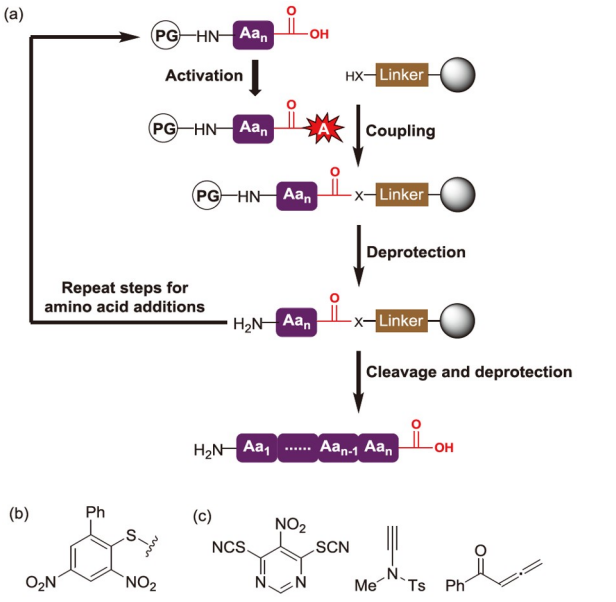

Figure 1 (a) Solid-phase peptide synthesis. (b) DNPBS, a recent α-amino protecting group. (c) Recently developed coupling reagents (color online).

α-carbonyl vinyl esters of amino acids, can be easily prepared and stored for months. The synthetic application of the ynamide- and allenone-mediated peptide bond formation has been showcased in the syntheses of linear peptides [41], cyclic peptides [42], cyclodepsipeptides [43], peptide thioesters [44], and thioamide-containing peptides [45,46], and peptide segment condensation [21–23].

The operational simplicity of Fmoc SPPS has led to the development of automatic peptide synthesizers. Early models required 1–2 h per coupling and deprotection cycle [47]; more recent developments, including the use of microwaves, have shortened cycle times to as short as four minutes [48]. Recently, Pentelute *et al.* [49] invented the automated fast-flow peptide synthesizer for the rapid synthesis of peptides with real-time quantitative monitoring, allowing for the stepwise total chemical synthesis of peptides up to 164 amino acids in length with a cycle time of ~2.5 min per amino acid. This flow-based SPPS technique enabled the synthesis of several mirror-image targets used for mirror-image screening to discover D-peptide drugs [50].

One shortcoming of Fmoc SPPS is its inherent unsuitability to synthesize peptides incorporating hydrophobic sequences or those prone to aggregation on the resin [51]; attempts to access such "difficult peptides" using SPPS often result in truncated products and/or those incorporating deletions. To obviate this problem, various innovative backbone modification strategies to inhibit hydrogen-bonded interchain association and disrupting peptide conformation have been developed, such as those based on the introduction of a pseudoproline dipeptide [52], 2-hydroxy-4-methoxybenzyl [53] or *O*-acyl isopeptide [54] group (Figure 2) [55].

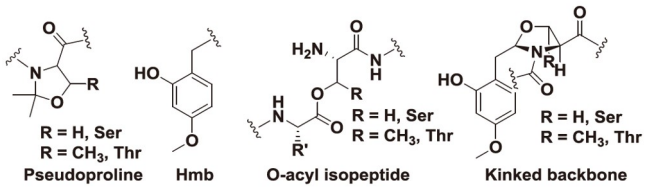

Figure 2 Structures of several representative backbone modifications.

Zheng *et al*. [56] developed an improved method of installing a 2-hydroxy-4-methoxybenzyl group at any primary amino acid, including highly sterically hindered ones (*e.g*., Val, Ile), and Li *et al*. [57] reported a kinked backbone modification strategy using *N*,*O*-benzylidene acetal dipeptides as building blocks.

2.2 Chemical ligation methods

2.2.1 Native chemical ligation

The convergency of peptide synthesis can be improved by native chemical ligation (NCL) [58], wherein a C-terminal peptide thioester reacts with an N-terminal cysteinyl peptide to give a larger peptide incorporating a native amide bond at the ligation site. The initial capture step in NCL is a thiol-thioester exchange reaction between the thioester of one peptide and the cysteine thiol functionality of a second to give a thioester-linked intermediate which undergoes a spontaneous intramolecular *S*,*N*-acyl transfer to give a larger peptide with a native peptide bond to cysteine (Figure 3a) [59]. NCL was invented by Kent *et al*. [58] in 1994 and is a ground-breaking method for chemical protein synthesis that has been widely used [3,8,60–63].

Although the low abundance of Cys residues in natural proteins has limited the application of NCL, the method has been extended using a thiolated amino acid-mediated ligation followed by desulfurization [64]. Several thiol-containing amino acids have been used in this ligation-desulfurization sequence, including the recently developed β-thiolysine derivatives (Figure 3b) [65–67]. Alternatives to the original radical-based desulfurization (tris(2-carboxyethyl)phosphine (TCEP)/VA-044/tBuSH) protocol have been developed as well. For example, Li *et al*. reported a P-B peptide desulfurization using TCEP/$NaBH_4$ [68], finding that tetraethylborate ($NaBEt_4$) can mediate superfast radical-based desulfurization within 15–30 s without the need for heating, irradiation, an inert atmosphere, mercaptan additives, or complicated equipment [69]. In addition, Stockdill *et al*. [70] reported a phosphine-only photodesulfurization using phosphine and near-ultraviolet light.

Removable thiol-based auxiliaries that mimic a Cys peptide in NCL, such as the acid-sensitive 1-aryl-2-mercaptoethyl auxiliary, have also been developed (Figure 3c), though they are often limited to ligations at glycine [71,72].

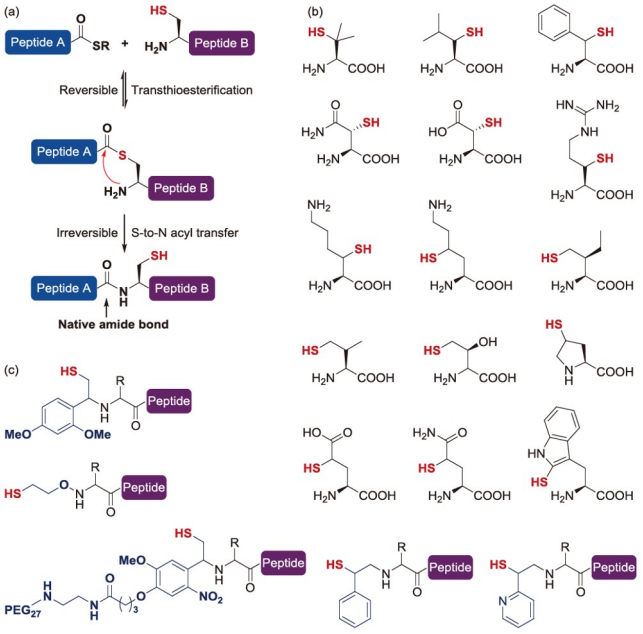

Figure 3 (a) Native chemical ligation. (b) Non-native thiolated amino acids. (c) Auxiliaries (color online).

Becker *et al*. [73,74] reported a PEGylated photocleavable auxiliary mediated ligation at glycine for the synthesis of complex glycosylated peptides. Auxiliaries capable of ligating at residues other than glycine include the new auxiliaries developed by Seitz *et al*. [75,76], which can even be used at junctions containing proline or valine.

2.2.2 Peptide hydrazide ligation

A long-standing problem of native chemical ligation is that peptide thioesters are challenging to make due to their lability in the Fmoc-based solid-phase peptide synthesis. To circumvent this problem, a plethora of methods have been explored to prepare peptide thioester equivalents or surrogates compatible with the reaction conditions of Fmoc-SPPS [38,77–81]. In this context, peptide hydrazide ligation, which was originally developed by Liu *et al*. [82–87] becomes a transformative method for chemical protein synthesis. Peptide hydrazide ligation relies on two counterparts that are both nucleophilic in nature and chemically inert to each other, namely, a C-terminal peptide hydrazide and an *N*-Cys peptide. To enable the ligation between these two nucleophiles, the hydrazide is first activated through a highly chemoselective oxidative reaction using sodium nitrite to give a high-energy intermediate of acyl azide, which is immediately subjected to thiolysis *in situ* to give the corresponding thioester that can ligate with the N-terminal Cys peptide (Figure 4) [54].

An important feature of the peptide hydrazide ligation is that peptide hydrazides can be very easily prepared by Fmoc SPPS, ideally starting from freshly-prepared (2-Cl)-Trt-$NHNH_2$ resin, which can be obtained from commercially

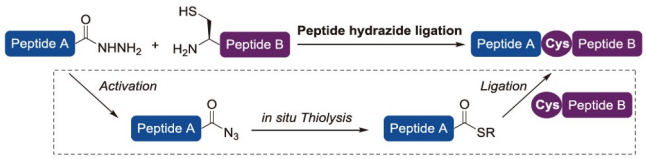

Figure 4 Peptide hydrazide ligation (color online).

available (2-Cl)-Trt-Cl resin [88,89]. Alternatively, the hydrazine-modified resin can be prepared as a Fmoc-protected form for long-term storage and better loading quantification [90]. Peptide hydrazides can also be prepared from recombinantly expressed proteins, such as those obtained using intein fusion, allowing for convenient chemical synthesis of large-sized proteins. Liu *et al*. [91] reported the generation of protein α-hydrazides through hydrazinolysis of a protein bearing a genetically incorporated α-oxoester in its backbone. A small molecule reagent to chemically activate a cysteine in a recombinant protein has also been developed; this activated cysteine is then converted into the corresponding peptide hydrazide by hydrolysis [92,93]. Other enzymatic methods to obtain peptide hydrazides include the sortase and peptidyl-α-hydroxyglycine amidating lyase/peptide amidase approaches [94–96].

A modified $NaNO_2$ activation protocol compatible with an N-terminal thiazolidine group has also been developed to convert the corresponding peptide hydrazide into the thioester in trifluoroacetic acid (TFA) [97]. In this method, acetylacetone was used to convert the peptide hydrazide into the corresponding acyl pyrazole. Then, thiolysis was carried out to give a peptide thioester for ligation. Acyl pyrazoles are mild acylating agents and are compatible with the N-terminal Thz moiety, enabling multiple sequential ligations without intermediate purification steps [98].

Peptide hydrazide ligation has become the most popular ligation method in modern chemical protein synthesis due to its flexibility and the relative ease with which the ligation partners can be prepared and activated. It has been used to prepare numerous small to large proteins, including those bearing post-translational modifications, mirror-image proteins, protein probes, and isotope-labeled proteins for structural and functional proteomics studies. Recent applications of peptide hydrazide ligation methods include the total chemical synthesis of targets comprised of ca. 500 amino acids [99–104] (*e.g.*, 441-residue Tau, 456-residue hexa-Ub, 472-residue tetra-Ub-α-globin, and 467-residue N-terminal Pfu).

2.2.3 Seleno-mediated chemical ligation

NCL can be accelerated using peptide selenoesters in place of peptide thioesters due to the enhanced reactivity of selenoesters compared with thioesters—for example, a C-terminal peptide prolyl selenoester ligated nearly 350 times faster than its corresponding thioester [105]. The N-terminal Cys in NCL can also be changed to the N-terminal selenocysteine (Sec) amino acid for ligation (Figure 5). In Sec-based ligation, deselenization of a Sec residue can be performed by reduction with TCEP and a hydrogen donor, such as dithiothreitol (DTT). Deselenization was compatible with unprotected Cys residues [106].

The diselenide selenoester ligation (DSL) invented by Payne *et al*. [107] involves the chemoselective ligation of an unprotected peptide selenoester and an unprotected diselenide peptide. The high reactivity of the C-terminal selenoester and N-terminal Sec enables ligation to proceed rapidly, even at low concentrations (*e.g.*, μM and even nM) and between sterically hindered junctions [108]. DSL is also useful for ligating poorly soluble peptides to afford hydrophobic proteins, such as lapidated proteins, without the need for any solubility tags or hybridizing templates [109]. Ligation at Sec has also been accomplished using an N-terminal Sec-containing segment expressed in *E. coli* [110].

2.2.4 Chemical ligation based on peptide salicylaldehyde esters

The Ser/Thr ligation (STL), developed by Li *et al*. [111–114], relies on a reaction between a C-terminus salicylaldehyde ester and a peptide bearing an N-terminal Ser or Thr residue (Figure 6). The ligation generates an *N*,*O*-benzylidene-acetal-linked intermediate, which transforms into the desired ligated peptide under acidic conditions. The β-hydroxyl amine unit of the N-terminal Ser/Thr can chemoselectively form an oxazolidine with the aldehyde to generate the *N*,*O*-benzylidene acetal after irreversible 1,5 O-to-N acyl transfer.

Furthermore, Li *et al*. [115] reported the cysteine/penicillamine ligation (CPL) using the reaction between a peptide salicylaldehyde ester and either cysteine or penicillamine (Figure 7). The reaction generates the *N*,*S*-benzylidene acetal intermediate after irreversible 1,5 O-to-N acyl transfer. Subsequent acidolysis released the ligated peptide, which can be converted into the peptide with Xaa-Ala/Val ligation sites by desulfurization. One unique feature of CPL is that the ligation can be performed at highly hindered ligation sites, including "Pro-Val".

The requisite peptide salicylaldehyde esters for STL and CPL can be chemically synthesized *via* both Fmoc-SPPS and Boc-SPPS [97–100]. Recently, He *et al*. [116] reported a facile and general approach to prepare peptide salicylaldehyde esters from peptide hydrazides *via* a sequential nitrite oxidation and phenolysis sequence using 3-(1,3-dithian-2-yl)-4-hydroxy-benzoic acid. Ubiquitinated Gadd45a, ubiquitin-like protein 5 (UBL-5) and a myoglobin variant were successfully synthesized using this new strategy—the first synthesis of expressed protein salicylaldehyde esters that significantly expands the synthetic toolbox of STL/CPL for

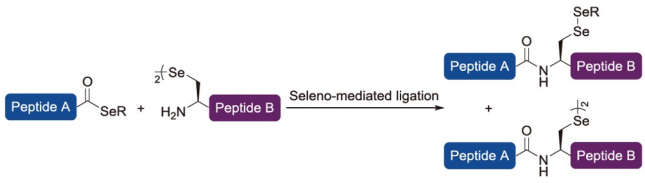

Figure 5 Seleno-mediated ligation (color online).

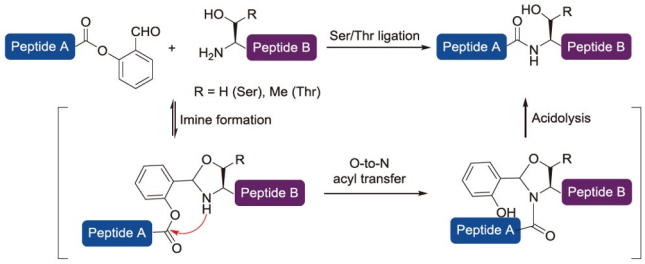

Figure 6 Serine/threonine ligation (STL) (color online).

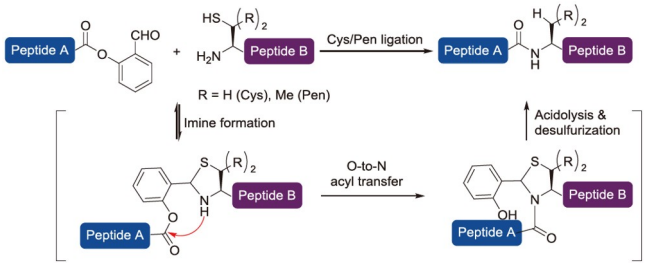

Figure 7 Cysteine/penicillamine ligation (color online).

chemical protein synthesis.

2.2.5 KAHA ligation and others

Another ligation useful for chemical protein synthesis is the KAHA ligation technique developed by Bode's group [117,118], which involves the coupling of peptide α-keto acids and hydroxylamine peptides, generating an amide bond under aqueous acidic conditions without the need for a protecting group or coupling agent [119]. Furthermore, the Staudinger ligation has been demonstrated as another effective strategy for constructing native amide bonds [120,121]. This chemoselective reaction involves the interaction between the phosphine component and the azide moiety, resulting in the formation of an iminophosphane intermediate. This intermediate spontaneously undergoes rearrangement, leading to the generation of the native peptide through acyl migration and hydrolysis. However, the Staudinger ligation has not been widely used for chemical protein synthesis due to its intrinsic limitation. The historical development and contemporary applications of the Staudinger ligation have been comprehensively reviewed recently [122].

2.3 Enzymatic peptide ligation

Peptide ligation can also be accomplished using enzymes [123], such as sortase A [124], butelase-1 and OaAEP1 [125]. These enzymes typically cleave a recognition sequence and form a peptide acyl-enzyme intermediate [126], which undergoes nucleophilic attack by the N-terminal amine of the second substrate to create a peptide bond (Figure 8) [127]. However, far fewer natural peptide ligases than proteases are known, and proteases often favor hydrolysis over aminolysis, making them less efficient for ligations [128]. Efforts have been made to engineer proteases into ligases with better aminolysis-to-hydrolysis ratios [129]. These engineered ligases have been widely used in protein synthesis, cyclization, N-terminal modification, and C-terminal modification [130,131].

2.3.1 Peptidyl asparaginyl ligases

Peptide asparaginyl ligases (PALs) can catalyze transpeptidation reactions involving a consensus Asx-Xaa1-Xaa2 tripeptide motif [132], but these reactions are reversible [133]. Strategies to address this reversibility include a cascade enzymatic reaction by Xia *et al.* [134], wherein the PAL is combined with glutaminyl cyclase to give a leaving group incompatible with PAL-mediated transpeptidation (Figure 9a). An alternative quenching approach was proposed by Rehm *et al.* [135], who disabled the reversibility of the ligation reaction by complexing the cleaved tripeptide with a metal (Figure 9b).

PALs have also been used to introduce the C-terminal Leu-ethylenediamine into synthetic peptides and recombinant proteins, thereby enabling the sequential C-to-C ligation of up to three proteins (Figure 10a) [136], and accomplishing the site-specific modification of target proteins at internal locations incorporating an isopeptide-linked glycylglycine moiety (Figure 10b) [137], or incorporate various small nucleophiles such as cysteamine at C-termini (Figure 10c) [138]. PALs can also be used to cyclize bioactive peptides, especially disulfide-rich peptides, with minimal ligation scars and simplified formation of precise disulfide bonds [139]. Noteworthy targets for cyclization include antimicrobial peptides and angiotensin I-converting enzyme inhibitory peptides [140]. Other research in the field of PALs has focused on discovering novel PAL variants [141].

2.3.2 Sortase A

Sortase A (SrtA)-mediated ligation is widely used for selective ligation in complex cellular environments [84], though limited by the requisite C-terminal LPXTG motif and incomplete substrate conversion due to its reversibility. To address these issues, Zuo *et al.* [142] demonstrated an efficient and irreversible ligation method using SrtA, involving a protein with C-terminal thioester bonds and a protein bearing

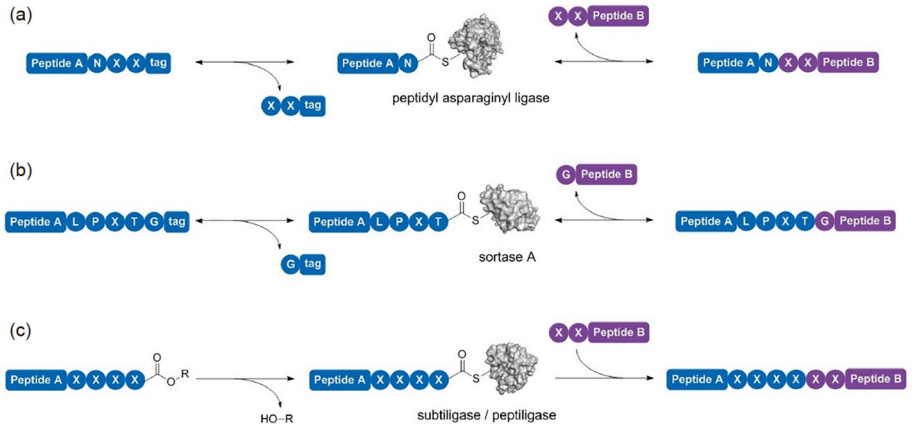

Figure 8 Peptide ligation catalysed by (a) peptide asparaginyl ligases, (b) sortase A, and (c) subtiligase or peptiligase (color online).

an N-terminal Gly (Figure 11a). This approach exhibited broad sequence compatibility, even with unconventional ligation sites. In addition, Podracky *et al*. [143] evolved a sortase variant with a preference for LMVGG substrates

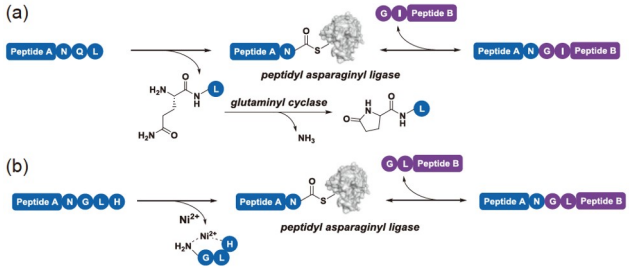

Figure 9 Favouring PAL-catalysed peptide conjugation by introducing (a) glutaminyl cyclase and (b) nickel ions (color online).

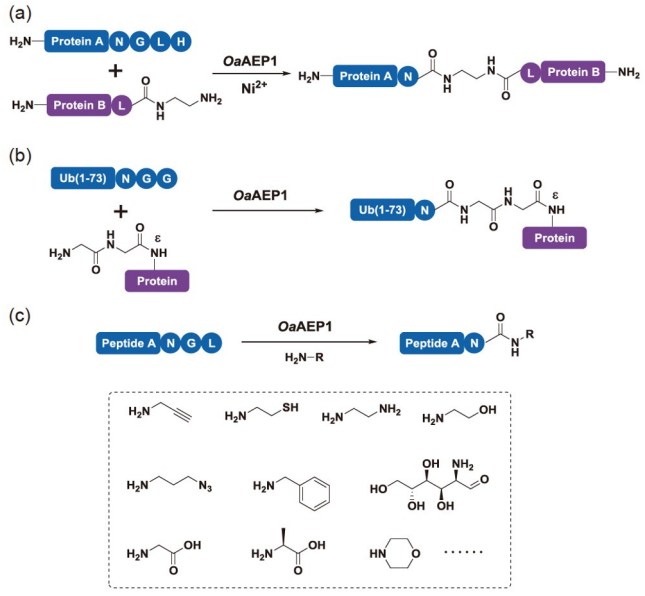

Figure 10 PAL examples. (a) C-to-C protein ligation. (b) Site-specific ubiquitylation using genetic-code expansion and *Oa*AEP1. (c) Protein C-terminal modification with diverse amines (color online).

(Figure 11b). However, the wild-type SrtA exhibits reactivity solely towards the N-terminal oligoglycine moiety, typically composed of tri-/penta-glycines. To expand the substrates scope of SrtA, Chen *et al*. [144] evolved a "promiscuous" SrtA variant (mgSrtA) capable of labeling the exposed N-terminal monoglycine residue, which is much more abundant in nature.

More recently, Thompson *et al*. [145] introduced trans-peptidase-assisted intein ligation (TAIL), using SrtA-mediated ligation to construct an active split-intein fusion for protein modifications in the chromatin environment (Figure 11c). Hofmann *et al*. [146] explored lysine acylation using conjugating enzymes (LACE) with SrtA, yielding dual-modified Fab fragments in near-quantitative yield (Figure 11d). Fottner *et al*. [147] developed a chemoenzymatic strategy for ubiquitylating and SUMOylating proteins (Figure 11e).

2.3.3 *Subtiligase and peptiligase*

Subtiligase was introduced by Wells *et al*. [129a] and is a versatile tool for protein synthesis. Peptiligase, a Ca^{2+}-independent subtilisin variant, was subsequently developed by Janssen *et al*. [129b]. This breakthrough marked a significant stride towards the industrial production of pharmaceutical peptides [148]. To enhance substrate versatility, Janssen *et al*. [149] designed omniligase-1 through rational design and mutagenesis. Concurrently, Weeks and Wells [150] proposed an alternative strategy in which peptide ligase specificity for *N*-termini is accomplished through proteome-derived peptide libraries. These broad-spectrum ligases have expanded the range of possible enzymatic ligation sites and enabled the large-scale production of important biomacromolecules [151].

Despite these advancements, the preparation of protein ester substrates suitable for peptiligase-mediated ligation remains challenging [60]. Wu *et al*. [152] engineered a peptide amidase to functionalize C-terminal amide groups

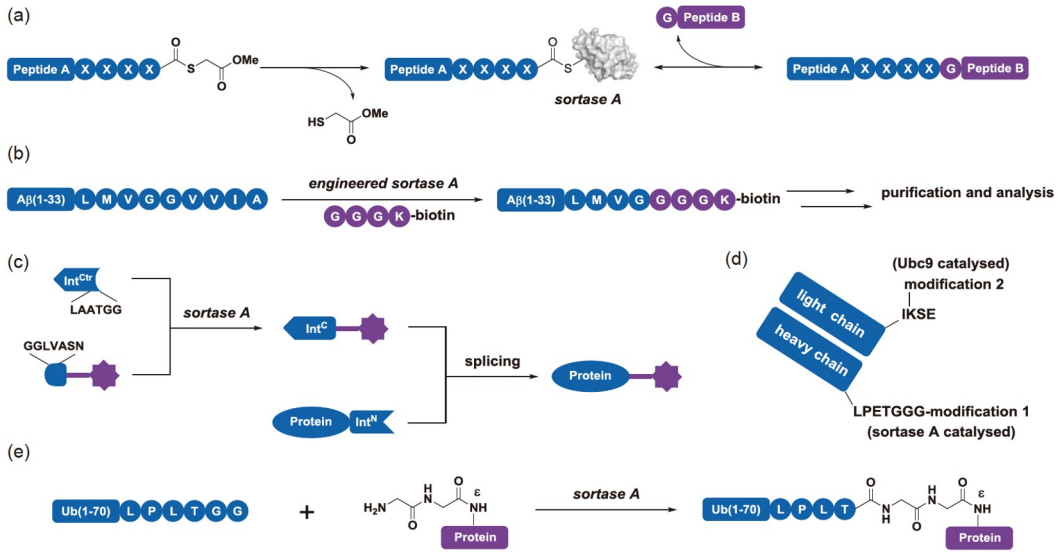

Figure 11 Sortase A examples. (a) Thioester-assisted sortase-A-mediated ligation. (b) Modification of amyloid-β (Aβ) protein by an engineered sortase variant. (c) Combination of intein-mediated protein splicing and sortase-mediated peptide ligation. (d) Dual modification of trastuzumab antigen-binding fragment by using sortase A and E2 small ubiquitin-like modifier conjugating enzyme Ubc9. (e) Site-specific ubiquitylation using genetic-code expansion and sortase (color online).

selectively. Subsequently, peptidyl-glycine hydroxylating monooxygenase and peptidyl-α-hydroxyglycine amidating lyase were introduced to process recombinant proteins bearing C-terminal glycine residues. Combined with omniligase-1, these enzymatic components gave rise to PALME (Figure 12), a platform that enables enzymatic protein synthesis without ligation site restrictions [153].

2.4 Templated ligation strategies

Templated ligation strategies can enable ligation at low concentrations by increasing the effective concentration of the reactive partners. The first templated native chemical ligation used secondary structure interactions between peptides to bring them into proximity [154,155]. Base-pairing

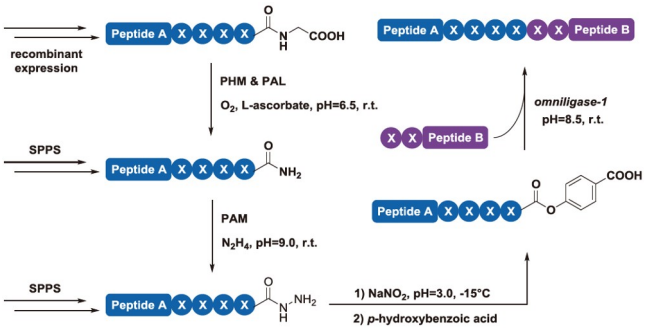

Figure 12 The PALME platform for traceless protein synthesis. Peptidyl-glycine hydroxylating monooxygenase (PHM), peptidyl-α-hydroxyglycine amidating lyase (PAL), peptide amidase (PAM) and omniligase-1 are used sequentially for the C-terminal activation and ligation of both proteins (prepared by recombinant expression) and peptides (prepared by SPPS) (color online).

noncovalent interactions between nucleic acids and peptide nucleic acids can also bring the reactive partners into proximity and mediate ligation at nM concentrations [156–158]. Other strategies include those of Melnyk *et al*. [159], which relies on phosphate-guanidinium electrostatic interactions, and Bode's group [160], who reported a templated amide-forming ligation between acylboronate and hydroxylamine groups using streptavidin-desthiobiotin as the template.

Although ligation templated by noncovalent interactions is effective in many contexts, it cannot be used with the strongly chaotropic solvents typically required for peptide solubilization, and such methods may leave their interaction pairs as a "ligation scar" in the product. To address these issues, covalent bond-mediated ligation strategies such as traceless click-assisted NCL [161] and backbone-installed split intein-assisted NCL [162] have been developed. These methods can be performed smoothly at micromolar concentrations in strongly chaotropic conditions.

2.5 Solubilizing-tag strategies

An ongoing challenge in the chemical synthesis of proteins is their hydrophobicity, which often leads to very low ligation yields due to the poor solubility of the proteins and their tendency to aggregate [163–165]. Various strategies to address this problem have been reported, which can be divided into two main categories: (1) adding organic solvents, chaotropic agents, or surfactants to the reaction mixture [166]; and (2) modifying the peptide backbone or side chain [167].

Temporary solubilizing modifications such as hydrophilic tags have revolutionized the use of chemical ligation on "difficult sequences" by improving the handling and purification of poorly-soluble peptides during ligation (Figure 13). Tags can be installed onto the *N*-terminus, C-terminus, amino acid side chains, or backbone amide. Disotuar *et al*. [168] used an N-terminal solubilizing tag for the insulin A chain, and several examples of C-terminal tags have been reported [169–171]. More recently, side-chain or backbone solubilizing tags have attracted attention. Kay *et al*. [172] reported the use of polylysine tags connected to a Ddae-linker to synthesize the 97-residue co-chaperonin GroES. Side chain thiol groups can also bear tags, such as the polyarginine tag used for the chemical synthesis of SUMO-2-Lys63-linked diubiquitin hybrid chains and HMGB1 protein [173,174]. Tsuda *et al*. [175,176] described a method using trityl-Lys (Trt-K) solubilizing tags to assist NCL. The enzyme or photo-removable solubilizing tag at Gln/Asn was developed for the synthesis of hydrophobic proteins [65,66,177]. Removable backbone modification strategies have been developed and used to prepare a series of hydrophobic membrane proteins not readily accessible by recombinant protein expression, such as the core transmembrane domain Kir5.1 (64−179), the influenza A virus M2 with phosphorylation or S-palmitoylation, the cation-specific ion channel p7 with site-specific NMR isotope labels, S-palmitoylated interferon-induced transmembrane protein 3 and even mirror-image proteins for phage display (Figure 14) [146,178–186]. Additionally, Li *et al*. [187–189] reported the ligation embedding aggregation disruptor to prepare interleukin-2, PD-1 immunoglobulin and extracellular domains.

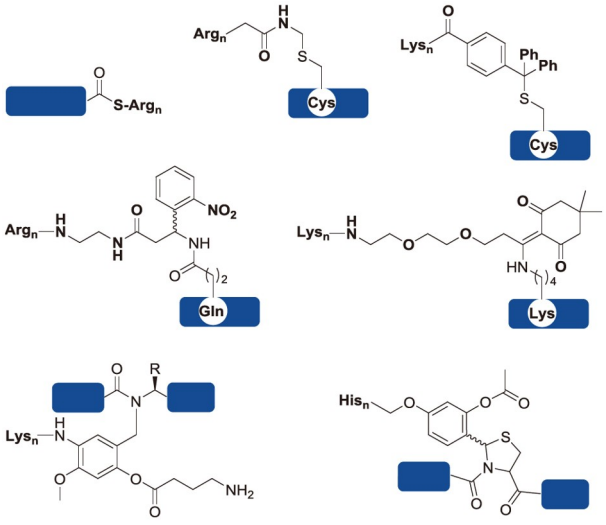

Figure 13 Examples of removable solubilizing tags (color online).

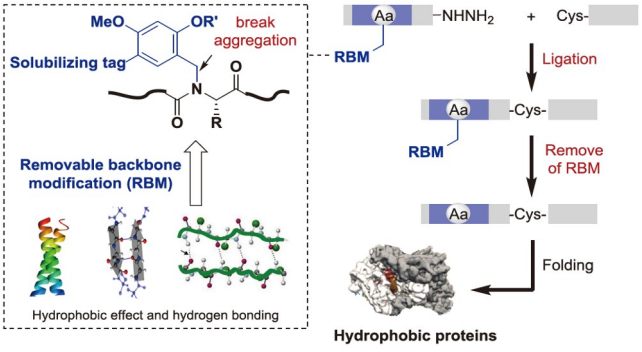

Figure 14 Removable backbone modification (color online).

2.6 Multiple-segment ligations

Chemical ligation is usually carried out using short 30–50 amino acid peptides obtained by solid-phase peptide synthesis, and therefore, the sequential ligation of multiple peptide segments is usually needed if a 100–150 amino acid protein functional domain is the target. Tremendous efforts have been devoted to developing sequential N-to-C and/or C-to-N NCL, STL, DSL, or KAHA ligations by temporarily masking or precisely controlling the reactivity of the C- or N-terminal reactive groups of the middle peptide segments. However, the need for intermediate purification and handling steps, both of which lower the overall yield, is severely limiting. Solutions to this problem include the one-pot multiple-segment ligation and solid-supported chemical ligation [190,191].

2.6.1 Solution-phase one-pot ligation

The C-to-N one-pot ligation requires that the N-terminal Cys of the middle peptide segment be protected with a group such as a thiazolidine (Thz), which can be removed after a one-pot, three-segment ligation using Pd [192], and after a one-pot, four-segment ligation using a 2-aminobenzamide-based aldehyde scavenger [193]. Other protecting groups include the trifluoroacetamidomethyl (Tfacm) group [194], acetamidomethyl (Acm) group [195], and 7-(piperazin-1-yl)-2-(methyl)quinolinyl (PPZQ) group [196] for thiol group protection, which can be removed by pH change, Pd(II) and photolysis, respectively. N-terminal amino protecting groups such as p-borobenzyloxycarbonyl group (Dobz) [197], allyloxycarbonyl (Alloc) [198a], and 9-fluorenylmethyloxycarboyl (Fmoc) [198b] have all been used for one-pot multiple segment ligation (Figure 15).

N-to-C sequential ligation studies have yielded interesting findings. For instance, NCL can be kinetically controlled by exploiting the different reactivities of highly reactive aryl thioester or fluoroalkyl thioesters and alkyl thioesters [199,200]. Additionally, the orthogonal thioester precursors cysteinyl valine ester (CPE) and *N*-alkylcysteine (NAC) were used for a one-pot N-to-C ligation [201]. Recently,

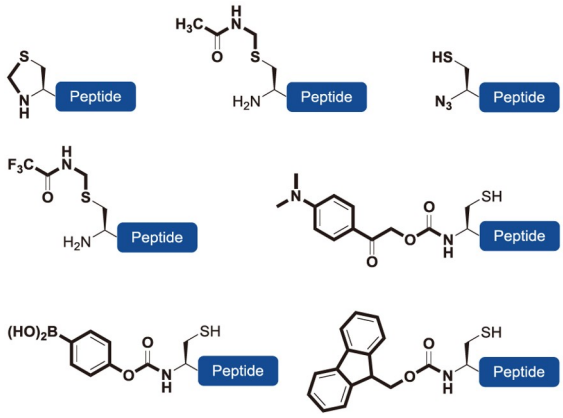

Figure 15 Representative Cys protecting groups for one-pot sequential ligations in the C-to-N direction (color online).

Dawson *et al.* [98] reported that C-terminal peptide hydrazides could be converted into their corresponding thioesters with stoichiometric acetyl acetone (acac), allowing one-pot multiple ligations.

The toolbox of chemical protein synthesis can be further expanded by combining different peptide ligation techniques. For example, Li *et al.* [202] reported that a peptide bearing a C-terminal thioester and N-terminal Ser or Thr as the middle peptide segment can undergo one-pot STL and NCL, as showcased in their synthesis of GlcNAcylated interleukin-25. Another example is the convergent synthesis of trifunctional Ubc9 variants by combining STL, NCL and KAHA ligation [203]. Recently, Zheng *et al.* [204] used a combined N-to-C sequential NCL and STL strategy to prepare complex protein samples such as S-palmitoylated proteins.

Metanis *et al.* [205] accessed proteins bearing non-strategically placed Cys residues *via* a sequential, C-to-N NCL and Sec-mediated ligation, the key to which is the use of a masked selenazolidine and selective deselenization. Human enzyme phosphohistidine phosphatase was accessed using this method. Another DSL and NCL combination strategy was developed by Hanna *et al.* [206] to access di- and tri-palmitoylated variants of the *Mycobacterium tuberculosis*-associated antigen protein ESAT6.

2.6.2 Solid-phase chemical ligation

Solid-phase chemical ligation (SPCL), where the nascent peptide is attached to a solid support *via* a linker at either the N or C terminus, can, in principle, speed the ligation process because the purification of ligation intermediates to remove unreacted peptides, reagents and by-products can be easily accomplished by simple washing and filtration, without the need for multiple high performance liquid chromatography (HPLC) purification and lyophilization steps which can lower yields and are time-consuming. Chemical ligation on a solid support can be conducted in either N-to-C or C-to-N directions and requires a suitable linker to release the desired product once the ligation steps have been completed (Figure 16). SPCL was first reported by Kent *et al.* [207], who described the chemoselective assembly of unprotected peptide segments on water-compatible solid support. Since then, a variety of linkers have been developed, facilitating cleavage of the final ligated peptide using acids [189,208], bases [209,210], nucleophiles [211], translation-metals [212], photolysis [213], or enzymes [214]. The total synthesis of proteins such as C5a, MIF, ubiquitin derivatives, thioredoxin-1, histone H2B, and truncated MUC1 VNTRs have been accomplished by SPCL. However, the performance of SPCL must be improved before it can be used to synthesize larger proteins.

2.7 Protein folding

Protein folding is another major synthetic challenge. Many proteins can be folded *via* direct dilution or gradient dialysis strategies, where the linear synthetic peptide is dissolved under denaturing conditions such as guanidine or urea, then dialyzed or diluted into a refolding buffer. However, some proteins are difficult to fold, such as disulfide-rich proteins, which are prone to misfold, and those having complex conformations. Such difficult-to-fold proteins are prone to aggregation and precipitation, lowering fold efficiency; a few cannot be folded *in vitro* at all.

Several new strategies have been developed for protein folding. For example, a single cystine-to-diaminodiacid replacement can significantly improve the folding of disulfide-rich proteins [215,216], as can small molecules, ultraviolet-light, and palladium, which have enabled the ultrafast folding of disulfide-rich proteins with high chemo- and regioselectivity [217].

Protein folding methods used by cells have also inspired new *in vitro* protein folding strategies. For example, inspired by the observation that molecular chaperones such as GroEL/ES mediate folding by preventing protein aggregation, Kay's group [218] developed a GroEL/ES-assisted protein folding strategy (Figure 17), which enabled the folding of both L-type and D-type chaperone-dependent protein (DapA). Cells sometimes also attach saccharides onto full-length protein sequences to facilitate protein folding in cells; Liu *et al.* [219] developed a removable glycosylation-assisted folding strategy to fold disulfide-bonded proteins. This strategy utilizes chemical protein synthesis techniques to introduce the *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc) groups at the serine/threonine (Ser/Thr) sites of a target protein. The *O*-GlcNAc groups on the protein improved the folding of disulfide-bonded proteins by increasing the stability of the folding intermediates. After folding, the *O*-GlcNAc groups were completely detached by using *O*-

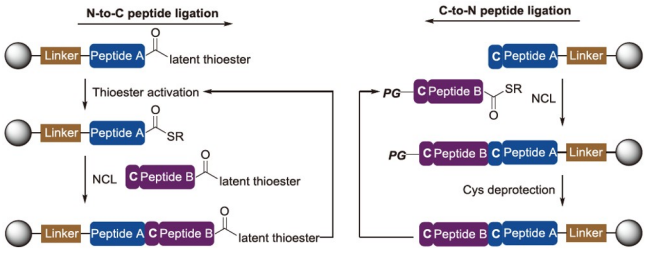

Figure 16 Solid-phase chemical ligation in N-to-C or C-to-N directions (color online).

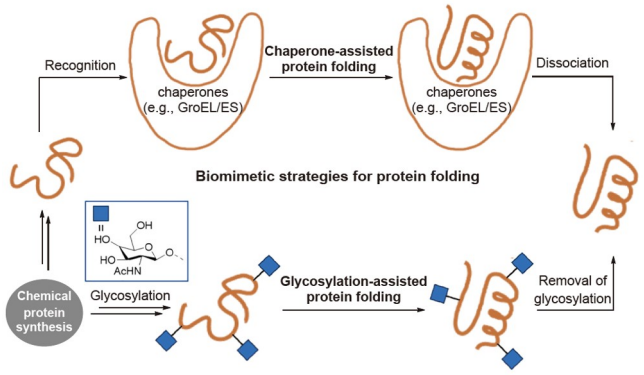

Figure 17 Biomimetic strategies for protein folding *in vitro* (color online).

GlcNAc glycosidase (OGA) to give the desired correctly folded protein. This study shows that biomimetic glycosylation modification can effectively assist in the folding of disulfide-bound proteins. Glycosylation also assisted the folding of difficult-to-fold proteins, including interleukin-17A [220] and SARS-CoV-2 spike receptor-binding domain [221], which cannot be folded *in vitro* using conventional folding strategies.

3 Protein synthesis through chemical modifications

Chemical modification of proteins is another approach for the generation of functional proteins [222–225]. Here, we survey recent representative advancements in the field of chemical protein modification, subcategorized by the properties of target amino acids.

3.1 Modification of nucleophilic residues

3.1.1 Cysteine as the target residue

Cysteine (Cys) is one of the most targeted residues due to its low natural abundance and high nucleophilicity [226,227]. Beier *et al*. [228] reported trifluoromethyl benziodoxol(on)es reagents, which have enhanced water solubility and enabled the conjugation of a range of functional groups to cysteine thiols. Subsequently, the same reagent was used to target less surface-exposed Cys residues, leading to stable vinylbenziodoxolone (VBX) products [229] amenable to further modification. Also, amphiphilic hypervalent iodine reagents were introduced for the lipidation of cysteine residues [230] (Figure 18a).

Reversible bioconjugation of Cys residues can be beneficial for certain applications. Wong *et al*. [231] reported the use of isoxazoliniums, which form stable phenylacyl thioether linkages with Cys residues and can be efficiently removed under UV irradiation. Another thiol-specific and traceless bioconjugation tool is 5-methylene pyrrolone moiety (5MP), which can undergo traceless cleavage under basic conditions through retro-Michael addition [232]. Zhou *et al*. [233] introduced a bromo substitution onto 5-methylene pyrrolones to improve its reactivity towards cysteine and allow multi-functionalization of a single cysteine as well as disulfide bridging bioconjugation (Figure 18b).

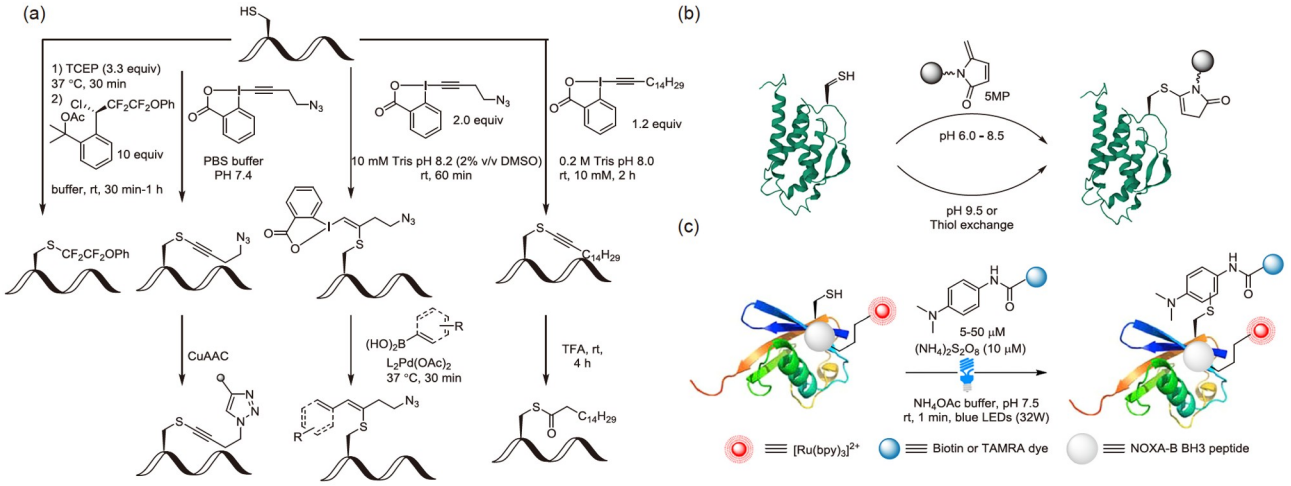

Figure 18 (a) Functionalization of hypervalent iodine reagents. (b) Thiol specific bioconjugation *via* Michael addition to 5-methylene pyrrolones. (c) SET-mediated ligand-directed labelling (color online).

Bernardes *et al*. introduced azabicyclic vinyl sulfones [234] and azanorbornadiene bromovinyl sulfones as thiol-selective Michael acceptors [235]. The obtained protein conjugates were stable in human plasma, even in the presence of elevated levels of glutathione. Matos *et al*. [236] harnessed electrophilic-unsaturated motifs to convert vinyl-pyridines and alkynyl-pyridines into highly reactive cysteine labeling reagents by quaternizing the nitrogen of the pyridine. Walsh *et al*. [237] heightened the electrophilicity of a vinylpyridine by replacing the pyridine scaffold with a pyrimidine. In addition to these examples, Becker *et al*. [238] reported a Pd-catalyzed Tsuji-Trost allylation method to chemoselectively prenylate proteins.

Finally, Hong *et al*. [239] introduced the first visible-light mediated thiol-ene click (TEC) strategy, utilizing a water-soluble, alkene-functionalized quinolinone (Q_{PEG}) as both the photocatalyst and cysteine labeling reagent. The electrophilic cysteine thiyl radical could be captured by electron-rich arenes. The reaction was employed in a ligand-directed cysteine labeling approach by the Wilson group (Figure 18c) [240].

3.1.2 *Lysine as the target residue*

Common Lys bioconjugation reagents such as activated esters, sulfonyl chlorides, and isothiocyanates [241] are often susceptible to hydrolysis in neutral buffers. Li *et al*. [242a] reported that *ortho*-phthalaldehyde (OPA) and its derivatives could chemoselectively and rapidly react with amino groups on peptides and proteins under physiological conditions (Figure 19a). Yao *et al*. [242b] reported that azaphilones have a high specificity for primary amine groups in a variety of peptides, proteins and lipids (Figure 19b). These activation-free azaphilone probes were remarkably stable and exquisitely chemo-selective. Chen *et al*. [243] pioneered the development of a light-induced cyclization reaction between the primary amine of lysine and *o*-nitrobenzyl alcohols (*o*-NBAs), which serves as a photoclick reaction (Figure 19c).

3.1.3 *Methionine as the target residue*

As milestone methionine bioconjugation methods, oxaziridine reagents [244] and hyper-valent iodine reagents [245] have been successfully developed and employed for methionine-specific modification of proteins. These methodologies have found applications in various down-stream biological studies, including proteomic investigations. Recently, MacMillan *et al*. [246] developed a photocatalytic method to deliver a stable conjugation product by converting the Met side chain to a carbon-centered α-thio radical, which is then trapped by alkylating reagents (Figure 19d).

3.1.4 *Serine as the target residue*

Serine and threonine residues are rarely selected as chemoselective bioconjugation sites primarily due to the weak

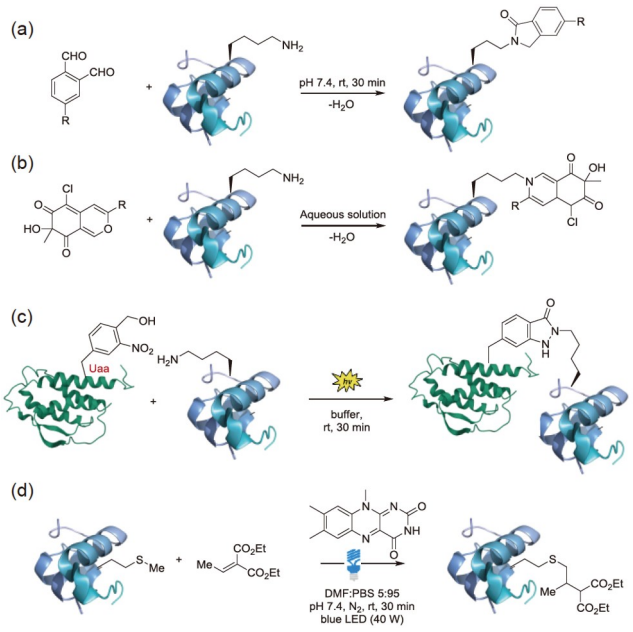

Figure 19 (a) Lys bioconjugation of proteins by *ortho*-phthalaldehyde (OPA). (b) Lys bioconjugation of proteins by azaphilones; (c) Light-induced labeling of Lys residues. (d) The alkylation of methionine with a lumiflavin photocatalyst (color online).

nucleophilicity of their hydroxyl side chains and the challenge of distinguishing them from other nucleophilic residues, especially under aqueous conditions. Addressing this issue, a collaborative effort involving the Eastgate, Dawson, Bernardes, and Baran's groups has led to the development of a platform based on a new class of phosphorus P(V)-based bioconjugation reagents. This study provides chemoselective, rapid and robust P(V) reagents designed for the direct functionalization of serine residues in peptides and proteins [247].

3.2 Modification of aromatic amino acid and glycine

3.2.1 *Tyrosine as the target residue*

Several methods have been developed to leverage the unique reactivity of the phenol side chain of Tyr residues for modification [222]. Lei *et al*. [248] introduced an electrochemical-based labeling strategy for proteins (insulin and myoglobin) containing Tyr residues using phenothiazine derivatives (Figure 20a). Francis *et al*. [249] discovered that tyrosine residues present at the N- or C-terminus (tyrosine tags or -GGY) of proteins can be readily oxidized by tyrosinase enzyme (abTYR) for subsequent reaction with a secondary amine. This work has expanded oxidative coupling chemistry through enzyme-mediated reactions (Figure 20b). MacMillan *et al*. [250] proposed that the surface accessibility and π-electronic properties of the multiple tyrosine residues present in a protein structure are different,

allowing for their selective modification by a phenoxazine dialdehyde under photocatalytic conditions (Figure 20c).

3.2.2 *Histidine as the target residue*

Classical strategies for site-selective histidine modifications rely on N-substitution reactions of the histidine imidazole group [251]. In 2019, Gopalakrishnan *et al*. [252] used sulfinate salts as radical precursors to accomplish the selective, late-stage modification of the histidine residues of unprotected peptides and introduced a wide range of aliphatic substituents at the C2 position of histidine (Figure 20d). In the same year, Wang *et al*. [253] reported a visible light-promoted histidine bioconjugation method by the chemoselective C–H alkylation of histidine residues using C-4-alkylated Hantzsch ester reagents (Figure 20e). Subsequently, Nakamura *et al*. [254] presented a histidine-selective modification using MAUra reagents previously developed for reactions involving tyrosyl radicals (Figure 20f).

3.2.3 *Tryptophan as the target residue*

Taylor *et al*. [255] employed an *N*-carbamoylpyridinium salt to transfer relevant reporter handles to the Trp residue of a protein upon irradiation with 302 nm light, a reaction fast and selective for tryptophan (Figure 21a). Later, Taylor *et al*. [256] extended this with a modified reacting agent, amenable to activation upon exposure to blue LEDs, that featured an electron-rich 6-methoxynaphthyl ring attached at the 4-position to the cationic pyridinium moiety (Figure 21b). In addition, Davis *et al*. [257] described a three-step sequence for producing functionalized tryptophan constructs using a ruthenium photocatalyst.

3.2.4 *Glycine as the target residue*

Glycine-specific C(sp^3)–H functionalization can enable the late-stage incorporation of unnatural amino acid residues. Xu *et al*. [258] reported a selective alkylation method of the *N*-α-arylated glycine residues of short peptides using redox-active esters and Katritzky salts as radical precursors.

It is noteworthy that beyond the conventional natural amino acid residues, a noncanonical amino acid, dehydroalanine (Dha), has been harnessed as a distinct handle for site-selective protein modification. A recent representative work comes from Davis's group [259], presenting a visible-light-mediated method for modifying dehydroalanine residues with a wide range of labels (Figure 22a). Moreover, Li *et al*. [260] pioneered a versatile approach for the late-stage modification of diverse Dha-containing peptides and proteins using a photo-initiated 1,3-dipolar cycloaddition reaction between Dha and tetrazoles under 302 nm photoirradiation. The incorporation of Dha and derivatives in proteins, along with its applications, have been reviewed elsewhere [261,262].

3.3 Peptide termini

Ethynylbenziodoxole (EBX) reagents have been employed for the decarboxylative photoredox-catalyzed alkynylation of amino acids [263]. In 2019, Waser *et al*. [264] introduced the first photo-mediated C-terminal decarboxylative alkynylation method using the *iso*-phthalonitrile organic dye 4CzIPN in combination with an EBX reagent to achieve C-terminal functionalization (Figure 22b). The same group subsequently reported the C-terminal decarboxylative modification of short peptide using visible light to generate N–O

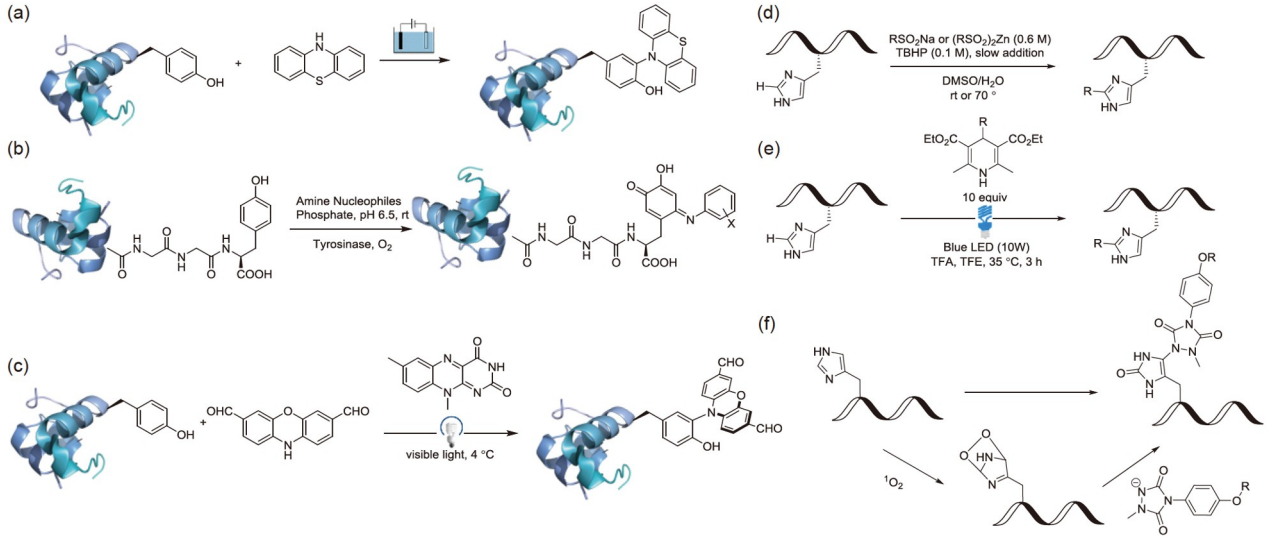

Figure 20 (a) Electrochemical oxidative tyrosine bioconjugation. (b) Tyrosinase-mediated oxidative coupling of tyrosine. (c) The photoredox-catalyzed tyrosine modification. (d) The modification of peptides at the C2 position of His. (e) Histidine-specific peptide modification *via* radical-mediated chemoselective C−H functionalization. (f) $^1O_2$-mediated histidine labeling (color online).

acetal conjugates (Figure 22c) [265].

Significant advancements have been made in the modification of the *N*-terminus of peptides. In 2021, Wang *et al.* [266] demonstrated a selective and rapid method for modifying the *N*-termini of proteins *via* the quinone-mediated oxidation of the N-terminal α-amine of complex peptides and proteins under physiological conditions (Figure 22d).

4 Peptide stapling and macrocyclization

Peptides can have a greater binding affinity and specificity for a target protein than small molecules but may also demonstrate poor proteolytic stability and membrane permeability, and the extra entropy associated with their flexible secondary structures can impede target binding. Macrocyclization is an important chemical strategy to overcome these limitations, and cyclic peptides are often less susceptible to proteolysis and more membrane permeable compared with their linear counterparts. [267–271]. Various strategies for peptide macrocyclization have been developed, including ring-closing metathesis [272,273], lactamization [274], thiol-ene chemistry [275], thiol/nitrogen-arylation/alkylation [276–281], azide-alkyne cycloaddition [282], metal catalyzed C–H activation [283] and the diaminodiacid (DADA) method [284–286] (Figure 23).

4.1 Ring-closing metathesis between olefinic side chains

In 2000, Schafmeister *et al.* [272] reported the synthesis of various α,α-disubstituted olefinic amino acids of different

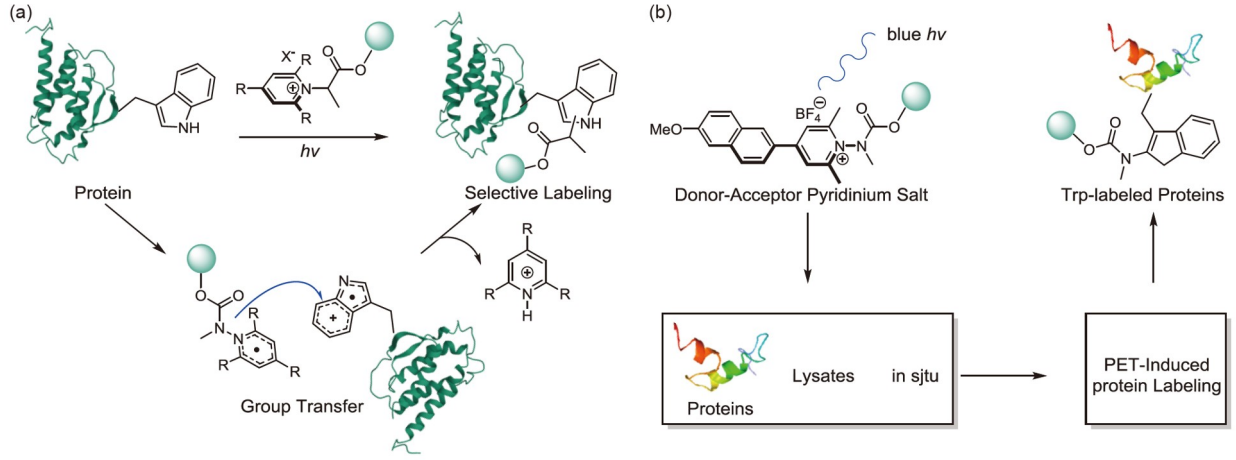

Figure 21 (a) Pyridinium salt reagents for Trp-selective protein modification. (b) Donor-acceptor pyridinium salts for photo-induced electron-transfer-driven modification of and proteomes of tryptophan in peptides, proteins (color online).

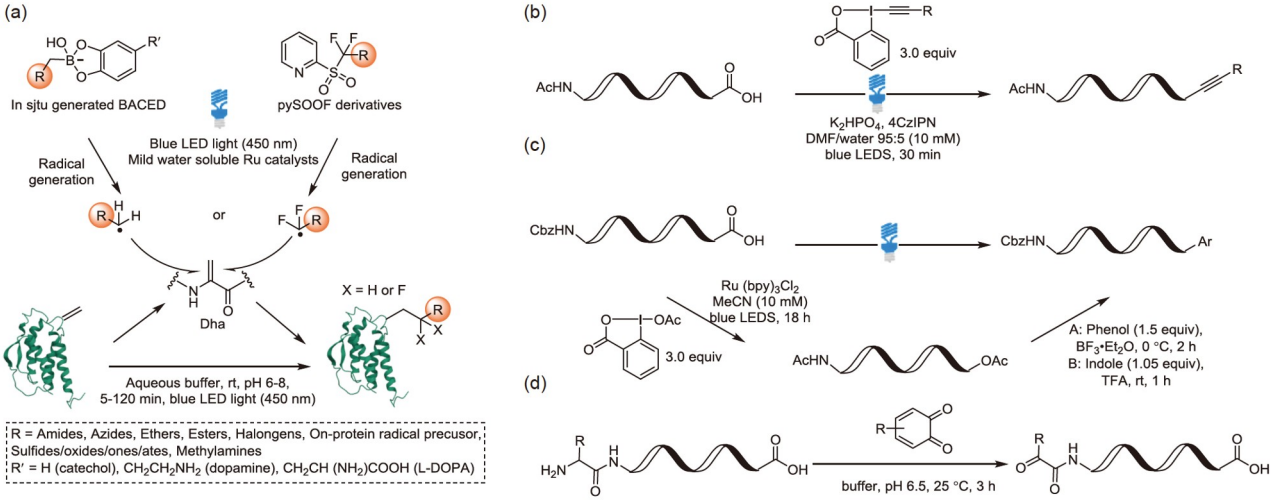

Figure 22 (a) Dha modification through the photocatalyzed generation of carbon-centered radicals. (b) Decarboxylative alkynylation using EBX reagents. (c) Decarboxylative introduction of proteinogenic indoles and phenols on the C-terminus of peptides. (d) Site-specific N-terminal protein modification *via* quinone-mediated transamination (color online).

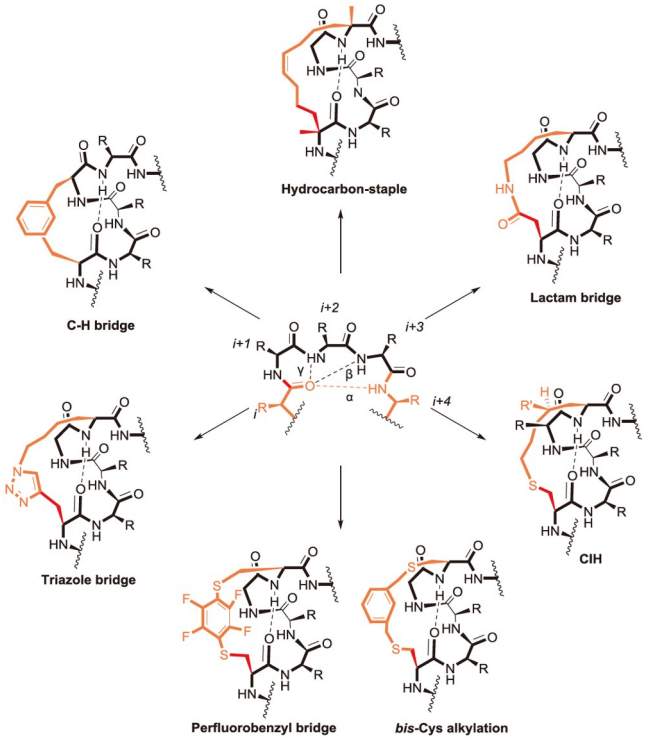

Figure 23 Several peptide stapling strategies [56] (color online).

lengths and stereochemistries [254], which could be stapled using a solid-phase Grubbs catalyst at the *i*, *i*+4 and *i*, *i*+7 positions, starting from S5-S5 and R8-S5 paired residues, respectively (Figure 24a). This chemistry has been successfully used to design various peptide inhibitors with improved proteolytic stability, membrane permeability, and biological activity [287–293]. One notable example is ALRN-6924, a hydrocarbon-stapled peptide antagonist of the oncogenic protein MDM2 and its homolog MDMX that functionally inhibits the tumor suppressor protein p53, and is now in clinical trials for advanced solid tumors and lymphomas [294–297].

Other types of stapling structures have also been developed. Speltz *et al*. [298] developed the olefinic-terminated amino acids bearing a methyl group in the γ-position and successfully applied them in the synthesis of stapled peptides (Figure 24b). Wilson and coworkers [299,300] removed the α-substituted methyl group previously incorporated to avoid the intrinsic helix-destabilizing effect and obtained the monosubstituted olefinic-terminated stapling amino acid (Figure 24c). This amino acid could be applied efficiently in Fmoc peptide synthesis and the resulting staple may lead to fewer steric clashes with the target compared with the staple it replaced [301].

Hu *et al*. [302] designed amino acids incorporating both the native side chain and the alkenyl arms needed for ring-closing stapling chemistry (Figure 24d). Introducing these amino acids into the β-catenin-binding domain of axin (469–482) led to new stapled peptides with high α-helicity, strong proteolytic stability and good cell permeability. Oba *et al*. [303] introduced cyclic α,α-disubstituted olefinic-terminated amino acids into arginine-rich peptides and provided an extra staple (Figure 24e). Hu *et al*. [304,305] designed the stapled peptide-based PROTAC, which consisted of a stapled peptide motif specifically targeting the protein and another peptide recruiting E3 ligase. These peptides were double-stapled for improved helical content and proteolytic stability [306].

4.2 Lactamization

Lactamization-based peptide stapling also calls for selective protecting and deprotecting groups for the amino and carboxyl groups [307]. Li *et al*. [308,309] developed a strategy for helix nucleation using an N-terminal cross-linked aspartic acid *via* facile macro lactamization in high yield and purity (Figure 24f). The group also designed a strategy to stabilize the peptide in the N-terminal sulfonium salt center [310] and developed peptides to degrade ERα [311,312]. Wan *et al*. [313] have reported reversible peptide stapling, which involves macrocyclization between two amino groups and decyclization *via* dual 1,4-elimination.

Wang *et al*. [314] reported a peptide macrocyclization and stapling strategy based on a fluorine thiol displacement reaction (Figure 24g). This peptide stapling strategy was initiated from benzyl thiol and an α-fluoroacetamide and led to lactamized peptides, the cellular uptake of which was higher than peptides stapled using all-hydrocarbon methods.

4.3 Thiol-ene and thiol-yne hydrothiolation

Cysteine is well suited to biocompatible cross-linking reactions due to its nucleophilic sulfydryl group. In 2015, Wang and Chou [315] reported a peptide stapling and macrocyclization methodology using thiol-ene reactions between two cysteine residues and a diene (Figure 25a).

In 2016, Li *et al*. [316] synthesized peptides with a chiral center in the stapling bridge using the thiol-ene reaction. The cell permeability, serum stability, and binding affinity toward ER-α of the products were found to depend on the stereochemistry of this chiral center (Figure 25b), the introduction of which increased the rigidity of the peptide and stabilized it to an α-helix [317].

Li *et al*. [318] also developed a UV irradiation-based thiol-yne method for stabilizing peptides in an α-helical conformation. An alkyne group introduced at an appropriate site in the form of an unnatural amino acid underwent the thiol-yne reaction with cysteine at the *i*, *i*+4 site when exposed to 365 nm UV irradiation (Figure 25c). This strategy could stabilize the peptide into a regular α-helical conformation at

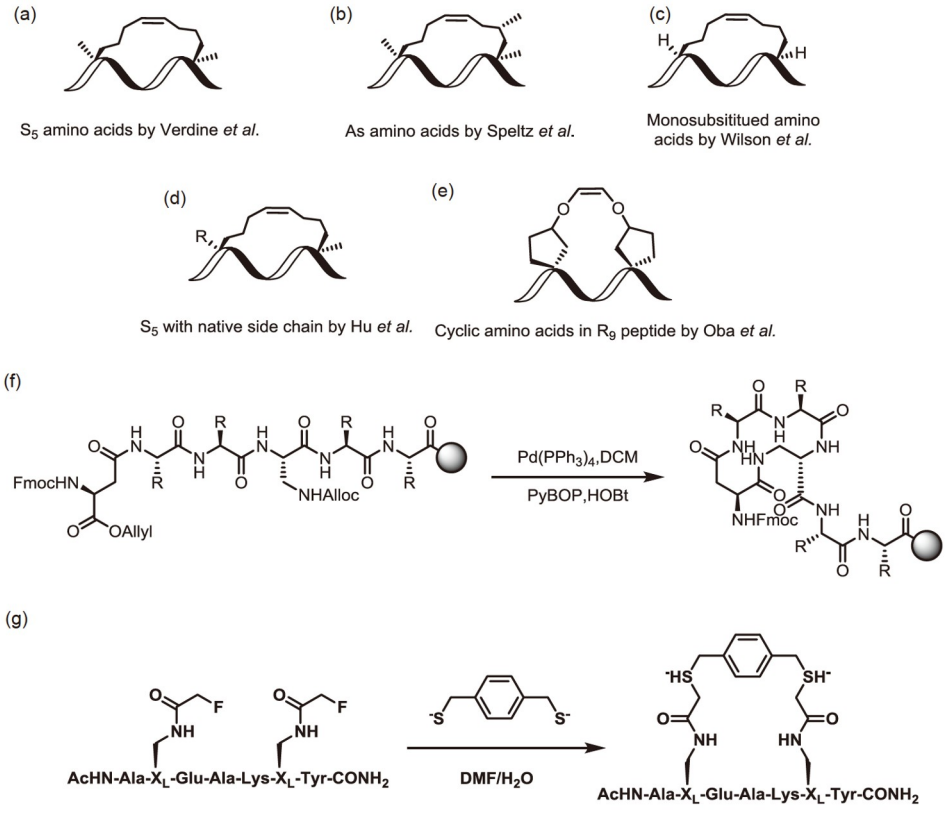

Figure 24 (a–e) Ring-closing metathesis (RCM) mediated peptide stapling of olefinic-terminated residues. (f) *i*, *i*+3 TD stapling strategy. (g) Fluorine thiol displacement reactions (FTDRs).

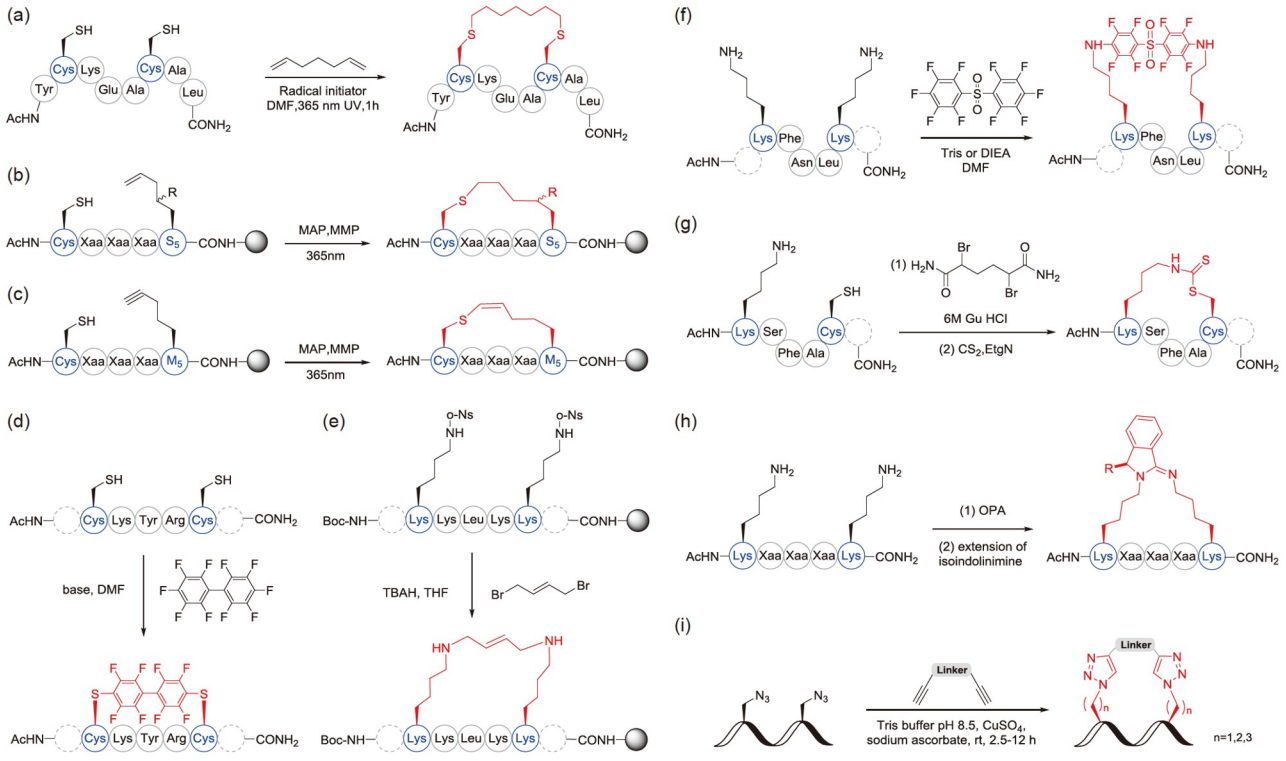

Figure 25 (a–c) Thiol-ene and thiol-yne hydrothiolation peptide stapling. (d–h) Thiol/Nitrogen-arylation/alkylation *via* nucleophilic substitution (S_N). (i) Click-based azide-alkyne cycloaddition (color online).

different temperatures and pH, and the reaction proceeded cleanly and was complete within about 10 min. The thioether cross-linker increased the serum stability and cell penetration of the stabilized peptide, as demonstrated by serum stability assay and confocal microscopy.

4.4 Thiol/nitrogen-arylation/alkylation *via* nucleophilic substitution (S_N)

Thioether staples can be formed by reacting bis-alkylators with paired Cys side chains at the *i*, *i*+4 position [248]. Alternatively, thiol-arylation can be applied to construct thioether staples of unprotected peptides bearing Cys residues using the perfluoroaromatic staples (Figure 25d) [259,260]. Zhang *et al*. [319,320] reported the peptide stapling of amphiphilic anti-microbial peptides *via* the *N*-alkylation reaction of two tethered ε-amino groups of a Lys residue (Figure 25e) [261]. *N*-arylation stapling reactions between decafluoro-diphenylsulfone (DFS) and a dichlorotriazine-based aromatic electrophile (DCT) with Lys-Lys pairs at the unprotected peptides were investigated, and *i*, *i*+7 stapled peptides successfully obtained (Figure 25f) [262].

Lu *et al*. [321] developed a peptide stapling strategy based on dithiocarbamate chemistry, linking the side chains of Lys and Cys paired residues at the *i*, *i*+4 position of unprotected peptides in solution (Figure 25g). Treatment of the Cys-containing peptide with 2,5-dibromohexadiamide afforded a DHA moiety, which undergoes a three-component reaction with the amino group of Lys residue and exogenous CS_2 to yield a stapled peptide. Chen *et al*. [322] revealed that OPA readily condensed with two alkyl amino side chains of the lysine residues to form a class of underexplored isoindolin-1-imine stapled peptides under mild aqueous conditions (Figure 25h).

Thiol/nitrogen-arylation/alkylation reactions biocompatible with phage-display systems have also been successfully developed and shown to facilitate the convenient selection of functional peptides derived from the phage-produced random peptide libraries. One early example was reported by Heinis *et al*. [323], who established bicyclic peptide libraries using a phage display system. Recently, Wu *et al*. [324] developed a bioorthogonal strategy relying on the condensation of 2-((alkylthio)(aryl)methylene)malononitrile with N-terminal cysteine for the site-specific peptide cyclization on the surface of phages, which has been effectively employed to select protein binders. Gao *et al*. [325] reported a cysteine-directed proximity-driven amine-selective cyclization strategy to construct bicyclic peptide libraries. In addition, Derda *et al*. [326] introduced a strategy involving the cyclization of peptides with two cysteine residues on the phage surface with 1,5-dichloropentane-2,4-dione for the late-stage modifications of unnatural fragments, which increased the diversity of phage-display libraries.

4.5 Click-based azide-alkyne cycloaddition

Spring *et al*. [327] developed an *i*, *i*+7 double-click-based peptide stapling technique and used it to synthesize peptide inhibitors of the p53-MDM2 interaction. The "click" reaction could trigger either one-component or two-component peptide stapling, with one-component initiated from a combination of both ω-azido- and ω-yl-α-amino acids, and two-component stapling using two repeated ω-azido- or ω-yl-α-amino acids (Figure 25i) [328–331].

4.6 Metal-catalyzed C–H activation

Transition metals have been used to staple peptides by catalyzing an intramolecular C−H activation [253,332]. The first example of this reaction was published by Lavilla *et al*. [333] in 2015 (Figure 13). Albericio, Wang and others [334–343] developed Pd-catalyzed late-stage peptide macrocyclization methods through N-terminal of amino acid β-C(sp^3)–H arylation, aryl-aryl crosslinking *via* arylation of C(sp^2)–H, aryl-alkene crosslinking, *etc*. (Figure 26).

External groups, such as 8-aminoquinoline, pyrimidine, pyridine, picolinamide and *N*-P^{t}Bu$_2$, have been used to direct the late-stage modification and macrocyclization of peptides in C–H activation reactions (Figure 27) [344–361]. C–C crosslinked peptide macrocycles, which exhibit diverse structural and functional properties, have been successfully synthesized by these methods. For example, Chen *et al*. [349] reported the synthesis of a highly constrained peptide macrocycle *via* Pd-catalyzed C–H activation. Other groups have reported C–C crosslinked cyclic peptides that are cytotoxic and capable of self-assembly and those that are cytotoxic [339].

In 2017, Albericio *et al*. [283] applied this technique to staple peptides by the C–H activation of a terminal alanine residue followed by cross-coupling with an iodo-phenylalanine derivative. Zheng *et al*. [362] constructed a new kind of stapled peptide using late-stage peptide substitution-cyclization *via* maleimidation on C(7)–H Trp. Moreover, tryptophan-substituted maleimide served as an effective click functional group capable of rapidly reacting with sulfhydryl groups [363].

5 Biological applications of chemical protein synthesis

5.1 Semi-synthetic methods and applications of site-specifically phosphorylated proteins

Protein phosphorylation occurs in almost every process of the cell life cycle and regulates the function of more than 1/3

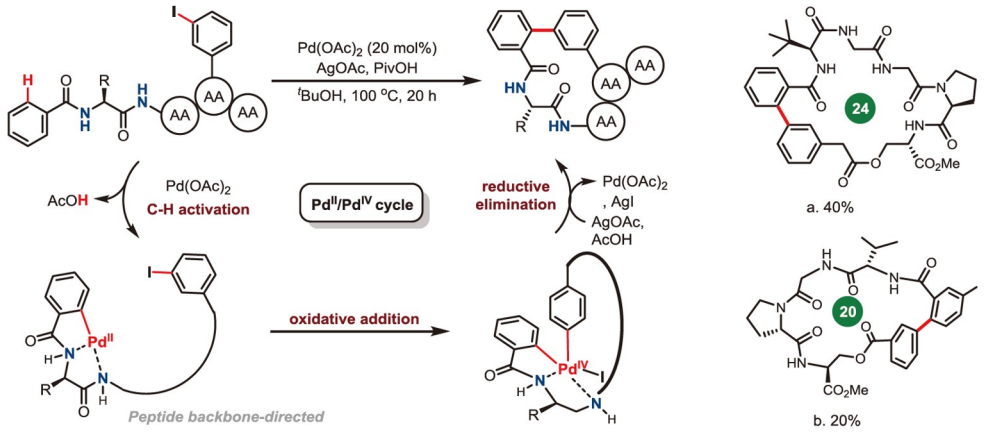

Figure 26 Backbone-directed C–H activation for peptide macrocyclization (color online).

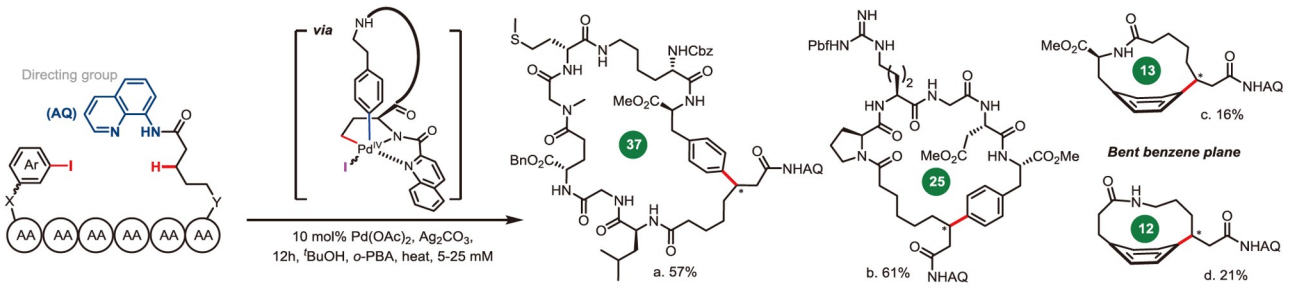

Figure 27 AQ-directed C–H activation for peptide macrocyclization (color online).

of cellular proteins [364–367]. Abnormal phosphorylation, notably of protein kinases, can lead to disease [368–371]. Despite its extensive study, however, many regulatory mechanisms that include phosphorylation, particularly those that work in cooperation with other post-translational modifications, are still unclear.

α-Synuclein [372] is an important phosphorylated target due to its role in the pathogenesis of Parkinson's disease (Figure 28), and several *in vitro* chemical synthetic strategies have been developed to generate synuclein phosphorylated at specific positions [373,374]. In general, these strategies split the sequence into multiple fragments, with the peptides containing the phosphorylated position synthesized by solid-phase peptide synthesis (SPPS), and the rest of the native sequence generated through expression in *E. coli*. The ligation between the two protein/peptide segments requires one coupling partner to have a (mutated) cysteine at the N terminal, and the other to be a thioester protein/peptide. The synthesized thioester peptide can be generated using the *N*-acylurea [375] or hydrazide methods [55,57]. The expressed thioester protein is generated *via* protein splicing, a naturally occurring thiolysis reaction in which an intein fragment is excised from the sequence [376].

The following procedure for semi-synthesizing pSer129 α-synuclein comprises three main stages. Throughout the different stages, the S-N acyl transfer reaction is crucial for the removal of temporally required fragments from the sequence by producing a thioester or a native amide bond, as specific requirements dictate. In the first stage, recombinant protein (sequence 1–106) bearing a purification tag and intein fragment is expressed. The purification tag is subsequently removed *via* protein splicing, resulting in a recombinant thioester protein. Meanwhile, the phosphorylated peptide (sequence A107C-140, containing pS129) is synthesized using standard Fmoc SPPS. Native chemical ligation between the thioester protein and the synthesized peptide led to a ligated product, from which the sulfhydryl group on Cys107 was removed to get the Ala107 needed for the native α-synuclein sequence, and hence a chemically well-defined homogeneous α-Syn with PTMs.

Ras is a family of phosphorylated proteins associated with more than 20% of cancers [378], the activities of which are significantly impacted by phosphorylation and lipidation modifications, which affect their membrane and/or chaperone binding ability. Studying the physiological function of PTMs of Ras can guide the development of targeted anticancer drugs against Ras. In 2017, Chen's group [379] successfully synthesized a fully functional, phosphorylated, farnesylated, and methylated K-Ras4B protein through chemical synthesis and protein ligation (Figure 29). Methyl

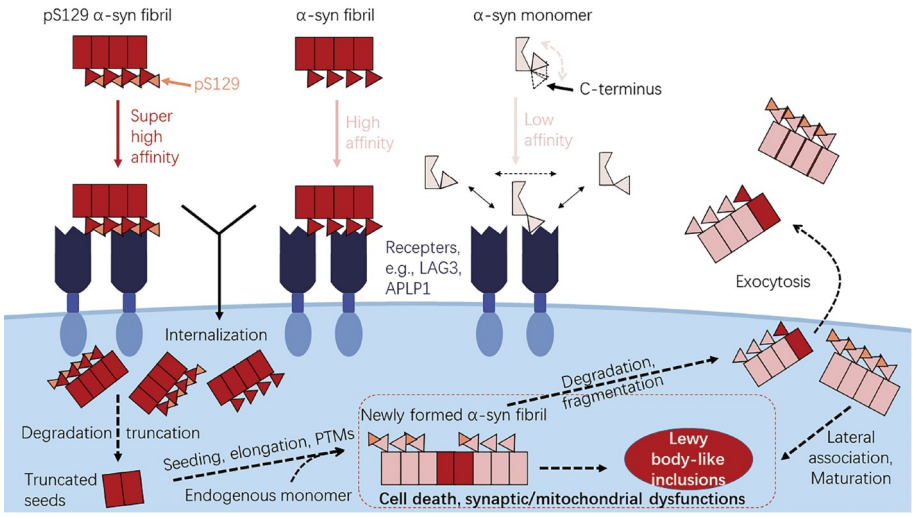

Figure 28 Schematic depiction of the pathological transmission of α-synuclein [377]. The pS129 fibril, wild-type fibril and monomer of α-synuclein exhibit different affinities on binding membrane receptors, such as LAG3 and APLP1. Among these, the pS129 fibril demonstrates the highest affinity, while the monomer exhibits the lowest affinity due to the structural instability at the C-terminus. Fibrils taken up by the cell are degraded, resulting in the generation of C-terminus-truncated seeds. These seeds are responsible for triggering the formation of intracellular α-synuclein fibrils and subsequent exocytosis of fibrils, ultimately leading to the formation of Lewy body-like inclusions and neuronal dysfunction (color online).

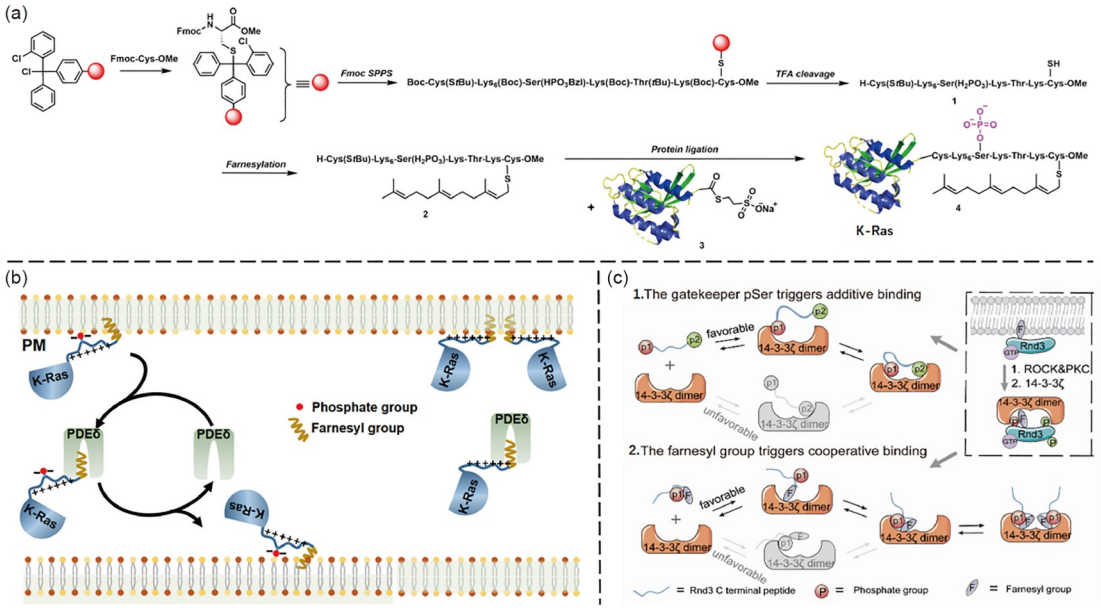

Figure 29 Synthesis and application of phosphorylated and farnesylated proteins. (a) Synthesis of phosphorylated and farnesylated K-Ras4B Protein-OPSF [379]. (b) Membrane binding of K-Ras4B simultaneously bearing phosphate, farnesyl, and methyl modifications [379]. (c) Proposed multivalent binding models between Rnd3 with phosphorylation and farnesylation and 14-3-3ζ [382] (color online).

peptides were synthesized beginning with Fmoc-Cys-OMe *via* standard SPPS, while phosphorylation was introduced using phosphorylated serine building blocks. Afterward, the crude peptide was removed from the resin using a TFA cocktail and an S-farnesyl modification installed by direct reaction trans,trans-farnesyl bromide. After purification, the peptide was ligated with expressed protein to obtain the fully modified protein. Using this semi-synthesized protein, Chen *et al*. [380] found that Ser181 phosphorylation weakened the interaction between K-Ras4B and the plasma membrane but did not affect its binding to the chaperone protein PDEδ. Chen *et al*. [380] also designed and synthesized a K-Ras4B-targeting peptide inhibitor, memrasin, which weakens the activity of K-Ras4B selectively by directly inhibiting the interaction between K-Ras4B and plasma membrane.

Phosphorylation and adjacent farnesylation of protein Rnd3 are known to enhance its binding to 14-3-3ζ [381], but the impact of polyphosphorylation on multivalent interac-

tions and the cooperative mechanism between phosphorylation and farnesylation remains to be elucidated. Chen *et al*. [382] used SPPS followed by late-stage biorthogonal modification of the C-terminal domain of Rnd3 bearing various phosphorylation and lipidation modifications, which were used to elucidate the influence of multiply charged phosphates and hydrophobic lipids on the multivalent binding thermodynamics and kinetics between Rnd3 protein and its chaperon 14-3-3ζ. Remote multi-phosphate modifications mediate this multivalent protein-protein interaction (PPI) through a gatekeeper-triggered additive mechanism, whereas adjacent phosphate and farnesyl modifications cooperatively contribute to the PPI *via* an induced fit mechanism. This study investigated the thermodynamic and kinetic processes of the interaction between Rnd3 and 14-3-3ζ and clarified the impact of phosphorylation and farnesylation on the binding mechanism. This study also establishes a useful new model to determine the effect and contribution of multiple synergetic modifications on the multivalent interactions [383,384].

Liu *et al*. [385] synthesized homogeneously phosphorylated and/or ubiquitinated H2AX proteins *via* expressed protein hydrazinolysis followed by DBA coupling. In contrast to methods used in cell biology and genetics, the effects of each post-translational modification of histone on the binding of 53BP1 with nucleosomes were quantitatively analyzed by chemical biology methods.

Scheffner *et al*. [386] used genetic codon expansion technology to obtain phosphorylated and non-hydrolyzable phosphorylated Ub and NEDD8. Ubiquitin is a well-known covalent modifier of proteins, and the diversity of ubiquitination is expanded by its phosphorylation [387,388]. However, there are many ubiquitin-like proteins (Ubls) whose phosphorylation effect remains unclear. This study elucidated the function of phosphorylated NEDD8, a Ubl. Parkin, a ubiquitin E3 ligase, can be allosterically activated by phosphorylated NEDD8 (pNEDD8) *via* protein interactomes differently to phosphorylated ubiquitin. Additionally, pNEDD8 preferentially interacts with Hsp70 family and significantly stimulates the activity of the Hsp70 ATPase.

Deshmukh *et al*. [389] utilized an *in vitro* kinase-catalyzed phosphorylation modification method to prepare tyrosine phosphorylation apoptosis-linked gene 2-interacting protein X (ALIX) for a study that demonstrated that PTM can regulate ALIX condensates such that tyrosine hyperphosphorylation leads to decondensation of the condensates, whereas dephosphorylation allows their reformation. These results suggest that the phase separation of PTM-mediated ALXI has an impact on cellular ACBs' biogenesis and stability and even governs abscission timing.

The reversibility and instability of natural phosphorylated proteins or peptides greatly complicate the study of abnormal phosphorylation *in vitro*. One approach is to develop phosphatase-stable, non-hydrolyzable phosphorylated protein or peptide mimics, for example, by inserting into proteins and peptides phosphorylated amino acid mimics. Chen *et al*. [390] synthesized an *N*-Fmoc protected CH_2-pThr mimic suitable for use in peptide synthesis using a palladium-catalyzed γ-methyl C–H activation reaction and the Appeland Michaelis-Becker reactions. A peptide containing a CH_2-pThr mimic binds more strongly with 14-3-3ζ than its phosphorylated counterpart and is, therefore, a powerful tool for biological research and further drug design in the cellular environment [391].

Chen *et al*. [392] also incorporated a phosphine ligand into proteins using P3BF, a borane-protected non-canonical amino acid capable of overcoming the sensitivity of phosphine to oxygen. Using this protein, they successfully established a novel one-pot deprotection and metal coordination strategy to allow for facile synthesis of palladium-bound protein phosphine under aerobic conditions and solved the first high-resolution structure of a protein containing a unique P–B bond. Genetic engineering methods are expected to be combined with this extraordinary phosphine-metal chemistry to enable the rational design of novel artificial enzymes or functional metalloproteins in the future.

5.2 Chemical glycoprotein synthesis and applications in exploring glycan functions

Glycosylation is another common posttranslational modification (PTM) in eukaryotes [393] and plays fundamental roles in protein quality control and stabilization and cellular adhesion, recognition, and signaling [363,394]. However, our understanding of glycan functions at the molecular level is lacking, in part due to the microheterogeneity of the appended carbohydrate motifs [395]. Chemical synthesis can help surmount these challenges by enabling the preparation of homogeneous glycoproteins, [396,397], usually by the ligation of glycosylated peptide segments. A glycoamino acid, usually bearing a monosaccharide, is incorporated to the designated site through SPPS [398] (Figure 30a), then elaborated to the desired glycan chain using enzymatic methods prior to its ligation into the final protein (Figure 30b, c). After reconstitution under suitable folding conditions, a series of proteins with varied glycan structures can be obtained (Figure 30d). Using this approach, Kajihara *et al*. [399] investigated the effect of an N83-triantennary sialylglycan in erythropoietin compared with the biantennary-modified counterpart (Figure 30e). Payne *et al*. [400] synthesized four differentially glycosylated variants of human interferon γ and evaluated their antiviral activities. Unverzagt *et al*. [401] prepared a small library of nine interleukin 6 glycoforms and assessed the protein's serum clearance rate in mice.

One recent illustrative example involves the exploration of

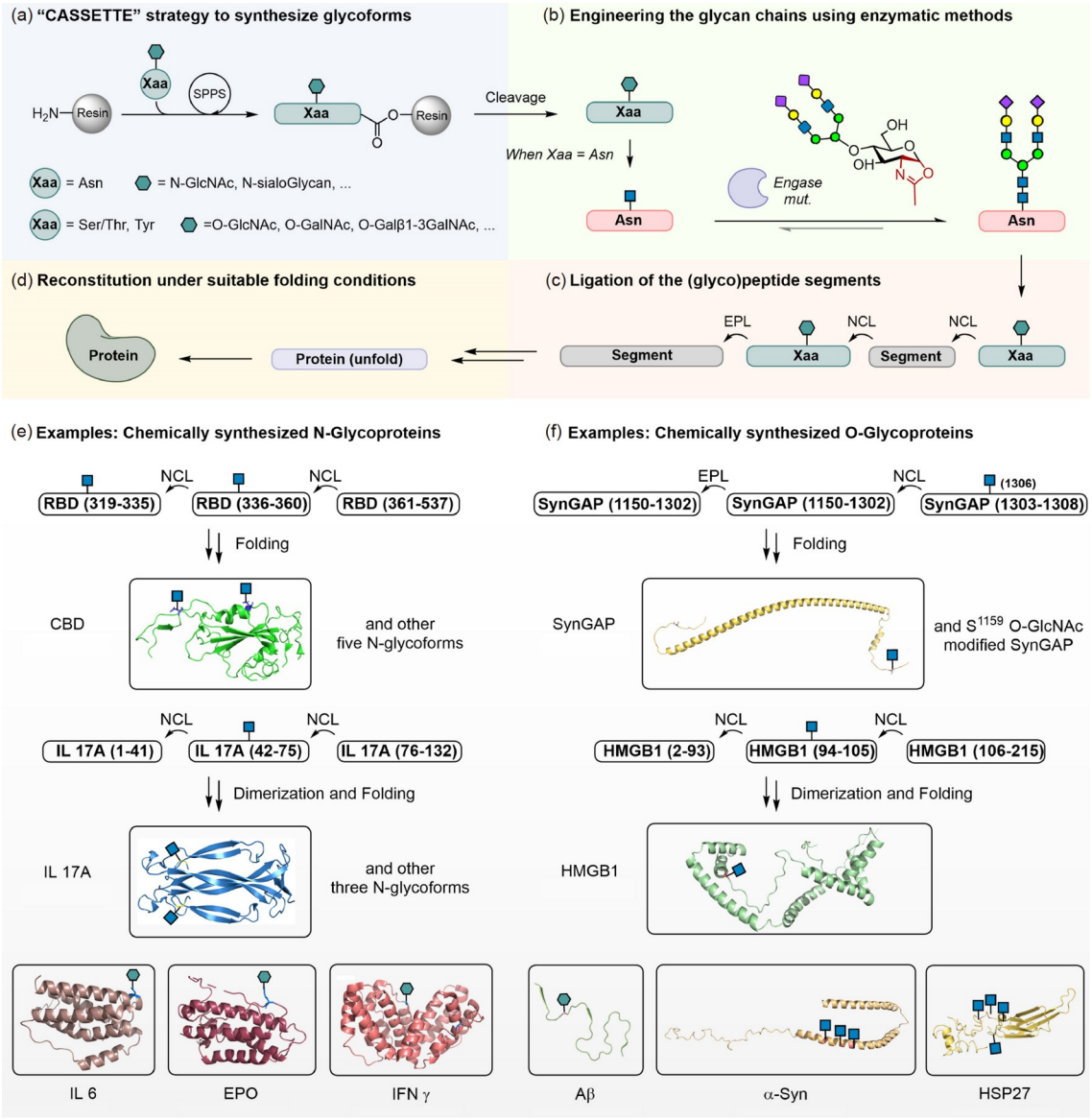

Figure 30 General strategies and recent examples of homogeneous glycoproteins prepared through chemical synthesis (color online).

N-glycosylation in interleukin-17A [220]. In a synthetic strategy that integrates SPPS, chemoenzymatic glycan modification and chemical ligation, three homogenously glycosylated IL-17A proteins were prepared. The mono- and di-saccharide-modified forms were derived from glycopeptide segments were prepared by submitting a glycosylated Asn building block to SPPS, while the undecasaccharide-modified form was prepared from the corresponding glycopeptide, in turn, derived from the monosaccharide-modified precursor. Through a comparative analysis of these three IL-17A glycoforms in a normal human dermal fibroblast (NHDF) assay, dose-dependent interleukin-6-inducing activities were observed in all instances. Notably, the sialyl undecasaccharide-modified IL-17A exhibits a considerably weaker stimulatory effect than its GlcNAc- or GlcNAc($\beta_{1\to4}$) GlcNAc-modified counterparts. Subsequent surface plasmon resonance (SPR) and hydrogen/deuterium exchange mass spectroscopic experiments further validate that the complex type *N*-glycan hinders the binding between IL-17A and its receptor IL-17RA, while the proteins with smaller-sized glycan display stronger binding affinities.

Another representative example is the synthesis and structure-activity relationship (SAR) exploration of glycosylated receptor binding domains (RBDs) derived from the SARS-CoV-2 spike protein [221,402–404]. Six distinct glycoforms of RBDs were generated, each incorporating homogeneous *N*-glycans at N331 and N343, complemented by an *O*-linked glycan at T323. The synthesis was achieved by employing the expressed protein ligation (EPL) strategy. Glycopeptide hydrazide segments corresponding to the RBD (319–360) and integrating the requisite *N*/*O*-glycans were chemically synthesized. Subsequent ligation with a re-

combinant *N*-cysteinyl segment, RBD II (361–537), followed by refolding, facilitated the production of the desired glycoproteins. The binding affinity of the synthetic RBDs with ACE2 was assessed through SPR experiments. Results indicate that all synthetic RBDs exhibit a comparable binding affinity to wt-RBD. Moreover, when evaluating the binding of the anti-SARS-CoV-2 RBD monoclonal antibodies (mAbs) CB6 and AS35, the glycosylation pattern appeared to have minimal impact. In contrast, the binding affinities of these RBDs to mAb S309 displayed a distinct SPR profile: the synthetic RBDs lacking core fucose in the N343 glycosylation had significantly reduced affinity (1,000–6,000 fold) compared with wt-RBD.

O-glycan functions can also be probed using chemically prepared samples. By far, the most studied intracellular glycosylation is *O*-GlcNAcylation, a monosaccharide that attaches to Ser/Thr [405]. Several *O*-glycoproteins have been synthesized, including α-synuclein [406], heat shock proteins [407], and high mobility group box 1 [408], all of which were investigated for the roles of their *O*-GlcAcylations (Figure 30f). More recently, in SynGAP, a protein abundant in the postsynaptic density (PSD) of neurons, *O*-GlcNAcylation was found capable of regulating the liquid-liquid phase separation (LLPS) of SynGAP and PSD-95 [409]. Two homogeneously GlcNAcylated SynGAP CC-PBM domains (A1150-W1302) bearing definitive modifications at either S1159 or T1306 were synthesized by EPL. Compared with the non-*O*-GlcNAcylated and the S1159-*O*-GlcNAcylated SynGAP CC-PBM, which form sphere-shaped liquid droplets when mixed with PSD-95 PSG, the T1306-*O*-GlcNAcylated SynGAP CC-PBM could not induce liquid droplet formation. Further experiments confirmed that SynGAP T1306 *O*-GlcNAc could suppress LLPS of SynGAP/PSD-95 in a dominant-negative manner. Therefore, using synthetic glycoproteins, the ability of *O*-GlcNAcylation of SynGAP at T1306 to block its interaction with PSD-95 and, therefore, serve as a suppressor of LLPS of the SynGAP/PSD-95 complex was demonstrated.

Noticeably, atypical protein *O*-glycosylation could also be probed using chemical synthesis, such as the investigation by Li *et al*. [410a] on the lysine *O*-glycosylation of adiponectin collagenous domains. Moreover, in the neuronal protein-derived peptide amyloid β (Aβ), a Tyr-*O*-glycosylation was found to be capable of modulating Aβ aggregation into fibrils. To understand the mechanistic role of this unusual *O*-glycosylation in the aggregation process, three homogeneously modified isoforms of Aβ (1–42) bearing Tyr10 *O*-GalNAc, Galβ1-3GalNAc, and sialyl Galβ1-3GalNAc modifications were chemically synthesized and their fibril-forming abilities compared [410b]. It was discovered that the *O*-glycans significantly affected the aggregation rate of Aβ42, with larger *O*-glycans, slowing fibril formation. The homogeneous glycopeptides also enabled the 3D structures of the Aβ fibrils to be determined. Specifically, cryo-electron microscopy (cryo-EM) structure of fibrils derived from Galβ1-3GalNAc-modified Aβ42 indicates that this *O*-glycosylation redirects Aβ peptide to form a new fibril polymorphic structure that is less stable. Results from various biochemical assays further demonstrated that this Galβ1-3GalNAc modified fibril was more vulnerable to an Aβ-degrading enzyme, insulin-degrading enzyme (IDE).

5.3 Chemical synthesis and biological application of mirror-image proteins

Chemical protein synthesis is the only way to prepare mirror-image proteins, which are composed entirely of D-amino acids instead of the canonical L-amino acids and cannot be produced by recombinant protein expression. The linear sequence of the mirror-image protein is usually assembled through native chemical ligation of multiple peptide fragments and then folded *in vitro* to form the biologically active mirror-image protein. L-proteins composed of L-amino acids are easily degraded by naturally occurring proteases. In contrast, mirror-image proteins are difficult to recognize and degrade by natural proteases. This unique biochemical property makes mirror-image proteins a useful biological tool with applications in drug discovery, protein crystallography, and the development of mirror-image biochemical systems [411,412].

The original studies on mirror-image proteins began with Kent *et al*. who pioneered the chemical synthesis of mirror-image enzymes to develop and explore the biochemical systems based on biomolecules of opposite chirality. As early as 1992, Kent *et al*. [413] accomplished the first synthesis of a biologically active mirror-image enzyme (D-HIV-1 protease), and this research opened the studies on mirror-image biological systems. The linear sequence of D-HIV-1 protease consisting of 99 amino acid residues was directly assembled by Boc SPPS and then folded *in vitro* to generate the bioactive D-HIV-1 protease. Kent *et al*. used this mirror-image protease to show that mirror-image enzyme can only cleave D-peptide ligands, but not the enantiomeric L-peptide ligands, laying the principles for mirror-image biology that subsequent studies have amply demonstrated in various examples. For instance, in 2016 using the method of peptide hydrazide ligation, Liu and Zhu [9] synthesized a mirror-image of African swine fever virus polymer X (ASFV pol X) composed of 174 amino acid residues. D-ASFV pol X was assembled by a three-segment peptide hydrazide ligation strategy in a total separation yield of 1.8%, in which the key peptide thioesters were generated by nitrite oxidation of peptide hydrazides. D-ASFV pol X used L-DNA as a template for mirror genetic replication and transcription, orthogonal to the natural L-ASFV pol X. D-ASFV pol X lacks heat resistance and thus cannot be used

for high-temperature PCR. To address this shortcoming, Klussmann *et al*. [414] prepared D-Dpo4-3C composed of 352 residues by a four-segment peptide hydrazide ligation strategy with a total separation yield of 0.07%. Note that D-Dpo4-3C can be used for standard PCR and has been successfully used to construct a mirror-image gene of the protein Sso7d [415]. Furthermore, Zhu *et al*. recently successfully synthesized high-fidelity mirror-image Pfu DNA polymerase [24a] and T7 RNA polymerase [24b] using the technology of peptide hydrazide ligation and split-protein assembly. In addition, Jiang *et al*. [416] prepared a mirror-image PET hydrolase through five-segment peptide hydrazide ligation, which could effectively degrade plastics such as PET, polybutylene terephthalate, and polybutylene succinate.

Another use for mirror-image proteins is mirror-image screening to discover D-peptide ligands as drug candidates. L-peptides composed of L-amino acids are easily degraded by proteases *in vivo*, have poor plasma stability, are usually unsuitable for oral administration, and can easily activate the human immune system, leading to an immune response. In contrast, D-peptides or proteins are barely recognized by proteases *in vivo*, have high plasma stability, and do not elicit an immune response. To accelerate the discovery of D-peptide ligands, Kim *et al*. [417] proposed a mirror-image phage display system using chemically prepared D-Src-SH3, the mirror image of the Src kinase domain SH3, which they used to discover a D-cyclic peptide ligand capable of binding Src-SH3 with a K_D value of 63 μM.

Such early mirror-image phage display studies were generally restricted to small proteins due to the difficulties of accessing large D-proteins. More recently, however, the emergence of native chemical ligation has greatly expanded the technique. In 2012, Kent *et al*. [418] prepared D-VEGF *via* a three-segment native chemical ligation and oxidative folding, which they then used to discover a D-peptide ligand, RFX001, capable of binding L-VEGF with a K_D value of 85 nM. Similarly, Liu and Gao prepared $^{D}IgV^{PD\text{-}L1}$ consisting of 124 residues [185] and $^{D}IgV^{TIGIT}$ consisting of 111 residues [184] by a four-segment peptide hydrazide strategy. Through mirror-image phage display, they found a ^{D}PPA-1 with a K_D value of 0.51 μM to hPD-L1 and ^{D}TBP-1 with a K_D value of 2.79 μM to hTIGIT. Finally, a mirror-image protein-based information barcoding and storage technology wherein D-amino acids are used to encode information into mirror-image proteins that are chemically synthesized has also been developed [419].

5.4 Development and application of cell-permeable ubiquitin (Ub) probes for the analysis of Ub-related enzyme activity in live cells

Ubiquitination is a highly conserved post-translational modification in which the 76-amino-acid protein ubiquitin (Ub) is covalently attached to the lysine side-chain amino group of the substrate protein by E1-E2-E3 enzymes (Figure 31a) [420,421]. Deubiquitinating enzymes (DUBs) reverse this process by removing Ub or polyUb chains from substrate proteins, and regulating various biological processes [422–424]. DUB and E1-E2-E3 enzymes regulate almost all cellular processes in eukaryotes, and dysregulation of their activity can lead to various diseases. They are potential targets for drug development, especially in autoimmune disorders and cancer. Ub-based chemical probes have been developed to monitor DUB and E1-E2-E3 activity, most of which comprise a Ub conjugate module [425], reporting group and warhead. The Ub conjugate module of the probe is recognized by the enzymes, the warhead covalently captures the active cystine, and the reporter group is for the identification of substrate [426–429]. These probes, which can be constructed in a chemically flexible and atomically tailored manner, have been widely used in *in vitro* settings, but their application requires lysis of cells, which typically leads to dilution of the cytoplasmic and nuclear proteins, leading to potential dissociation of protein complexes necessary for Ub-related enzymatic activity [430–435]. To address this problem, the most modern probes use protein cell delivery vehicles such as cell-penetrating peptide (CPP) and phase-separating peptide to analyze Ub-related enzymes in live cells (Figure 31b, c) [436–446].

Zhuang *et al*. [447] developed the first membrane-penetrating Ub probes by coupling a CPP containing TAT sequence, a widely used cationic transmembrane peptide derived from the trans activator of transcription protein of human immunodeficiency virus, or cyclic polyarginine (cR10), an engineered cell-penetrating peptide with cyclic polyarginine structure, to the N-terminal of Ub-propargylamide (Ub-PA) or Ub-vinyl methyl ester (Ub-VME) probes. The cR10-modified probe exhibited enhanced intracellular delivery efficiency. Subsequently, the authors showed, using chemical proteomics techniques, that the cR10 probe captured nearly three times as much DUB in live cells as in lysates.

To further analyze the activity of Ub-related enzymes in specific cellular processes, probes capable of penetrating live cells and being activated on-demand have been developed. Brik *et al*. [448] reported a Thz-protected Ubv2.3-aldehyde probe incapable of labeling DUBs *in vitro* until Pd-mediated cleavage of Thz. The authors found that the activated Ubv2.3 probe inhibited the enzymatic activity of USP2a, promoting the degradation of USP2a's substrate MDM2. Zhuang *et al*. [449] developed another class of caged Ub probes that could be activated by UV irradiation. A tetrazole-derived warhead and cR10 were installed onto the C and N termini of Ub, respectively. Upon activation by 365 nm UV light, the inert tetrazole group was converted to nitrilimine intermediate,

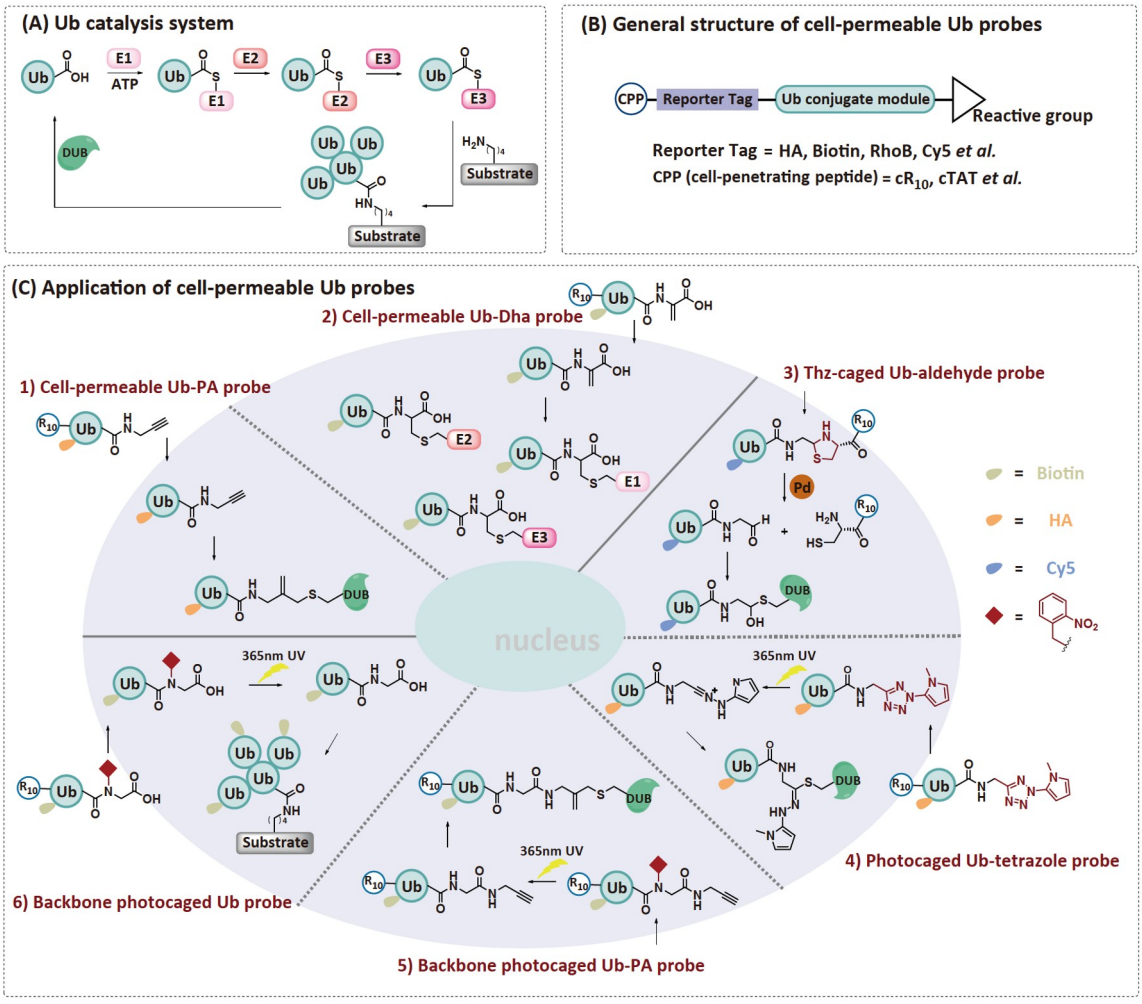

Figure 31 (a) Ub catalysis system, actions of E1, E2, E3 and DUB. (b) The main components of cell-permeable Ub probes. (c) Application of cell-permeable Ub probes. 1 and 2 are cell-permeable Ub probes, 3-6 are on-demand activation Ub probes (color online).

which then captured the active cysteine of DUBs. Compared with no UV treatment, 15 DUBs were significantly enriched in live cells after activation. The authors also used this probe to study DUB activity in different phases of the cell cycle.

The above on-demand activation probes enable the time-resolved monitoring of DUB activity through masking activity of the warhead, but more widely used probes such as Ub-PA and diUb-Dha are difficult to temporarily mask using this strategy. To solve the problem, Li *et al*. [450] temporarily blocked the activity of the probe by replacing the glycine at position 75 of Ub with sterically bulky 2-nitrobenzylglycine (Nbg) to impede the entry of Ub into the active pocket of DUBs. A cell-permeable photocaged Ub-PA synthesized based on this backbone modification strategy capable of labeling DUBs *in vitro* only after activation by 365 nm UV light was used in conjunction with chemical proteomics techniques to analyze fluctuations in DUB activity in live cells under oxidative stress. DUB activity was found to be reversibly deactivated under these conditions. To further demonstrate the versatility of the strategy, the authors synthesized a photocaged K48/63 diUb-Dha probe and achieved Ub chain-specific DUBs labeling in live cells. Finally, Li *et al*. [451] also prepared cell-permeable photocaged SUMO2-PA probes to monitor the activity of Sentrin-specific proteases in real time.

In contrast to deubiquitination, ubiquitination involves several enzymes and therefore, the temporary masking of newly occurring ubiquitination events in living cells is difficult. Li *et al*. [452] prepared cell-permeable photocaged Ub to screen newly occurred ubiquitination events using a backbone modification strategy. The authors loaded a 2-nitrobenzyl group on the amide backbone of the Ub C-terminus 75 or 76 glycine by chemical synthesis, such that the Ub variant could be loaded onto substrates by E1-E2-E3 only after light activation. Fluorescence imaging and immunoblotting showed that the cell-permeable photocaged Ub could be activated in live cells. The authors then used the probe to investigate levels of newly ubiquitinated substrates

in cells under oxidative stress; 264 proteins, mainly those involved in RNA splicing, RNA/protein translocation, and protein translation, were found to be significantly enriched. These studies show that the backbone modification strategy can be used to synthesize probes capable of the time-resolved profiling of DUBs and ubiquitinated substrates in different cellular states.

5.5 Development of adjuvant-protein conjugate as a potent protein-based vaccine

Protein-based conjugates have witnessed broad applications in biomedical research and pharmaceutical developments, exemplified by innovations like antibody-drug conjugates and various lysosome-targeting protein degraders [453,454], extensively reviewed elsewhere [455,456]. In this review, constrained by length considerations, our discussion is specifically centered on protein-based vaccines.

Vaccines incorporating protein subunits as antigens have attracted attention due to their favorable safety profiles and the relative ease with which they can be produced, stored and transported [457]. However, the weak immunogenicity of protein antigens is challenging and can necessitate the use of an adjuvant to enhance the immune response (Figure 32a) [458–460]. Adjuvants used in protein vaccines include aluminum-containing adjuvants [461], toll-like receptor (TLRs) agonists [462], stimulator of interferon genes (STING) agonists [463], and invariant natural killer T (iNKT) cell agonists [464]. These adjuvants act as "danger signals" that can effectively activate the innate immune system and greatly enhance the immunogenicity of antigen proteins [465,466]. Nevertheless, the co-administration of adjuvants and proteins as a physical mixture might cause adjuvant dispersion from the injection site.

The covalent conjugation of adjuvants and antigens is a highly promising strategy for developing vaccines that effectively promote targeted immune responses against specific antigens without additional safety concerns [467,468]. Advantages associated with such conjugate vaccines include chemical controllability, reproducibility, uniform composition, and the co-delivery of antigen and immunostimulant to the same antigen-presenting cell (APCs), effectively activating APC-mediated antigen presentation [442]. Li *et al*. [469] prepared a construct with covalently conjugated iNKT cell agonist αGalCer and MUC1 antigen, demonstrating a significantly enhanced immunogenicity of the vaccine. Zhao *et al*. [470] coupled the TLR2/6 agonist fibroblast stimulating lipopeptide 1 (FSL-1) to the antigen, inducing a robust antigen-specific immune response. This strategy is particularly effective for amphiphilic or hydrophobic adjuvants, such as the iNKT cell agonist αGalCer [444]. The amphiphilic structure of the adjuvant-antigen conjugate can effectively promote the self-assembly of antigen and adjuvant, improve the transmembrane capacity of adjuvant, and enhance antigen uptake by APCs [442,443]. For hydrophilic adjuvants that need to function intracellularly, such as the STING agonist cyclic di-GMP [471], the high water solubility of the resulting adjuvant-antigen conjugates hinders their transmembrane transport and functionalization.

Three strategies to construct adjuvant-protein conjugates are in current use: (1) random coupling based on protein residues; (2) site-specific conjugation; and (3) conjugation based on glycans on glycoproteins. In the random coupling strategy (Figure 32b), covalent bonds are formed directly

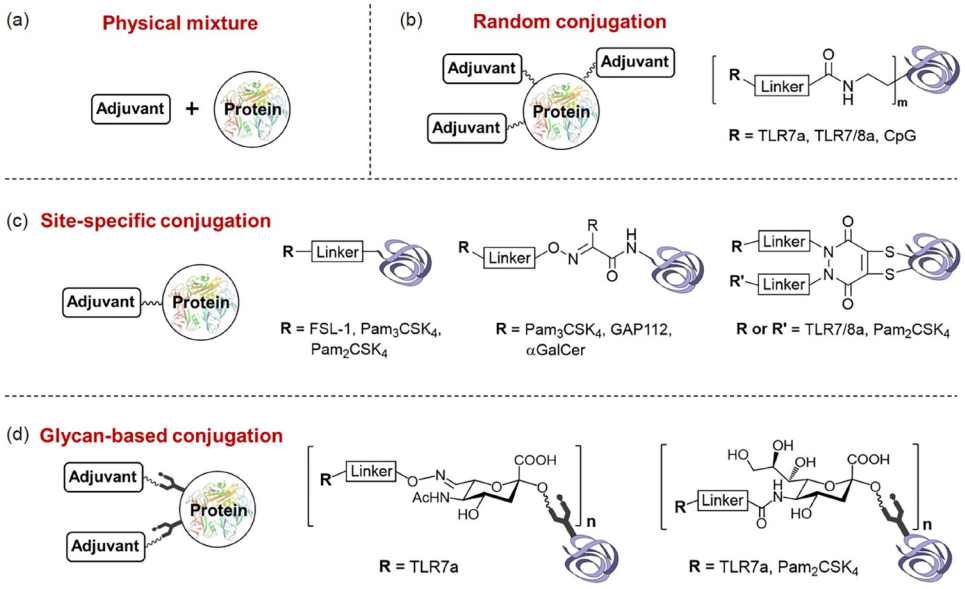

Figure 32 Protein vaccine design and adjuvant-protein conjugating strategies. (a) Conventional protein vaccine with add-mixed adjuvant. (b) Random conjugation of adjuvant to the protein side chain, represented by lysine residue. (c) Site-specific conjugation of adjuvant to protein terminus and disulfide bond. (d) Glycan-based conjugation of adjuvant to sialic acid of glycoprotein (color online).

between the adjuvant and certain reactive amino acid side chains (such as lysine amino groups) of the protein [472]. For instance, Guo *et al*. [473] conjugated a TLR7 agonist to lysine residues on the SARS-CoV-2 Spike S1 protein *via* an *N*-hydroxysuccinimide ester strategy to give a vaccine that elicited robust humoral and cellular immune responses, and high-titer neutralizing antibodies against SARS-CoV-2 and its variants of concern (VOCs). This adjuvant-protein construction strategy has also been applied to carrier proteins to enhance the immunogenicity of haptens [474,475].

The site-specific conjugation strategy can address the deficiencies due to random conjugation (Figure 32c). Moyle *et al*. [476,477] used the EPL strategy to attach three distinct TLR2 ligands onto recombinant protein antigens *via* enzymes, leading to a potent antigen-specific immune response. Payne *et al*. [478] covalently coupled immune adjuvants to the terminus of a synthetic peptide, thereby eliciting a potent immune response [188]. Utilizing a pyridoxal 5′-phosphate mediated transamination reaction to efficiently introduce built-in adjuvant into specific sites [479], Guo *et al*. conjugated TLR1/2 agonist Pam3CSK4 [480], TLR4 agonist GAP112 [481], and iNKT cell agonist αGalCer [482] to the *N*-terminus of the SARS-CoV-2 spike RBD protein, respectively. In addition, Boons *et al*. [483] developed an approach for the site-selective derivatization of the protein based on the selective reduction of a disulfide bridge of protein followed by a reaction with functionalized dibromopyridazinediones to form adjuvant-protein conjugate.

Conjugating the adjuvant to the protein *via* its glycan moieties is another method (Figure 32d). Guo *et al*. [484] reported a vaccine platform that involves the covalent linkage of adjuvants to sialic acid residues on the glycan chains of glycoproteins. The aldehyde structures generated by periodate oxidation of the *N*-glycans on the receptor-binding domain (RBD) could enhance antigen uptake by antigen-presenting cells (APCs) *via* scavenger receptors [485]. Furthermore, Boons *et al*. [486] developed a conjugation method to covalently link TLR7/8 agonists to *N*-glycans located on viral glycoproteins.

5.6 Applications of synthetic ubiquitin-modified nucleosomes

Chromatin is the complex of DNA and protein responsible for integrating genetic information and maintaining genome stability in eukaryotes [487–489]. The primary protein components of chromatin are four types of histones (H2A, H2B, H3, and H4) and the linker histone (H1), which undergo dynamic decoration with a multitude of post-translational modifications, such as acetylation, methylation, phosphorylation, and ubiquitylation. Notably, ubiquitylation stands out as a particularly intriguing PTM due to its larger size and increased complexity compared with the other modifications. Histone ubiquitination modification can serve as a signal integration platform, mediating different downstream biochemical events by recruiting different effector proteins [490–492]. For example, K13 or K15 ubiquitination of histone H2A, namely H2AK13/15Ub, can recruit BRCA1/BARD1 and 53BP1 in response to DNA double-strand damage repair, initiate the DNA repair process and maintain genome stability [493,494]; H2AK119Ub can recruit the polycomb repressive complex PRC2 to mediate transcription repression and gene silencing processes [495,496]; H2AK125/127/129Ub can recruit the chromatin remodeling enzyme SMARCAD1 to promote DNA damage repair by homologous recombination [497]; H2BK120Ub can recruit the methyltransferase COMPASS complex and Dot1L and activate its effects on methylation of H3K4 and H3K79, thereby activating transcription [498,499]. One of the main difficulties in studying post-translationally modified nucleosomes is the acquisition of pure samples having a precise structure [500–502]. Traditional protein expression technology is poorly suited to the synthesis of site-specific modified proteins, and chemoenzymatic methods are often limited by difficulties in reassembling enzyme complexes [503–505].

A hallmark of epigenetic modifications, H2B monoubiquitylation (H2Bub; modified on K123 in yeast or K120 in mammals), plays essential roles in regulating assorted nuclear processes, including gene transcriptional activation, higher-order chromatin organization, and the DNA damage response. Cellular levels of H2Bub are precisely and dynamically regulated and depend on a balance in the activities of ubiquitin (Ub) installation by Ub-modifying enzymes (Bre1-Rad6 [506] in yeast or RNF20/RNF40-hRAD6A [507] in humans), and its removal by deubiquitylating enzymes (Ubp8 and Ubp10 in yeast [508], and USP22 [509,510] in mammals). Morgan *et al*. [511] used a semi-synthetic strategy to obtain a Xenopus laevis nucleosome core particle (NCP) containing two copies of H2B with ubiquitin attached to K120 of H2B *via* a nonhydrolyzable dichloroacetone (DCA) linkage and study the catalytic mechanism of Spt-Ada-Gcn5 acetyltransferase (SAGA) deubiquitinating module containing Ubp8. The crystal complex structure of a nucleosome with H2BK120Ub bound by the SAGA revealed the mechanism by which it excises H2Bub by recognizing the H2A-H2B acidic patch, H2B C-terminal helix, and Ub in the nucleosome core particle (NCP).

Nonetheless, the elucidation of the structural mechanism for the site-specific ubiquitylation on H2B has long been hindered by the challenges associated with the capture of the transient and dynamic protein complexes formed during this unique ubiquitylation event. Deng *et al*. [512,513] developed a chemical trapping strategy integrating mechanism-inspired conjugation and split-intein splicing technology and successfully captured the transient cryo-EM structures of Bre1-

or RNF20/RNF40-mediated Ub transfer from Rad6 or hRAD6A to nucleosomal H2B. This work revealed a critical role of nucleosomal DNA in mediating E3 ligase recognition for H2Bub and established a framework for understanding the oncogenic mutations of human RNF20/RNF40 (Figure 33).

H2BK120 monoubiquitination (H2BK120ub1) is a prerequisite for the efficient methylation of H3K79 by the unique non-SET domain-containing histone methyltransferase DOT1L *in vitro*. The essential histone H3 lysine 79 methyltransferase Dot1L regulates transcription and genomic stability and is deregulated in leukemia, but the mechanism by which Dot1L is stimulated by H2BK120Ub is not understood. Chatterjee *et al*. [514] developed a synthetic route that directly attaches the Ub protein onto the H2B proteins with a disulfide linkage (H2BssUb, ss represents disulfide linkage). The expressed full-length Ub was modified with a C-terminal aminoethanethiol linker, which could join H2B with K120C mutation through a disulfide linkage. The same strategy was used to prepare the NCP with H2BK120Ub (UbNCP) and yield the cryo-EM structure of the DOT1L-UbNCP complex which revealed the crosstalk mechanism between H2BK120Ub H3K79 methylation [515,516]. Further replacing H3K79 with norleucine to trap DOT1L in the active state, Worden *et al*. [515] reported the cyro-EM structure of DOT1L revealed that DOT1L and H4 tail induce conformation change of H3 and make H3K79 accessible to DOT1L. Xue *et al*. [517] also used DCA to obtain a cryo-EM structure of H2BK120Ub and reported that H2BK120Ub promoted H3K4 methylation through complex formation with MLL proteins and their regulatory factors (WDR5, RBBP5, ASH2L and DPY30).

Histone PTMs like H2BK34Ub can also stimulate DOT1L. Chu *et al*. [518] developed two strategies to prepare the H2BK34Ub and independently obtained the rough cryo-EM structure of H2BK34Ub-NCP. Ai *et al*. [519] further optimized the synthesis strategy with lysyl auxiliary-mediated site-specific ubiquitination to prepare natural H2BK34Ub and H2BK120Ub more efficiently. The authors reconstituted the DOT1L stimulation effect of both H2BK34Ub-NCP and H2BK120Ub-NCP. Further cryo-EM structure studies showed that H2BK34Ub-NCP induces the DOT1L orientation closer to the H3K79, which is similar to the mechanism of H2BK120Ub. An *in vitro* assay comparing H2BK34Ac-NCP, H2BK34Sumo-NCP and H2BK34-aeea-Ub-NCP (aeea, 2-(2-aminoethylamino)ethanol) showed the ubiquitin fold and linking mode are important for the DOT1L activation effect.

5.7 Revealing the mechanism of the modulation of membrane proteins using synthetic peptide ligands

Most naturally occurring peptides target the proteins located on the cell surface, including ion channels, transporters, and receptors. Venomous creatures deploy a wide array of toxic peptides active against a range of targets for prey capture or defense; most of the toxic peptides are disulfide-rich polypeptides composed of 10–80 amino acid residues that adopt highly ordered 3D structures and potently modulate the activity of specific classes of cell membrane targets. A variety of disulfide-rich peptide toxins with different structure scaffolds have been prepared by chemical synthesis and used to study the mechanism by which ion channels are modulated. Combined with technologies such as cryo-EM, a variety of channels complexed with peptide toxins have been determined (Figure 34). Sun *et al*. [520,521] determined the cryo-EM structure of human acid-sensing ion channel subtype 1a (ASIC1a) complexed with the synthetic snake toxin mambalgin-1, revealing the inhibited conformation of hASIC1a upon toxin binding [522]. Moreover, mabaligin-1 incorporated with 19F-labeled L-4-trifluoromethyl-phenylalanine (19F-tfmF) was synthesized and used for structural and physiological studies, revealing that mambalgin-1 preferentially binds ASIC1a in a closed state and inhibits the channel through a closed-state trapping mechanism, causing an analgesic effect. Additionally, μ-conotoxin KIIIA bound

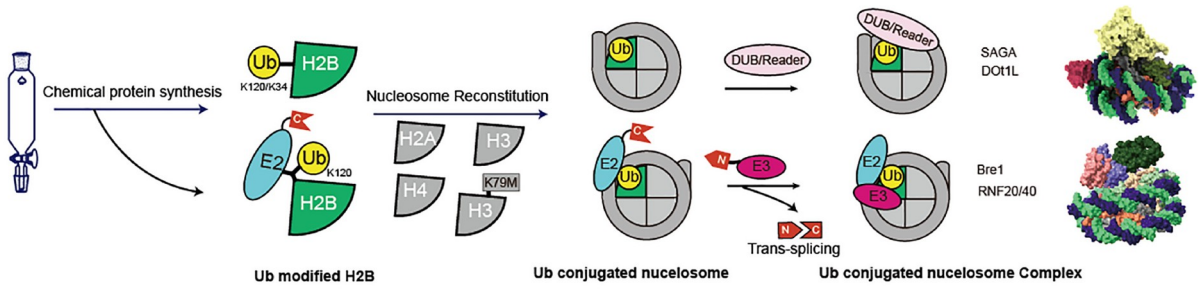

Figure 33 Synthetic ubiquitinated nucleosomes enable the elucidation of mechanistic insights into nucleosome ubiquitination or deubiquitination. Site-specific ubiquitination adding, reading, or removing processes on nucleosome play critical roles in epigenetic regulation. However, studying the molecular mechanisms of these processes is challenging because it is difficult to capture complexes of ubiquitinated nucleosomes having precise structures. Chemical protein synthesis strategies have resulted in precise structures of ubiquitinated histones, facilitating the generation of ubiquitin-conjugated nucleosome complexes and elucidation of the final complex structures (color online).

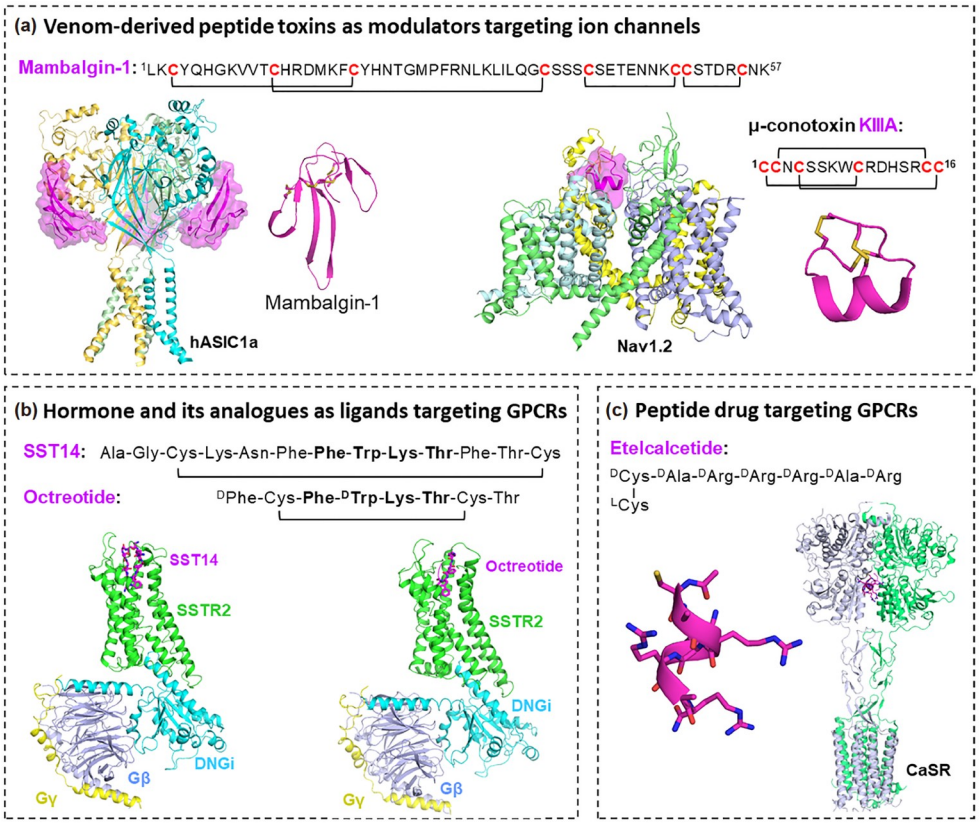

Figure 34 Representative structures of membrane proteins resolved using synthetic peptides as modulators or ligands. The sequences of peptides are indicated. The structures of peptide ligands are shown in magenta. (a) Disulfide bond rich peptides derived from venom and used to study the structure and function of ion channels: snake toxin mambalgin1 targeting human acid-sensing ion channel subtype 1a (hASIC1a) (PDB: 7CFT) [521]. Cone snail toxin KIIIA targeting voltage-gated sodium channel 1.2 (Nav1.2) (PDB: 6J8E) [523]. (b) Ribbon diagram representation of the cryo-EM structures of SST14 and octreotide bound to somatostatin receptor 2 (SSTR2) (PDB: 7XAT, 7XAU). (c) Cryo-EM structure of class C GPCR calcium-sensing receptor (CaSR) complexed with peptide drug etelcalcetide (PDB: 7M3J) (color online).

to voltage-gated sodium channel 1.2 (Nav1.2) [523], spider toxin ProTx-II bound to Nav1.7 [524], DkTx bound to transient receptor potential cation channel subfamily V member 1 (TRPV1) [525] have also been reported. Gao *et al.* [526] revealed the molecular basis for the specific pore blockage of ziconotide by solving the structure of the U.S. Food and Drug Administration (FDA)-approved, venom-derived painkiller bound to its target human Cav2.2 channel.

More than a hundred G protein-coupled receptors (GPCRs) activated by endogenous peptide ligands have been characterized in humans and are recognized as drug targets [527], and the structures of several peptide ligands bound to GPCRs have been solved. The endogenous cyclic peptide SST14 was reported to stimulate somatostatin receptors (SSTRs) for hormone release, cell growth arrest and cancer suppression. Bo *et al.* [528] determined the cryo-EM structures of human SSTR2 bound with SST14 and its short cyclic SST analogues (octreotide or lanreotide), revealing that interactions between β-turn residues in SST analogues and transmembrane SSTR2 residues in the ligand-binding pocket are crucial for receptor binding and functional stimulation of the two SST14-derived cyclic octapeptides.

Tachykinins are a family of neuropeptides distributed in the mammalian nervous system. The tachykinin peptides substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) share a conserved C-terminal motif. NKA preferentially activates NK2R, while SP and NKB preferentially bind the tachykinin receptors NK1R and NK3R, respectively. Sun *et al.* [529,530] determined the cryo-EM structures of the NK3R bound to NKB, SP and senktide, as well as NK2R bound to NKA. Gao *et al.* [531] reported the structures of active-state calcium-sensing receptor (CaSR) in complex with clinically used peptide drug etelcalcetide, an octapeptide composed of D-amino acids, providing a detailed structural framework for CaSR activation and the rational design of therapeutics targeting this receptor. Representative structural studies of peptide ligands and GPCR also include neurotensin at the NTS1R [532]; synthetic endorphin-derivatives at the μ-opioid receptors [533]; glucagon and glucagon-like peptide 1 (GLP-1) analogues at the glucagon receptor [534,535] and GLP1R [536,537], respectively; parathyroid hormone at the PTH1R [538]; and thyrotropin-releasing hormone (TRH) and its analogue taltirelin at thyrotropin-releasing hormone receptor [539].

6 Conclusions and perspectives

To summarize, synthetic organic chemistry enables access to proteins that are not attainable through biology. Chemical synthesis of proteins tests the limits of what can be rationally synthesized by modern chemistry and how we can develop biochemical systems that go beyond the biological limits. Using the methods developed over the past years, we can now chemically synthesize proteins containing up to 500 amino acids in single-chain protein molecules and even larger proteins containing over 1,000 amino acids in multi-component protein complexes. The ability of chemical protein synthesis to engineer proteins with unprecedented precision and diversity sets the stage for researchers to unravel the mysteries of biology and design solutions for pressing challenges in medicine, biotechnology, and beyond. Although much progress has been made, an infinite number of massively complex proteins having many unknown functions remain to be explored, calling for even more innovative strategies. Moreover, while chemical protein synthesis plays a vital role in elucidating protein functions and interactions and has already aided in the unraveling of intricate cellular mechanisms, the extent to which synthetic proteins *in vitro* can represent their native forms in a live organism remains an open question—the answer to which may require a new perspective and even a new research paradigm, such as chemical protein synthesis in living cells. Finally, with the potential to create proteins that push the boundaries of natural diversity, chemical protein synthesis should play more significant roles in drug discovery and development, creating therapeutic proteins with tailored properties, enhancing efficacy, and minimizing side effects. Overall, the integration of chemically synthesized proteins into biomedical research will foster new frontiers in drug discovery, diagnostics, and therapeutic interventions, stimulate new discoveries and development from bioorganic chemistry and chemical biology, and demonstrate the importance, power, and value of synthetic chemistry in solving cutting-edge problems in molecular biology and biomedicine.

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Conflict of interest The authors declare no conflict of interest.

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