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More than add-on: chemoselective reactions for the synthesis of functional peptides and proteins

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The quest to enlarge the molecular space of functional biomolecules has led to the discovery of selective, mild and high-yielding chemical reactions for the modification of peptides and proteins. These conjugation methods have recently become even more advanced with the advent of modern biochemical techniques such as unnatural protein expression or enzymatic reactions that allow the site-specific modification of proteins.Within this overview, we will highlight recent examples that describe the site-specific functionalization of proteins. These examples go beyond the straightforward attachment of a given functional moiety to the protein backbone by employing either an innovative linker-design or by novel conjugation chemistry, where the modification reaction itself is responsible for the (altered) functional behaviour of the biomolecule. The examples covered herein include 'turn-on' probes for cellular imaging with low levels of background fluorescence, branched or cleavable polymer–protein conjugates of high stability within a cellular environment, the installation of natural occurring posttranslational modifications to help understand their role in complex cellular environments and finally the engineering of novel antibody drug conjugates to facilitate target specific drug release.

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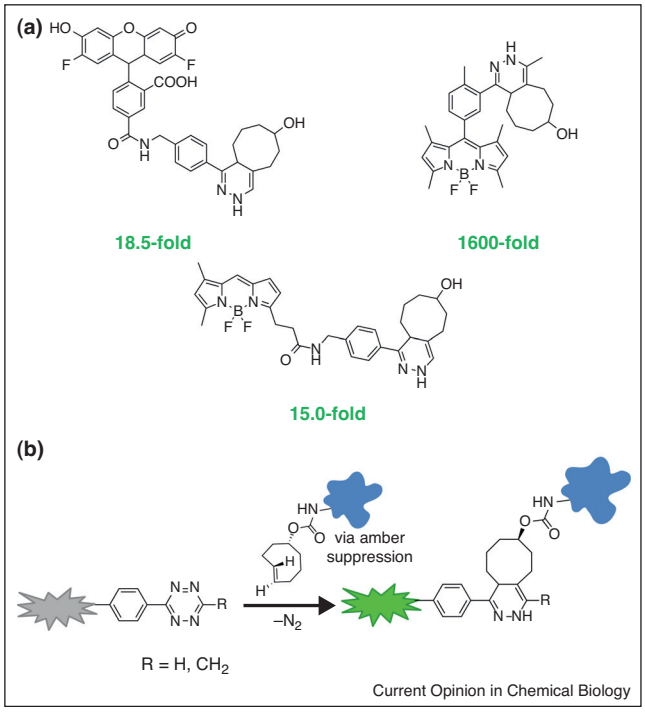
Introduction

Demands for the understanding of biological processes and for the treatment of complex molecular dysfunction are constantly rising. This trend is even further accelerated by the need for personalized medicine to cope with individual physiological variation. The chemical modification of functional biomolecules is one way to help address these challenges. Despite the many advances in this area within recent years, there is still a great need to develop effective probes for protein labelling, in order to advance the understanding of naturally occurring post-translational protein modifications and to introduce moieties for intracellular stabilization. Furthermore, methods for the specific targeting of bioactive peptides, proteins and other biologically relevant molecules need to be enhanced for successful biological and in particular intracellular applications. Over the years, mostly unselective coupling methods to natural amino acids and reactive side chains have been expanded by the incorporation of unnatural functional groups into biomolecules. Techniques including solid phase peptide synthesis, expressed protein ligation, genetic code expansion, or metabolic engineering allow the site-specific placement of unique chemical handles in a polypeptide sequence or even in a complex cellular surface. These bioorthogonal functionalities can then be further addressed by appropriate chemical reactions [1,2]. From the many successful chemoselective attachments of functional entities to peptides and proteins, this review will highlight recent examples in several areas of modern chemical biology, that either take advantage of the specific molecular composition of the formed conjugate or that install multifunctional linkers into a protein architecture.

'Turn-on' fluorescent labels

Since it was shown that the green fluorescent protein (GFP) from *Aequorea victoria* remains active when expressed in eukaryotic and prokaryotic cells, it has intensively been used for labelling and localization of proteins in living organisms [3]. Although fusion of GFP to a protein of choice is straightforward, its photobleaching properties and the unknown resulting changes of the treated system due to the size of the GFP protein restrict its applications. Organic compounds on the contrary are small and often have better photophysical properties [4]. One major drawback in the use of classical fluorophores for live cell and fixed cell imaging is the high background fluorescence and consequent low signal-to-noise ratio. Therefore, a lot of effort has been made to develop 'turn-on' fluorophores that are non-fluorescent until they are conjugated to the molecule of interest. A phosphine probe that has been modified with a fluorescence resonance energy transfer (FRET) quencher was one of the first examples suitable for live cell experiments. Upon Staudinger reaction with azides, the quencher gets cleaved and the fluorescence quantum yield increases 170-fold [5]. More recently, unsymmetrical aryl-tetrazines have been used in fluorogenic labelling reactions. When conjugated to certain fluorophores, the tetrazine

Figure 1



Turn on probes. **(a)** Fluorescent turn-on after inverse-electron-demand Diels–Alder tetrazine cycloaddition of *trans*-cyclooctenol and different tetrazine fluorophores. Turn-on ratios of up to 1600-fold can be applied in live-cell imaging where washing steps are unfavourable. **(b)** *Trans*-cyclooctene-lysine was incorporated into a protein using amber suppression methods. Rapid labelling with tetrazine conjugated fluorophores was performed in living cells [6••,7].

moiety can play an important double role by acting as a bioorthogonal reactant for selective biomolecule functionalization and by simultaneously quenching the fluorophore, which is then turned on by cycloaddition to strained alkenes [6••]. Furthermore, boron dipyromethene tetrazine fluorophores show up to 1600-fold fluorescence increase when coupled to *trans*-cyclooctene (TCO) derivatives (Figure 1) [7]. These properties are of huge advantage for live cell imaging by making intensive probe wash steps unnecessary. The unique chemistry of tetrazine probes enabled Lemke *et al.* to perform a pulse-chase dual labelling of the insulin receptor and virus-like particles in mammalian cells using two cyclooctynyl derivatives and selective tetrazine coupling [8•]. This strategy allows the rapid labelling of cellular processes at two specific moments in time.

Chemical installation of posttranslational modifications (PTMs)

PTMs such as phosphorylation, glycosylation and lipidation are nature's way of regulating protein function, transport and degradation. However, only for a few PTMs the biological functions are fully understood since their selective replication remains highly challenging. Over the last decade, many new (semi-)synthetic methods have been developed to access functional PTMs and mimics of PTMs in protein molecules [9–11].

One of the frequently occurring PTMs is the ubiquitination of proteins, which is used by eukaryotes as a marker before protein degradation by the proteasome [12]. In nature, ubiquitin is enzymatically conjugated to the protein via an isopeptide bond between the ε-NH_2 of specific lysine residues and the C-terminal carboxy group of ubiquitin. Ubiquitin itself has seven lysine residues which facilitates the formation of defined linear and branched ubiquitin chains, making the study of this complex PTM even more challenging. Many isopeptide variants of ubiquinated peptides and proteins have been synthesized using disulfide bonds, oxime bonds and triazoles to link the ubiquitin to the target protein [13–16]. However, generating proteins that carry site-specific native ubiquitinations is indispensible to mimic cellular processes. Native ubiquitination of histone H2 by expressed protein ligation revealed a direct correlation to an increased methylation of H3 [17••]. A combination of genetic code expansion, orthogonal amino acid protection and final aminolysis of ubiquitin thioester furnished a native di-ubiquitin-conjugate [18]; alternatively, peptide synthesis and sequential native chemical ligations provided access to ubiquitinated peptides with varying lengths of specific ubiquitin chains [19]. These studies showed that deubiquitinases have a higher activity towards shorter chains as well as Lys29-linked and Lys48-linked poly-ubiquitins. The genetically directed site-specific incorporation of δ-thiol-L-lysine and δ-hydroxy-L-lysine enables the generation of isopeptide bonds at flexible positions within proteins (Figure 2a) [20•]. Strieter *et al.* used an enzymatic reaction to generate allylamine containing ubiquitin and obtained a K48C-linked di-ubiquitin by thiol-ene coupling (Figure 2a) [21].

Glycosylation and phosphorylation have a high impact on protein function, localization and cellular signalling processes [22,23]. Davis developed two complementary methods for the chemical glycosylation of proteins by utilizing glycosyl thiols. The first strategy relies on the auxotrophic incorporation of L-homoallylglycine into a protein molecule, followed by a hydrothiolation reaction with a 1-glycosyl thiol [24]. In the second case, naturally occurring cysteines within a protein are quantitatively transformed into dehydroalanine (Dha) by incubation with *O*-mesitylenesulfonylhydroxylamine. The resulting alkene is used to generate S-linked glycoproteins by addition of nucleophilic glycosyl thiols [25•,26]. Furthermore, the Dha-approach has been used to install thiophosphates as phosphoserine mimics into different proteins like histone H3 [27].

In our own group, we have used the chemoselective Staudinger-phosphite reaction for the site-specific