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Bioorthogonal Reactions for Labeling Proteins

Kathrin Lang* and Jason W. Chin*

Medical Research Council - Laboratory of Molecular Biology, Francis Crick Avenue, Cambridge CB2 0QH, U.K.

Over the past 15 years a great deal of progress has been made on the discovery, rediscovery, and invention of bioorthogonal reactions between functional groups that do not react with biological entities under physiological conditions but selectively react with each other.[1] Strategies for labeling different classes of biomolecules have been developed by coopting the biosynthetic machinery of cells to introduce molecules containing bioorthogonal functional groups.[2] Tagging approaches have allowed some additional functional groups to be attached to proteins,[3] and genetic code expansion and reprogramming have facilitated the site-specific incorporation of unnatural amino acids bearing bioorthogonal functional groups into proteins in bacteria,[2a,b] mammalian cells,[4] and animals[5] via the discovery and synthetic evolution of orthogonal aminoacyl-tRNA synthetase/tRNA pairs and orthogonal ribosomes.[6] In addition, selective pressure incorporation and its derivatives have allowed the statistical labeling of proteins and proteomes with analogues of natural amino acids.[7] The incorporation of unnatural amino acids bearing bioorthogonal functional groups and their chemoselective labeling has great potential for imaging and controlling individual proteins and labeling proteomes, but the ability of investigators to leverage these approaches for biological discovery will be crucially dependent on the properties of the chemical reactions used.

The reactants in a bioorthogonal reaction should be kinetically, thermodynamically, and metabolically stable before the reaction takes place and not toxic to living systems. The reaction should yield stable covalent linkages with no or innocuous byproducts. Moreover, the two bioorthogonal moieties have to react selectively with each other under physiological conditions (ambient temperature and pressure, neutral pH, aqueous conditions), without either of them cross-reacting with the plethora of chemical functionalities found in living cells.[1b,c] Despite the challenges of meeting these criteria, a number of reactions have been developed that show good biocompatibility and selectivity in living systems (see Figure 1).[1b,c] Some of these reactions are chemoselective with respect to many but not all biological functionalities and have been used to label proteins *in vitro* and on the cell surface, while other reactions have additionally been used for the more challenging task of labeling proteins inside cells or living animals.

Most bioorthogonal reactions follow second-order kinetics, and their rates depend directly on the concentrations of both reaction partners as well as on the intrinsic second-order rate constant k_2 [M^{-1} s^{-1}] of the reaction. Rapid reactions with high second-order rate constants are therefore advantageous for labeling during biological processes that occur on a very short time scale or for the labeling of low abundance proteins. Lower abundance proteins can sometimes be labeled with a large excess of labeling reagent,[8] but this strategy may be practically limited by solubility, off target reactions and toxicity.

Bioorthogonal reactions for which one partner can be installed into proteins are summarized in Figure 1. Their second-order rate constants span 9 orders of magnitude with the fastest bioorthogonal labeling reactions reaching rates up to 10^5 M^{-1} s^{-1}, which approaches the rate constants for many enzymatic labeling approaches. Here we briefly introduce the bioorthogonal chemistries used for labeling proteins and comment on their utility for protein labeling before providing a perspective on future directions.

Amongst the first functionalities to be explored as bioorthogonal reporters were ketones and aldehydes.[9] Under acidic conditions (pH 4–6) their carbonyl groups react with strong α-effect nucleophiles such as hydrazines and alkoxyamines.[10] Ketone/aldehyde condensations show rather slow kinetics with second-order rate constants in the range of 10^{-4} to 10^{-3} M^{-1} s^{-1},[9,11] necessitating high concentrations of labeling reagent in order to achieve good labeling, which might be problematic in terms of toxicity and background signal. In general ketone/aldehyde condensations are best suited for *in vitro* or cell-surface labeling, because the reaction requires an acidic pH, which is difficult to obtain inside most cellular compartments. Furthermore, inside living cells, α-effect nucleophiles may undergo side-reactions with carbonyl-bearing metabolites.[1a,c]

A functionality that is essentially absent from biological systems and truly orthogonal in its reactivity to the majority of biological functionalities is the azide group.[12] Azide-bearing unnatural amino acids have been incorporated into proteins and used in a variety of chemical reactions.[2a,b,e,7] One potential limitation of the use of azides for protein labeling is that some unnatural amino acids bearing azides appear to be reduced in some proteins examined. Azide-modified proteins have been reacted with phosphines in Staudinger ligations.[13] This reaction has been used to label biomolecules in living cells and animals.[14] The Staudinger ligation, however, has slow kinetics: the reaction proceeds with second-order rate constants in the low 10^{-3} M^{-1} s^{-1} range.[15] In addition many of the phosphine reagents are oxidized by air or metabolic enzymes.

Azides can also react with terminal alkynes in [3 + 2] cycloadditions, catalyzed by Cu^I salts.[16] The CuAAC (Cu^I-catalyzed alkyne–azide cycloaddition) reaction proceeds considerably faster than the Staudinger ligation in physiological settings.[17] However, its reliance on the Cu^I catalyst is not without problems, since Cu^I may be toxic to living systems,[18] and decreasing the copper concentration is generally accompanied by a large decrease in reaction rate.[19] The development of tailored water-soluble Cu^I ligands[19c,20] and/or

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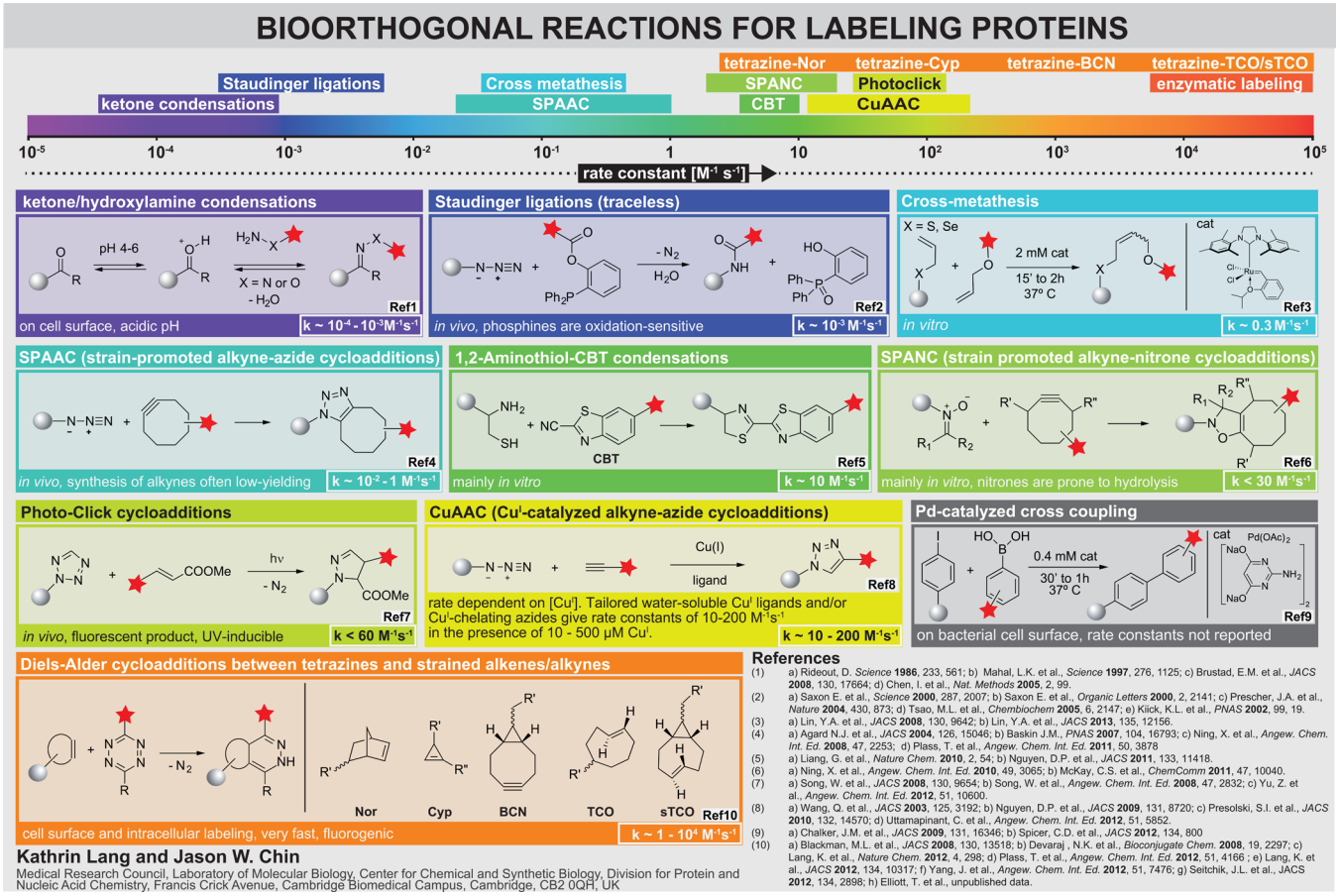

Figure 1.

the use of Cu^{I}-chelating azides[21] enables labeling of cell-surface proteins with rate constants of 10−200 M^{-1} s^{-1} in the presence of 10−500 μM Cu^{I}.[19b]

The alkyne−azide cycloaddition is accelerated with respect to the uncatalysed reaction by introducing ring strain into the alkyne (rather than using metal catalysis),[22] creating a reaction dubbed the "strain-promoted alkyne−azide cycloaddition" (SPAAC).[17b,23] A series of cyclooctyne-based probes have been developed that react with azides, and these reactions were used to label abundant biomolecules within complex biological systems, including live mammalian cells[23,24] and animals.[24,25] Reaction rates of 10^{-2} to 1 M^{-1} s^{-1} have been reported.[17b,26] Cyclooctyne derivatives can also be reacted with nitrones in a [3 + 2] cycloaddition (SPANC = strain promoted alkyne−nitrone cycloaddition).[27] These reactions are up to 30 times faster than the corresponding cycloadditions with azides.[27a] For *in vivo* applications, however, the stability of nitrones toward hydrolysis has to be further investigated.

Recently, metal-catalyzed reactions for forming new carbon−carbon bonds, including ruthenium-catalyzed olefin metathesis and palladium-catalyzed cross-metathesis have been explored for their chemoselectivity and utility in biological systems. Proteins carrying allyl sulfide or allyl selenide moieties react with allyl alcohols in the presence of a phosphine free Hoveyda−Grubbs second-generation catalyst.[28] Reaction rates of 0.3 M^{-1} s^{-1} have been reported for *in vitro* labeling of proteins.[29] Future developments may lead to ruthenium catalysts suitable for use in living cells. A water-soluble palladium catalyst for a Suzuki−Miyaura reaction was recently used for labeling of *p*-iodophenylalanine-bearing cell-surface proteins with boronic acid derivatives in *E. coli*.[30] Additional investigations will be required to determine the cell permeability and toxicity of the palladium catalyst for intracellular labeling, especially in mammalian cells.

The condensation of 1,2-aminothiol moieties with 2-cyanobenzothiazole (CBT) probes has been used to label proteins *in vitro*.[31] The reaction shows rate constants of ∼10 M^{-1} s^{-1} but is generally limited to *in vitro* applications, since the 1,2-aminothiol functionality forms adducts with metabolites in live cells and deprotection is required prior to labeling.

A light induced 1,3-dipolar cycloaddition reaction between tetrazoles and terminal alkenes to form fluorescent pyrazoline cycloadducts has been developed for use in biological settings.[32] Photoclick chemistry has been used to modify purified proteins *in vitro*, and also to visualize proteins in living cells.[32,33] Rate constants up to 60 M^{-1} s^{-1} have been reported.[32c] The major advantage of this reaction lies in its inducibility by UV-light, which may provide a tool for spatiotemporal initiation of labeling reactions in living systems. Development of new, highly reactive tetrazole reagents, that can be laser-activated at wavelengths that are less harmful to living cells will make this bioorthogonal reaction more attractive.[33b]

Inverse-electron demand Diels−Alder reactions between tetrazines and strained alkenes or alkynes (including norbornenes, cyclopropenes, bicyclononynes, and *trans*-cyclooctenes) yield dihydropyridazines or pyridazines with nitrogen gas as the only byproduct.[34] These reactions have recently been explored as chemoselective reactions for labeling and

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manipulating biomolecules in their native setting.[4c,35] The reactions are extraordinarily fast, showing rate constants up to 10^5 M^{-1} s^{-1},[35a,c,36] and can be made fluorogenic by conjugating tetrazines to some red fluorophores, where the fluorescence is quenched by the tetrazine, but activated upon cycloaddition.[35c,37] These characteristics have allowed the rapid and selective modification and imaging of proteins in live bacteria and mammalian cells.[4c,35c,38] Importantly, norbornene-, 1,3-disubstituted cyclopropene-, bicyclononyne-, *trans*-cyclooctene-, and tetrazine-bearing unnatural amino acids have been genetically encoded,[4c,35c,d,38,39] and the labeling of encoded alkenes and alkynes with tetrazine probes is quite specific with respect to proteomes tested.[4c,35c] The approach may also be extended to site-specific protein labeling in animals and provides a step-change in approaches to site specifically labeling proteins.

A range of bioorthogonal reactions, with rates spanning 9 orders of magnitude, have been used to label proteins *in vitro*, on the cell surface and inside living cells. Each reaction has its own strengths and drawbacks, and we anticipate that further reactions that complement the limitations of existing approaches will be explored for protein labeling. Ideally the rate of new reactions should be reported along with their discovery to enable simple comparison to existing methods. Evidence of quantitative and specific protein labeling, rather than simply fluorescent bands on gels, should be provided to show that competing reactions or reagent degradation do not compromise the ability to quantitatively label proteins.

While we have focused in this In Focus piece on progress in accelerating bioorthogonal reactions, many additional factors may contribute to the utility of labeling approaches, including (i) the simplicity and scalability of reagent synthesis, (ii) the ability of the labeling molecules to penetrate cells and compartments within cells, and (iii) the ability of labeling molecules to get into animals and specific tissues within animals. As we enter an era where the rates of emerging bioorthogonal reactions approach the rates of enzymatic labeling approaches, a more sophisticated, systematic, and quantitative investigation of the properties of the most promising bioorthogonal reactions is merited. In addition to asking about the chemoselectivity of a reaction with respect to the 20 amino acids or a single model protein, it is important to ask about the chemoselectivity with respect to organisms, cells, proteomes, genomes, transcriptomes, metabolomes, and the complement of lipids and carbohydrates. In addition to demonstrating that it is possible to selectively label a protein bearing a bioorthogonal group *in vitro* or when overexpressed in a cell, it is important to understand more quantitatively how the ability to specifically label proteins depends on the protein's abundance, method of incorporating the bioorthogonal group, permeability of reagents, and label used. We anticipate that a quantitative understanding of reaction specificity with respect to the molecules and systems of the biosphere will inform intelligent choices in leveraging the promise of bioorthogonal chemistry for transformational discoveries about the natural world.

AUTHOR INFORMATION

Corresponding Authors

*E-mail: klang@mrc-lmb.cam.ac.uk.
*E-mail: chin@mrc-lmb.cam.ac.uk.

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