Review

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Probing protein function by chemical modification‡

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Labeling proteins with synthetic probes, such as fluorophores, affinity tags, and other functional labels is enormously useful for characterizing protein function *in vitro*, in live cells, or in whole organisms. Recent advancements of chemical methods have substantially expanded the tools that are applicable to modify proteins. In this review, we discuss some important chemical methods for site-specific protein modification and highlight the application of established techniques to tackle biological questions.

Keywords: protein labeling; fluorescent proteins; chemical probes; native chemical ligation; expressed protein ligation; protein *trans*-splicing; chemoselective reactions; bioorthogonal reactions; *in vivo* chemical labeling; Rab GTPases; prenylation; post-translational modification

Introduction

Studying protein function *in vitro* or in the context of live cells and organisms is of vital importance in biological research. Genetic tags such as fluorescent proteins (FPs) are widely used to detect proteins. The 2008 Nobel Prize in Chemistry rewarded the discovery and use of GFP as a tagging tool in biological science. However, compared to chemical tags, FPs have several limitations. First, FPs are generally not sensitive to environmental parameters such as hydrophobicity, pH, and ion concentrations, because the chromophore is buried in a barrel structure [1]. Second, although different variants of FP are available through mutagenesis, they cannot compete with organic dyes in the flexibility of modification and spectral range [2]. Moreover, FPs have disadvantages in brightness and photostability and are therefore are not ideal for most single molecule studies. Third, FPs can only provide an optical read-out, whereas other detection modalities such as spin labels for electron paramagnetic resonance (EPR) spectroscopy have unique properties and can be used to monitor conformational change of proteins [3]. Fourth, genetic reporters are less amenable for temporal control. In living systems, the proteins are produced on the timescale of gene expression and mature gradually, presenting a disadvantage for monitoring rapid intracellular biochemical events. In contrast, protein activity can be precisely controlled by light using photocleavable chemical probes [4]. Finally, genetic tags cannot be used to label nucleic acids, glycans, lipids, and protein post-translational modifications. In contrast, chemical probes are able to achieve properties that are not readily possible when using fluorescent proteins, such as fluorophore-assisted light inactivation, real-time detection of protein synthesis, and multi-color pulse-chase labeling [5]. Many organic dyes are superior to fluorescent proteins in terms of brightness, photostability, far red-emission, environmental sensitivity, and potential for modifications to their spectral and biochemical properties. Moreover, chemical modification is widely used for protein immobilization on microarrays [6]. Altogether, chemical modification of proteins has become an important strategy for the study of protein function. The emerging chemical labeling techniques have significantly broadened the range of manipulating protein structures. Here, we summarize the advancement of chemical labeling methods and their application in biological research.

Native Chemical Ligation and Expressed Protein Ligation

Synthetic chemistry provides almost unlimited possibilities for modulation of the structure of a polypeptide chain in order to understand protein function. However, as proteins are large molecules, total chemical synthesis of proteins is a considerable challenge [7]. In the early 1990s, Kent and coworkers introduced the breakthrough approach of native chemical ligation (NCL), which is now a general method for chemical protein synthesis [8]. In this method, two unprotected synthetic peptide fragments are coupled together under neutral aqueous conditions with the formation of a native peptide bond at the ligation site.

The principle of NCL is depicted in Figure 1. The approach is based on the chemoselective reaction between a peptide containing a *C*-terminal thioester and another peptide containing an *N*-terminal cysteine. The initial chemoselective transthioesterification in NCL is essentially reversible, whereas the subsequent S → N acyl shift is spontaneous and irreversible. Thus, the reaction is driven to form an amide bond specifically at the ligation site, even in the presence of unprotected internal cysteine residues. NCL

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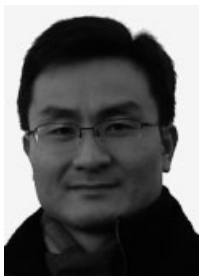
Biography

Roger Goody was born in 1944 in Northampton, England and studied at the University of Birmingham, England. He obtained his BSc in chemistry in 1965 and his PhD in 1968. After two postdoctoral positions, he completed his habilitation in biochemistry/biophysics at the University of Heidelberg, Germany and was nominated professor in 1990. Since 1993, he has been Director at the MPI of Molecular Physiology, Dortmund, Germany. In 2004, he became Professor of Biochemistry at the Ruhr-University, Bochum, Germany. His interests are in the area of phosphate-transferring proteins.



Yao-Wen Wu was born in 1980 in China. He received his BS in Chemistry from Sun Yat-sen University in 2001 and his MS in Organic Chemistry from Tsinghua University in 2004. After graduating as Dr rer. nat. (2008) at the Technical University of Dortmund working at the Max Planck Institute of Molecular Physiology in Dortmund and a postdoctoral period in cell biology at King's College London, he was appointed leader of an Otto Hahn group at the Max Planck Institute in Dortmund. His research interests are in chemical biology and biochemistry of membrane trafficking.

permits connecting two or more synthetic peptides to introduce functional tags into proteins. A number of refinements and extension in ligation methodology and strategy have been developed, such as extension of ligation site other than cysteine, auxiliary group-facilitated ligation [9,10], catalytic thiol cofactors [11,12], and kinetically controlled ligation (KCL) [13,14] (for a recent review see Ref. 15).

The scope of application of NCL was significantly widened upon introduction of the approach referred to as expressed protein ligation (EPL) from the Muir laboratory [16]. In this method, both fragments containing *C*-terminal thioester and *N*-terminal cysteine, respectively, can be produced recombinantly. Expressed protein ligation (EPL) emerged as a result of the advances in self-cleavable affinity tags for recombinant protein purification using intein chemistry [17]. Inteins are protein insertion sequences flanked by host protein sequences (*N*- and *C*-exteins) and are eventually removed by a post-translational process termed protein splicing [18]. Mutated inteins containing a *C*-terminal Asn to Ala substitution have been designed to keep their ability in the initial N $\rightarrow$ S acyl shift without further going through later steps of protein splicing [19]. Therefore, proteins fused to the *N*-termini of these engineered inteins can be cleaved by thiol reagents (such as 2-mercaptoethanesulfonate, MESNA) via an intermolecular transthioesterification reaction, releasing the α-thioester-tagged proteins (Figure 2).

The recombinant polypeptide α-thioesters can then be ligated with a synthetic peptide or recombinant protein containing *N*-terminal cysteine. EPL thereby allows site-specific introduction of probes (such as fluorophores and isotopes), post-translational modifications (such as prenylation, glycosylation, phosphorylation, and ubiquitination), incorporation of unnatural amino acids, and immobilization of proteins or peptides onto a chip (reviewed in Ref. 20). Moreover, sequential EPL strategies make it possible to introduce modification at any position in the protein sequence [21,22]. Therefore, EPL provides a platform that allows the application of powerful synthetic chemistry tools to proteins.

Recent examples of using EPL include the preparation of fluorescent prenylated Rab GTPases, key proteins involved in organizing intracellular vesicular transport. Rab GTPases are post-translationally modified by (usually) two geranylgeranyl groups at their *C*-terminus, which enables them to associate with membranes. Earlier, there were insurmountable difficulties in recombinant preparation of prenylated proteins and significant challenges in obtaining prenylated Rab GTPases in defined nucleotide-bound states. Therefore, it was technically difficult to analyze the interaction between GDP/GTP-bound prenylated Rab and its regulators Rab escort protein (REP) and GDP dissociation inhibitor (GDI). The EPL technique allowed the production of fluorescent prenylated Rab proteins by ligating synthetic prenylated peptides containing a fluorophore on the lipid or on the side chains of amino acids with recombinant Rab thioesters [23,24] (Figure 3). Dansyl and NBD fluorophores were introduced as reporters. This approach also enabled precise installation of GDP/GTP(or analog GppNHp) into Rab proteins to generate the 'off' and 'on' states, yielding homogeneous preparations of functionalized prenylated proteins in well-defined nucleotide-bound states, which was previously unfeasible. Such semi-synthetic Rab protein probes displayed significant changes in fluorescence intensity upon binding to REP and GDI, therefore enabling quantitative analysis of the interactions. These analyses led to the establishment of a thermodynamic model of Rab membrane recycling and a novel model of guanine nucleotide exchange factors (GEFs)-mediated Rab membrane targeting [25,26].

Inteins can also be split into two parts that function only when they interact with each other [27,28]. The precursor fragments can be fused to parts of a split intein, so that when these two pieces interact with each other, the resulting intein activity can mediate a *trans*-splicing reaction (Figure 4). The discovery of the naturally occurring split *Synechocystis (Ssp)* DnaE intein makes it possible to bypass denaturation and renaturation of the isolated precursor fragments. Protein *trans*-splicing is a useful approach to selectively ligate two polypeptides, thereby providing a valuable tool for protein engineering [29,30]. In particular, the split *Ssp* DnaB Int^N fragment with a length as short as 11 amino acids was capable of protein *trans*-splicing, which significantly facilitates the preparation of the modified *N*-terminal fragment by solid-phase peptide synthesis (SPPS) [29,31]. Protein *trans*-splicing has been employed to generate head-to-tail cyclic peptides and proteins [32,33], to selectively incorporate isotopes for NMR analysis [34,35], to fluorescently label proteins *in vitro* or in live cells [36,37], to assay protein–protein interaction *in vivo* [38–41], to detect biological events *in vivo* [42–44], and to selectively immobilize proteins onto a chip [45].

Chemoselective Chemistry

In general, the genetic code specifies 20 standard amino acids for proteins. Some of the residues are amenable for chemoselective modification under physiological conditions, for example,

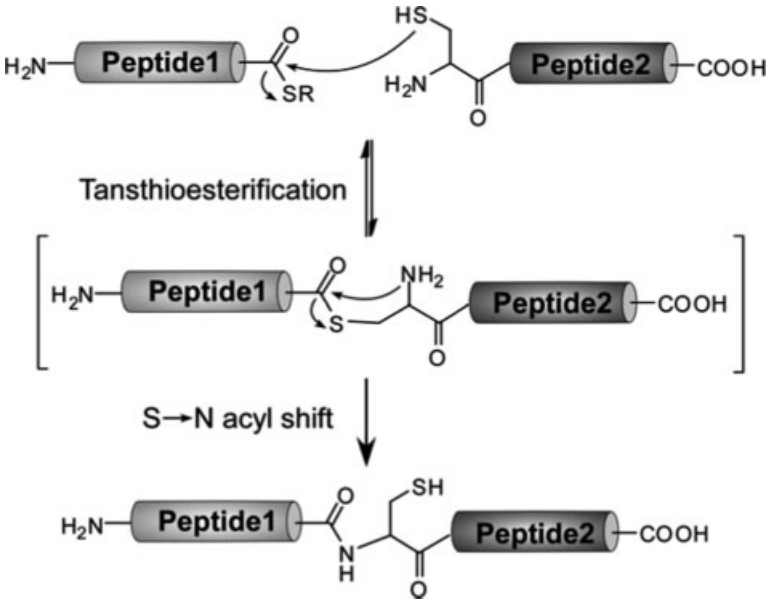


Figure 1. Principle of native chemical ligation.

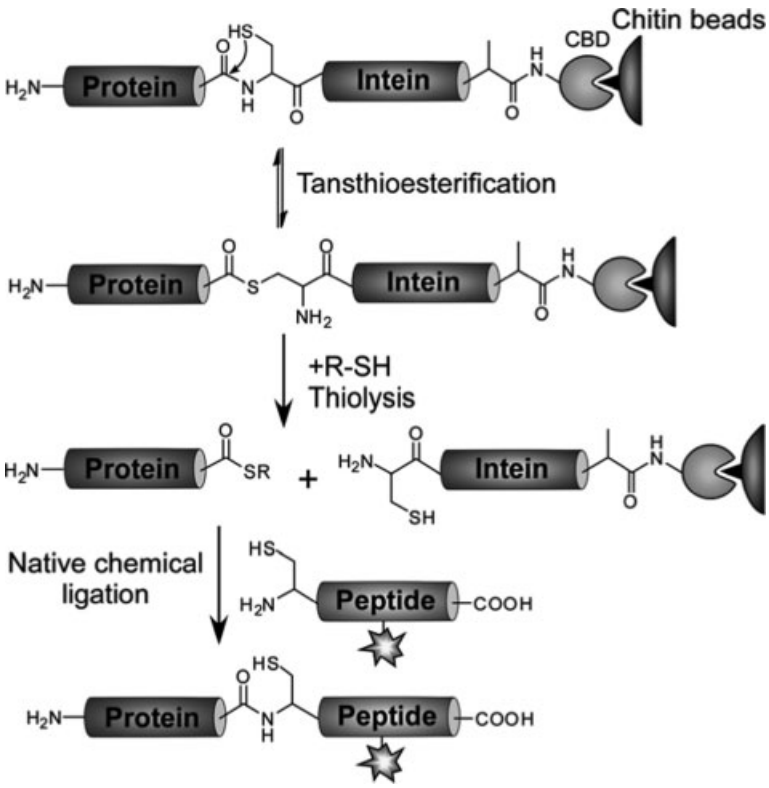


Figure 2. Principle of expressed protein ligation.

the sulfhydryl reagents iodoacetamide and maleimide modify cysteine, and succinimidyl esters react with lysine side chains or *N*-terminal primary amines. Such residue-specific reactions are widely used in tissue immunostaining via the conjugation of organic dyes to antibodies. Recent progress in bioconjugation techniques has expanded the range of modification residues to include tryptophan and tyrosine [46–49]. However, these residue-specific bioconjugation approaches are not selective when the object of study is biological systems instead of an individual protein.

Bioorthogonal chemistry allows specific, covalent attachment of chemical tags to biomolecules, without displaying cross-reactivity with other biomolecules *in vivo*. The term 'bioorthogonality' was introduced by Bertozzi's Lab when they first developed Staudinger ligation for selective labeling of glycans at the cell surface [50]. There are several advantages for bioorthogonal chemistry: (a) it is versatile and applicable to all kinds of biomolecules; (b) it can be used to label targets of interest in living cells and organisms; (c) the functional groups are small enough to

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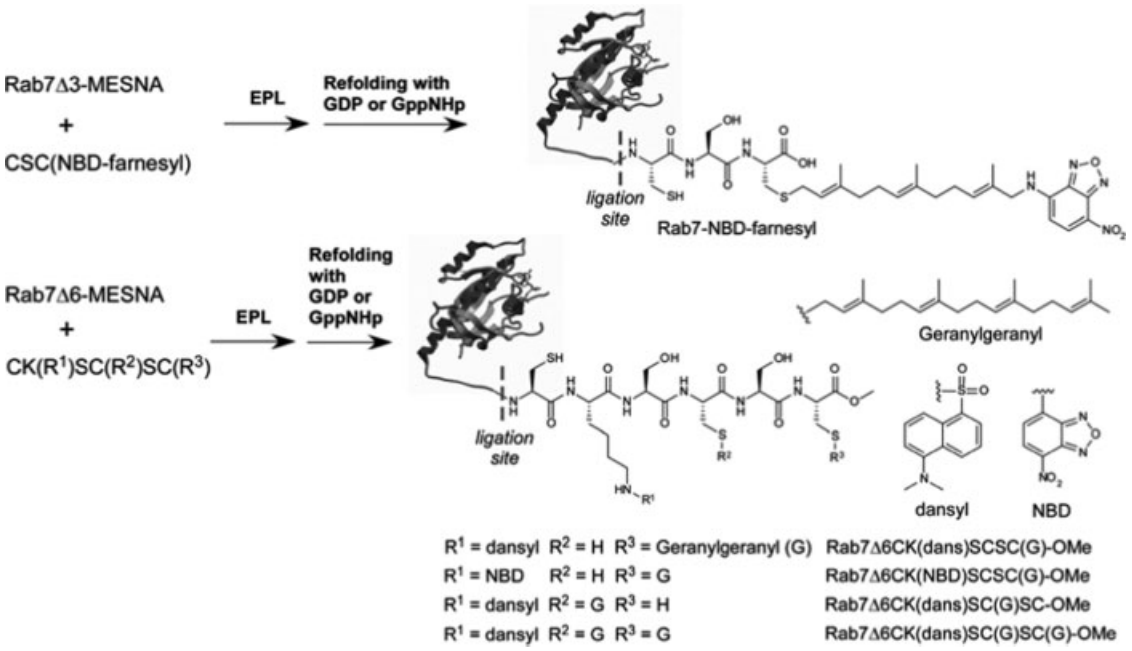


Figure 3. Construction of prenylated Rab proteins with a fluorophore on the lipid or on the side chain of amino acid.

Figure 4. Protein *trans*-splicing to ligate two fragments. The split intein *N*-terminal part (I_N) is fused to a tag and the split intein *C*-terminal part (I_C) is fused to the *C*-terminal protein fragment. The two pieces of the intein associate to reconstitute splicing activity.

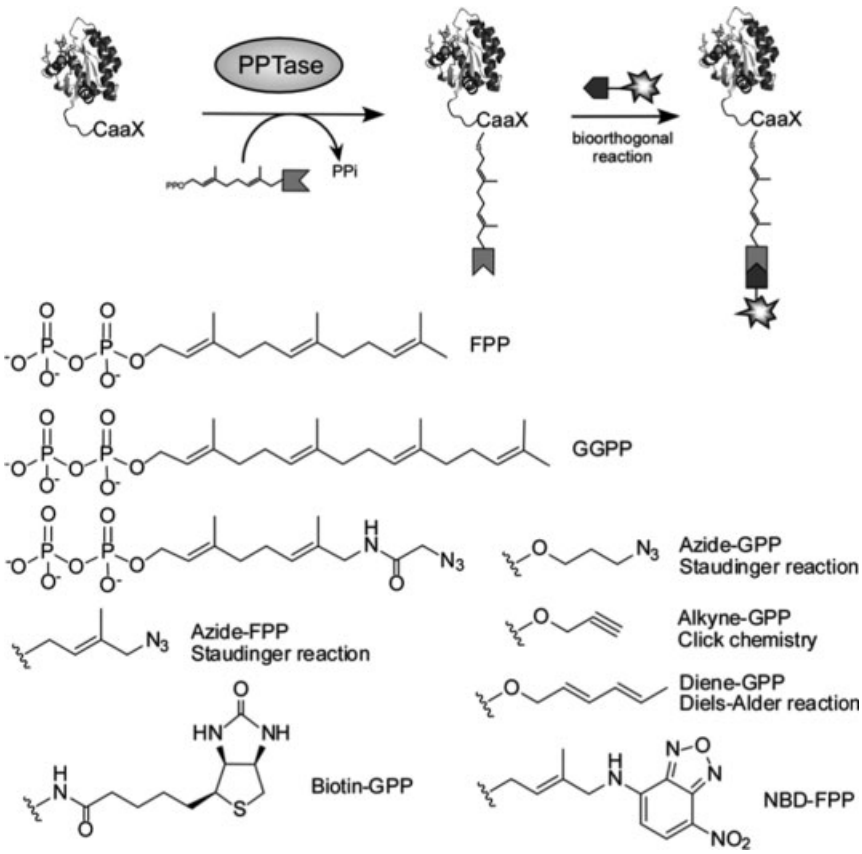
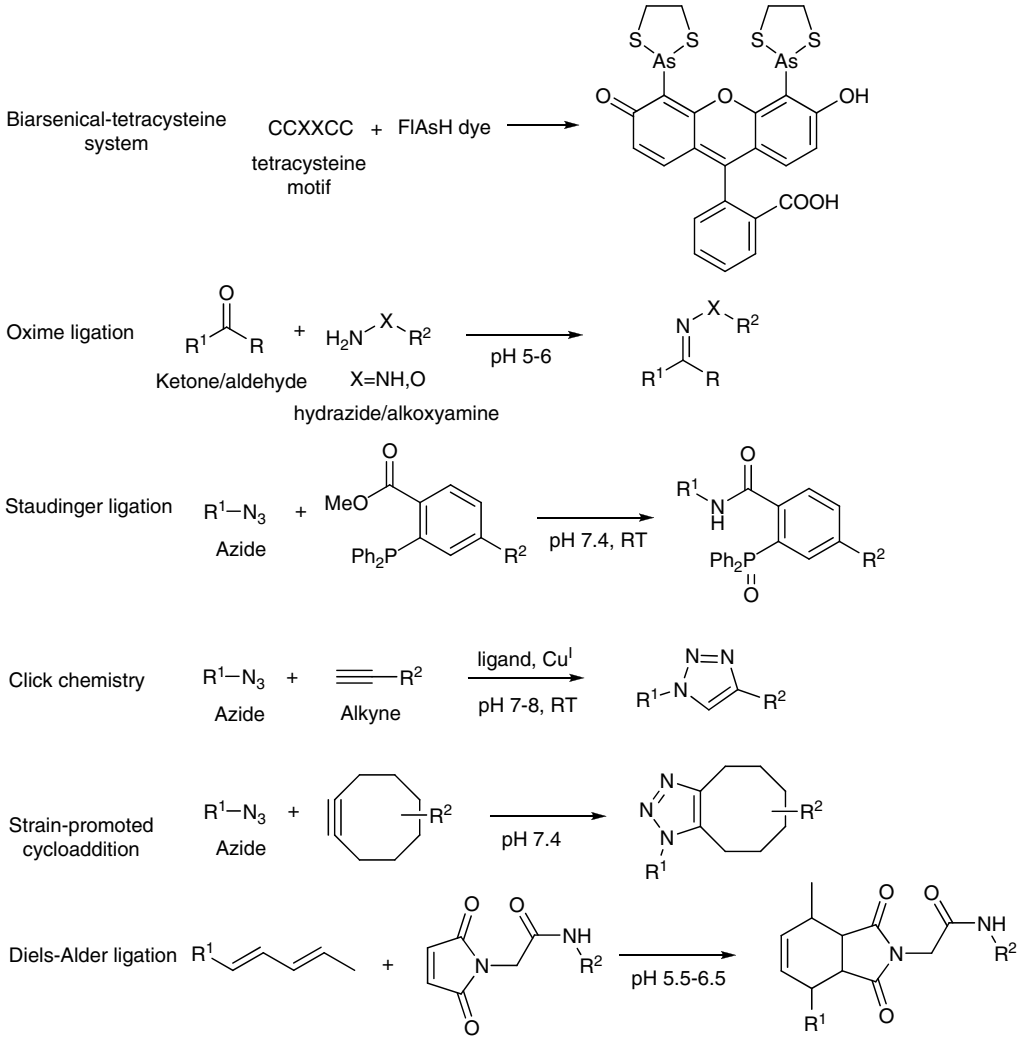


Figure 5. Labeling proteins through prenylation using modified isoprenoids.



Scheme 1. Bioorthogonal reactions: biarsenical-tetracysteine system [66], oxime ligation [67], Staudinger ligation [50], click chemistry [68], strain-promoted cycloaddition [69], and Diels–Alder ligation [70].

be tolerated by biosynthetic enzymes and could therefore be incorporated into metabolites or substrates in biological systems. Therefore, bioorthogonal chemistry has been successfully used in genome-wide protein profiling, including proteomic analysis of glycoproteins and glycosidases [51–53] and activity-based protein profiling (ABPP) for the identification of hydrolases, proteases, kinases, phosphotases, and glycosidases [54,55].

Widely used bioorthogonal reactions are listed in Scheme 1. Recent progress in bioorthogonal chemistry has been reviewed elsewhere [56,57]. Applying bioorthogonal chemistry for protein labeling involves the introduction of one of the functional pairs into a biomolecule and subsequent selective attachment of tags. This can be achieved by using the cellular biosynthetic machinery to incorporate unnatural amino acids [58] and modified monosaccharides [59], by nonsense suppression methodology [60,61], by enzyme-catalyzed modifications [62–64] (See Section on Chemical Labeling in Live Cells), and by semi-synthetic approaches as described above. A recent example includes the intein-mediated incorporation of an oxyamine moiety into the *C*-termini of proteins, which are amenable for subsequent conjugation with a fluorophore containing a ketone functionality [65].

One of the strategies for introducing functional groups to proteins includes using post-translational prenylation. Protein prenyltransferases (PPTases) are responsible for coupling soluble farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) moieties to the *C*-terminal cysteine(s) of proteins, leading to the formation of a stable thioether bond. Farnesyltransferase (FTase) and protein geranylgeranyltransferase type I (GGTase-I) recognize the *C*-terminal CaaX motif (C is cysteine, a is usually, but not necessarily, an aliphatic amino acid, and X can be one of the varieties of amino acids) in their substrates. Interestingly, PPTases can tolerate diverse modifications of their lipid substrates. Therefore, bioorthogonal groups or probes can be incorporated into proteins containing a CaaX tag via prenylation [71–73] (Figure 5). This strategy has been employed for proteomic analysis of prenylated substrates in cells [71,74], for identification of PPTases inhibitors [75,76], for protein immobilization [77,78], and for selective protein labeling [79,80].

Chemical Labeling in Live Cells

Biological systems are composed of interacting networks of biomolecules. Observation of many biologically relevant

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Table 1. Methods for chemical labeling in live cells

Tag (size in amino acids)	Small molecule probe	Enzyme required	Cellular labeling	Comments	References
Fluorogenic biarsenical and bisboronic reagents					
CCXXCC (6) tetracysteine	Biarsenical: FlAsH, ReAsH	No	Intracellular	Fluorogenic label; Nonspecific reactions and interactions; Cytotoxic in some cases.	66
SSXXSS (6) tetraserine	Bisboronic RhoBo	No	Cell surface	Fluorogenic Low; Cytotoxic; Off-target labeling of endogenous tetraserine motif.	86
Metal chelation					
6H (6)	Ni-NTA	No	Cell surface	Ni^{2+} is toxic to cells and quenches fluorescence.	87–89
6H (6)	Zn^{2+} complex HisZiFit	No	Cell surface	Noncytotoxic.	90
DDDD (4)	Zn^{2+} complex	No	Cell surface	Spectroscopic change upon chelation.	91–94
Enzymatic modification					
ACP (77)/A1 (12) or PCP(80)/ybbR (11) tag	Coenzyme A (CoA) derivatives	Phosphopantetheinyl transferases (AcpS or Sfp)	Cell surface	AcpS or Sfp catalyzes the attachment of a CoA-activated probe to the serine residue of the peptide tag. $k_{cat} = 0.03–0.3\ s^{-1}$, $k_{cat}/K_m = 0.25–4.2 \times 10^4\ M^{-1}\ s^{-1}$	95–98
AP tag (15)	Biotin ketone analog and subsequent oxime ligation	Biotin ligase (BirA)	Cell surface	Ketobiotin functions as a substrate for BirA and is incorporated into the lysine residue of the peptide tag; Minimal labeling time 20 min.	63
Q-tag (7)	Cadaverine derivatives	Transglutaminase (TGase)	Cell surface	TGase mediates the formation of amide bond between the amine of cadaverine and the glutamate residue of the peptide tag.	62,99
LAP tag (13–22)	Azido lipoic acids and subsequent strain-promoted cycloaddition, or coumarin	Lipoic acid ligase (LplA) and LplA mutant	Cell surface and intracellular	$k_{cat} = 0.048\ s^{-1}$ for the conjugation of azido lipoic acid and $k = 4.3 \times 10^{-3}\ M^{-1}\ s^{-1}$ for cycloaddtion; Coumarin addition by ^{W37V}LplA: $k_{cat} = 0.019\ s^{-1}$, $K_m = 50\ \mu M$; ^{W37I}LplA: $k_{cat} = 0.016\ s^{-1}$, $K_m = 261\ \mu M$.	64,100
CXPXR (6)	Oxyamine or hydrazide probes	Formylglycine-generating enzyme (FGE)	Cell surface	FGE catalyzes the transformation of a Cys of the peptide tag to a formylglycine.	62,101
LPXTG (5)	Polyglycine peptide probes	Sortase A (Srt A)	Cell surface	SrtA cleaves the peptide at the Thr-Gly site, and attaches a labeled polyglycine peptide; No limitation of the size of modification introduced.	102
Self-labeling enzymatic domains					
SNAP, CLIP tags (182)	Benzylguanine derivatives	Mutated O^6-alkylguanine-DNA alkyltransferase (hAGT)	Intracellular and cell surface	hAGT is alkylated through transferring the labeled benzyl group from O^6-benzylguanosine derivatives. $k_{obs} = 0.1–2.8 \times 10^4\ M^{-1}\ s^{-1}$	103,104
Halo tag (296)	Alkyl chloride probes	Mutated haloalkane dehalogenase (DhaA)	Intracellular and cell surface	The mutated DhaA is covalently modified by alkyl chloride probes. $k_{obs} = 2.7–8.5 \times 10^6\ M^{-1}\ s^{-1}$	105
Ligand-binding domains					
eDHFR (159)	Trimethylprim (TMP) conjugates	No	Intracellular	Tight and specific binding between *Escherichia coli* dihyrofolate reductase (eDHFR) and TMP ligand; Efficient labeling in cells within 10 min; Reversible labeling.	106
FKBP12 (108)	SLF′ conjugates	No	Intracellular	Tight and specific binding between FKBP12 mutant and SLF′ ligand.	107

events cannot be accomplished by merely studying individual biomolecules, but requires investigation in a functioning biological system. Thus, tremendous efforts have been made to visualize biological processes in live cells and whole organisms, usually requiring tracking of biomolecules in such environments. In addition to fluorescent proteins, chemical tagging methods have been shown to be valuable for labeling biomolecules in live cells and organisms [5,59,81,82]. For example, pulse-labeling of a protein of interest in cells can be easily achieved by applying a small molecule probe into the cell medium and subsequent wash-out. The newly synthesized proteins can be detected by adding a probe of different color. Such measurements have enabled the real-time observation of protein assembly, trafficking, and degradation [83–85]. An ideal chemical probe for *in vivo* labeling should follow several criteria: high sensitivity (S/N ratio) and specificity (bioorthogonality), fast reaction rate, low interference with function and localization of the target, and minimal cellular perturbation.

Although there have been significant advances in the methods for chemical labeling proteins *in vitro*, only few of them are applicable for *in vivo* labeling. This represents a great challenge for selective chemical labeling of proteins in their native environments. Recent advancements in *in vivo* chemical labeling techniques involve the combination of the specificity of genetically encoded tags and the flexibility of small molecule probes. Such amino acid sequences include tetracysteine/tetraserine motifs, metal chelation motifs, peptide tags for enzymatic modification, ligand-binding domains, and self-labeling enzymatic domains (Table 1). As summarized in Table 1, all these *in vivo* chemical labeling methods have their pros and cons in terms of the tag size, cytotoxicity, specificity, reaction kinetics, signal-to-noise ratio, and accessibility for intracellular labeling. Short peptide tags are possible; however, the specificity has to be conferred by using chelation or enzyme-catalyzed modifications, which usually leads to the problems of cytotoxicity and relatively slow reaction rates, respectively. Tagging systems using ligand-binding domains and self-labeling enzymatic domains are more straightforward and suited for intracellular labeling, whereas these relatively large tags may interfere with protein functions.

Perspectives

The methodologies of protein chemical modification have grown rapidly in the past few years. They have opened up new opportunities to tackle fundamental biological questions. However, there is still a great demand for labeling reagents that display fast kinetics and high specificity. Rapid reactions are important for the detection of biological events that usually occur on a short timescale. Future directions could include the improvement of conjugation chemistry and the discovery of efficient enzymes for modification. Such efforts may involve discovering new bioorthogonal chemistry in water, directed-evolution, and rational design of mutant enzymes.

Chemical labeling in live cells and organisms, albeit challenging, is essential for cell biological studies. Intracellular chemical labeling is more demanding than labeling at the cell surface due to the stringent conditions for chemical reactions inside cells and the problem of cell penetration. Many chemical labeling reagents are cytotoxic or have low cell-permeability, and are thus not suited for intracellular labeling. Therefore, chemical reagents for intracellular labeling with low cytotoxicity, good cell-permeability, fast reaction rates, and low background staining are required in the future. Further development of fluorescent probes may include photo-controllable reporters, ion sensors, two-photon fluorophores, and probes for small biomolecules such as cholesterol [108] and phosphatidylinositol 3-phosphate (PI3P) [109]. Besides organic dyes, many other imaging modes emerge as an interesting avenue, particularly for the diagnosis in whole animals and single molecule studies in cells. These include long-lifetime luminescent lanthanides [110], quantum dots [111], magnetic resonance imaging (MRI) contrasting reagents [112] and radionuclide-filled single-walled carbon nanotubes (SWNT) [113]. Chemical modification of proteins or other biomolecules is a fast-growing field that offers ample opportunities for chemical biologists to contribute to the expansion of novel chemical tools for biological studies.

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