Protein Modification

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Bioorthogonal Ligation in the Spotlight**

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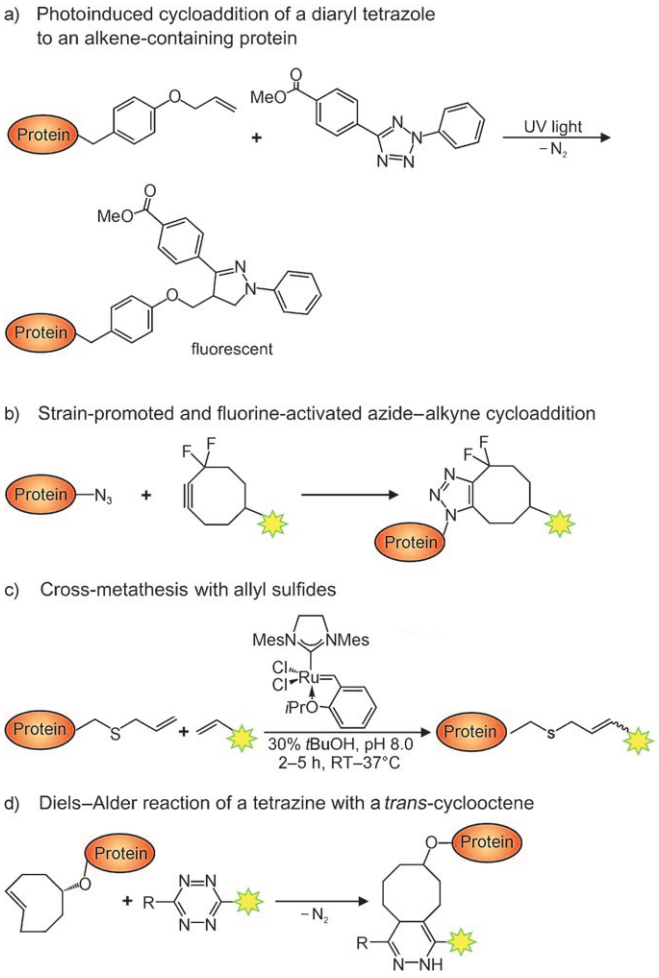
Chemical labeling strategies for the selective covalent attachment of synthetic groups to biopolymers, such as proteins, nucleic acids, lipids, and glycans, are the key to many applications in biotechnology, medicine, and basic research. The success story of the green fluorescent protein (GFP),[1–3] the discovery and development of which was recognized with the Nobel Prize in Chemistry in 2008, underlines the compelling advantage of a fluorescent tag that can be added selectively to a protein of choice, in this case to serve as a reporter of protein localization and dynamics. However, the use of a genetically fused protein tag has limitations, for example, when the aim is to mimic a posttranslational modification, or is even inappropriate, for example, with biomolecules such as glycans or lipids, which are not encoded directly. In such cases, a bioorthogonal ligation between a functional group in the biomolecule and an exogenous synthetic probe is the method of choice.[4–6] Several powerful new reactions for this purpose have been reported recently, including a photoinducible 1,3-dipolar cycloaddition developed by Lin and co-workers (Scheme 1a), which was shown to be useful for the selective functionalization of proteins in vitro and in live cells.[7,8]

What are the hallmarks of a bioorthogonal ligation reaction? Two functional groups need to react selectively with one another under mild conditions in a biological medium with the formation of a stable covalent linkage. These groups should be biologically inert to avoid cross-reactivity with other biomolecules, and ideally also biocompatible, that is, nontoxic. Furthermore, the reaction should be fast to be useful on the timescale of biological processes, and should proceed even at low concentrations to provide high sensitivity. Given the wealth of established organic transformations, it appears surprising that only a handful of reactions have so far been recognized as suitable for bioorthogonal ligation. These reactions include the nucleophilic addition of hydrazides or hydroxylamines to ketones or aldehydes,[9,10] the Staudinger ligation of an azide with a modified phosphine,[11] and the 1,3-dipolar cycloaddition of an azide and an alkyne, either catalyzed by Cu^{I}[12,13] or promoted by the ring strain of a cyclooctyne (Scheme 1b).[14,15] The



Scheme 1. Overview of some recently developed bioorthogonal reactions. Mes = 2,4,6-trimethylphenyl.

incorporation of these unique functional groups into the target biomolecule can be a challenge in itself and is a field of intense research. The approaches most often used involve metabolic labeling,[16–18] selective bioconjugation of natural functional groups, mutagenesis with nonnatural amino acids,[19] and other ligation reactions, such as expressed protein ligation (EPL).[20]

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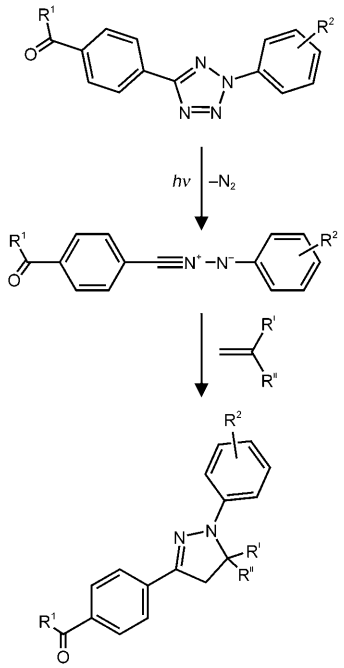
Why is there still an urgent need for new bioorthogonal ligation procedures despite these well-established reactions? One reason is that each reaction has strengths and drawbacks and may not be ideal for a specific application. For example, the copper(I)-catalyzed cycloaddition of azides with alkynes exhibits favorable robustness and reaction kinetics, which were exploited in proteomics approaches for probing enzyme activities in cell lysates[21] and for visualizing biomolecules in fixed cells.[22] However, it is usually not suitable for use in living cells or organisms because of the cytotoxicity of the copper catalyst. In contrast, the Staudinger ligation is based on nontoxic reactive groups, but has the disadvantages of a relatively slow reaction rate and competing oxidation of the phosphine reagents.[23] An important recent development was the introduction of novel strained and fluorinated cyclooctyne derivatives,[15] (Scheme 1b) the cycloaddition reactions of which do not require a cytotoxic copper catalyst and proceed at significantly higher reaction rates than those described for the Staudinger ligation. These reagents enabled improved imaging of dynamic processes in vivo, for example, the visualization of changes in glycan patterns during the development of the zebrafish embryo.[24] A further motivation for the development of new bioorthogonal reactions is that with more than one reaction available for a particular problem, one could in principle perform two or more reactions in parallel. In this respect, the generation of fluorescent proteins with different colors[1,2,25,26] has enabled the simultaneous observation of more than one protein as well as FRET (Förster resonance energy transfer) applications.

Lin and co-workers recently reported a photoinducible bioorthogonal ligation reaction which has the potential to become an important addition to the ligation toolkit.[7,8] An extra degree of experimental control is derived from the requirement for photoinduction. The reaction, first described by Huisgen and co-workers,[27] is a 1,3-dipolar cycloaddition between a nitrile imine and an alkene dipolarophile to afford a pyrazoline cycloadduct (Scheme 2). The nitrile imine is generated in situ by photolysis with UV light from a diaryl tetrazole, which undergoes cycloelimination with the release of nitrogen. Initial studies showed that this first reaction is very rapid with a first-order rate constant of $k_1 = 0.14\ s^{-1}$ ($t_{1/2} = 5.1$ s) and requires irradiation times of only a few minutes. The subsequent cycloaddition follows second-order kinetics with a rate constant k_2 of up to $11.0\ M^{-1}s^{-1}$, depending on the reactants used. It can thus be significantly faster than the Staudinger ligation and the optimized strain-promoted 1,3-dipolar cycloaddition ($k = 0.4$ and $7.6 \times 10^{-2}\ M^{-1}s^{-1}$, respectively[15,23]). The resulting pyrazoline adducts are fluorescent with emission maxima between 487 and 538 nm and high quantum yields. This property can be used to monitor the reaction progress directly. On the other hand, this fluorescent product might also be problematic if it interferes with other fluorophores used in the experiment.

To show the selectivity of the reaction in the context of proteins, the tetrazole moiety was incorporated into lysozyme through the acylation of lysine side chains and incorporated regioselectively into GFP by protein semisynthesis by using the EPL strategy.[7] Subsequent reactions with various acrylamide-based reagents were initiated by brief irradiation with



Scheme 2. Photoactivated 1,3-dipolar cycloaddition between a diaryl tetrazole and a substituted alkene.

UV light and were found to be highly specific. Alternatively, alkene-modified lysozyme was obtained by labeling with methacrylic anhydride and could be modified selectively with various tetrazole ligands, such as a PEG-substituted tetrazole.[7]

However, to benefit from the full power of this approach, general and specific methods are required for the incorporation of either the diaryl tetrazole or the alkene into biopolymers. In a first important step toward this goal, Lin and co-workers took advantage of a previously reported *E. coli* strain that incorporates *O*-allyltyrosine site specifically into proteins by the tRNA-suppressor technology.[28] In this way, an alkene-containing Z-domain protein was obtained and modified selectively by the photoinducible cycloaddition (Scheme 1a).[8] Furthermore, this reaction could be carried out successfully even in entire *E. coli* cells expressing the alkene-containing protein. Control cells expressing the wild-type Z-domain protein were not labeled (Figure 1). Thus, the reaction is potentially useful for the study of live cells. One of the most pressing next goals will be the extension of this promising method to yeast and higher organisms, for example, to mammalian cells. Another route for the incorporation of one of the functional groups into biomolecules could involve the feeding of amino acids containing an alkene group[29] and the processing of these modified amino acids by the machinery for protein biosynthesis. Suitably modified precursors for lipid and glycan biosynthesis could be processed in a similar way. Finally, one of the two functional groups could be attached to a protein fused with a tag amenable to chemical modification.[30–32]

Another important observation of Lin and co-workers was that the reaction with the alkene group of *O*-allyltyrosine proceeded significantly more slowly than the corresponding

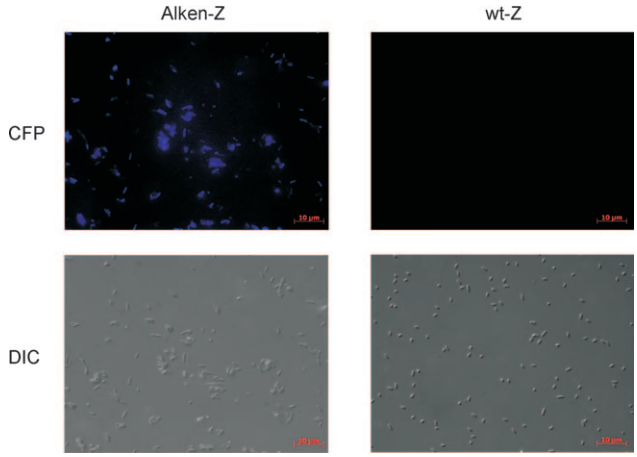


Figure 1. Selective functionalization of an alkene-containing Z-domain protein with a tetrazole (see Scheme 1 a) in *E. coli* cells: CFP-channel (top) and DIC-channel images (bottom) of bacterial cells expressing either the alkene-containing or wild-type (wt) Z-domain protein after treatment with the tetrazole (100 μM). Reproduced with permission from Ref. [8]. CFP = cyan fluorescent protein, DIC = differential interference contrast.

reaction with acrylamide derivatives.[8] By way of explanation, the authors suggested that the lower LUMO energy of the acrylamide led to a better overlap with the HOMO of the nitrile imine (LUMO = lowest occupied molecular orbital; HOMO = highest occupied molecular orbital). The selection of an appropriate nitrile imine/alkene pair might hold the potential for further optimization.

The special feature of the reaction presented herein is clearly its light dependence. This property opens the way for the precise spatial and temporal control of the ligation event and could thus provide a formidable tool for cell biology. However, it will also restrict the use of the ligation reaction to media and biological material that are transparent at the applied wavelength. By fine-tuning of the substituents on the diaryl tetrazole, it was already possible to shift the absorption maximum so that the reaction could be triggered with light with a wavelength of 365 nm, which is less harmful to cells.[33]

In summary, recent research efforts have yielded important developments for the selective modification of proteins and for bioorthogonal ligation. These developments broaden the scope of application of these reactions. Two further recent examples are also based on the reaction of an alkene moiety: The research group of Davis has started to exploit the potential of cross-metathesis for the modification of proteins functionalized with an allyl sulfide (Scheme 1 c),[34] whereas Fox and co-workers reported the extremely fast Diels–Alder reaction of tetrazines with *trans*-cyclooctenes for protein modification (Scheme 1 d).[35] There seems to be a bright and exciting future ahead for selective chemical protein modification.

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