Bioconjugate Chemistry

Review

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Developments in the Field of Bioorthogonal Bond Forming Reactions—Past and Present Trends

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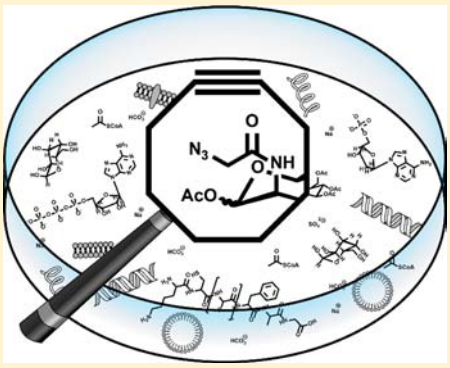
ABSTRACT: In response to the ever increasing need of chemical biology for new tools, a wide variety of new, highly selective reactions have been described. Herein we report a summary of recent developments and the historical background on bioorthogonal ligation reactions.



INTRODUCTION

Imaging living organisms became viable for the first time with the discovery of the green fluorescent protein (GFP) and its application as a protein tag in living organisms.[1,2] Despite the huge impact of GFP on the imaging of proteins, it has remained restricted to this class of biomolecules due to its nature as a fluorescent protein.[3,4] A work-around for this problem has been designed with the bioorthogonal ligation strategy. In a first step a small tag is introduced to the biomolecule of interest through metabolic or enzymatic labeling. Then, right before the imaging occurs, this tag is revealed via a bioorthogonal bond-forming reaction with a detectable moiety (Scheme 1).[5]

As this strategy solves one problem, it opens up a new challenge in another part of the process. More precisely, the requirements for a reaction to be suited for this process and to be considered a "bioorthogonal ligation reaction" are highly diverse and extensive. Namely, they have to be highly selective in order to specifically label only the intended target and minimize background labeling in a vastly diverse biological environment. Their products and educts have to be inert and remain stable toward any unwanted biological or chemical interactions. The reaction kinetics has to be favorable so that the ligation occurs before the reaction partners can be cleared from the biological system. Furthermore, it has to be biocompatible, meaning that it has to work at physiological conditions and no toxic component must be present. Last but not least, the functional groups for the ligation reaction have to be as small as possible and should be readily available to enable the incorporation into biomolecules. When considering these characteristics, the leap to "Click Chemistry" proposed by Sharpless et al.[6] in 2001 is small. "Click Chemistry" utilizes only a small number of hand-picked reactions with outstanding selectivity and efficacy, thus enhancing synthetic results. Soon, the idea proved its value and the buzzword "click" can today be found in such diverse fields as organic synthesis, chemical biology,[7] drug delivery,[8] and material science.[9]

INNOVATORS AND EARLY ACHIEVERS

As a matter of fact, even before the key phrase "bioorthogonal ligation" was coined, several reactions and applications have been explored that fulfill its characteristics. First experiments in this direction were carried out in order to label a scarce but naturally occurring functional group, which was then replaced by using two artificial groups in order to improve the signal-to-noise ratio.

Carbonyl Ligation. One of the first reports of a "bioorthogonal ligation" was published in 1986, more than 15 years before the phrase was defined in 2003. In his publication, Darryl Rideout describes the *in vitro* assembly of the cytotoxic *N*-decylidenimino-*N*′-1-octylguanidine (DIOG) from the non-harmful precursors *N*-amino-*N*′-octylguanidine (AOG) and decanal via hydrazone formation (Scheme 2a).[10] This method was soon adopted into the field of protein modification where additional partners like hydrazides, aminoxy, and cystamine groups aside from hydrazine were found to react specifically with aldehydes (Scheme 2b).[11]

With the scope of aldehyde as a bioorthogonal reporter tag widening, more and more attempts were made to introduce it selectively into proteins and biomolecules in general. Early approaches to introduce carbonyl functions into proteins by *N*-terminal transamination required to some extent harsh conditions such as acidic medium,[13] which are not compatible

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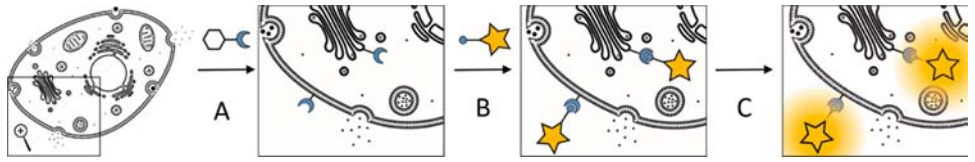
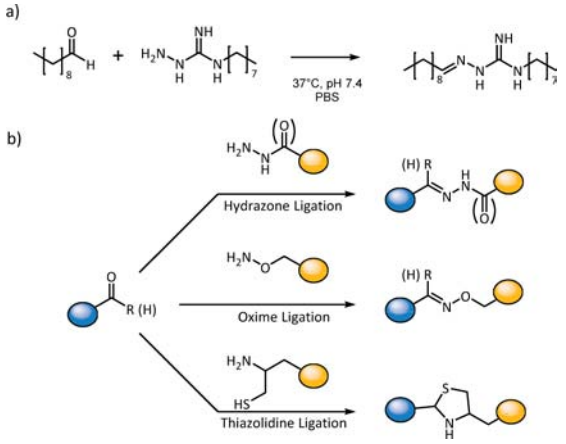
Scheme 1. Labeling Experiment Utilizing a Bioorthogonal Ligation Reaction to Introduce a Tag into Living Cells[a]



[a](A) Introduction and immobilization of the reporter inside a living cell by genetic, metabolic, affinity based, or other means. (B) Labeling of the immobilized reporter with a reactive probe bearing a detection tag by a bioorthogonal ligation reaction. (C) Revelation of the detection tag by an analytic method such as microscopy, gel electrophoresis, pull down, and so forth.

Scheme 2. Carbonyl Ligation Techniques[a]



[a](a) Originally reported toxin assembly by Rideout.[10] (b) Techniques utilizing carbonyl functions applied as bioorthogonal ligation reported in the literature.[12,11]

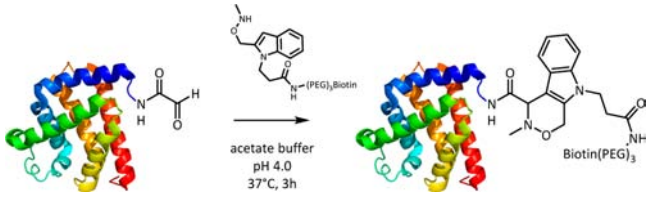
with all biomolecules or living organisms. Even when Dixon et al. achieved a transamination of a *N*-terminal amino group at room temperature,[14] this application was not adopted widely. In 2006, Francis et al. developed a protocol that achieved this under physiological conditions by studying the oxidative capacities of various aldehydes from which pyridoxal-5-phosphate emerged as the most suited.[15] Despite thi significant progress, there are still severe restrictions and drawbacks to this method, such as oxidative stress to the protein, varying yields, and confinement to N-terminal residues. Therefore, other options are explored at the moment, such as site specific incorporation by the biosynthetic pathway[16] or genetic encoding of aldehyde containing peptide sequences.[17]

A totally different approach to introduce carbonyl functions into biomolecules and an exemplification of the bioorthogonal potential of the hydrazone ligation was presented by Bertozzi et al. in 1997.[18] They supplied Jurkat, HL-70, and HeLa cells with a ketone containing mannosamine derivative, which was then, by the cells own biosynthetic pathway, incorporated into the cell membrane as sialic acid retaining the carbonyl function. These extracellular functions were then labeled by a biotin hydrazide probe and could be detected with a fluorescein containing avidin. Further studies reported the rate constant of this reaction to be 0.033 ± 0.001 M^{-1} s^{-1}.[19]

In 2006, Dawson and co-workers managed to enhance the reaction kinetics by addition of nucleophilic aniline, which formed a highly reactive protonated Schiff base that acted as an electrophile. After transamination with the reactive probe, the catalytic aniline was released. The application of the aniline Schiff base proved beneficial for both hydrazone[20] and oxime[21] ligation and was able to enhance their rate constants to 170 ± 10 M^{-1} s^{-1} for hydrazone and 8.2 ± 1.0 M^{-1} s^{-1} for oxime ligation.[22] The *in vitro* applicability of this aniline-catalyzed oxime ligation was shown in combination with a mild periodate oxidation, which generated the necessary cell surface aldehydes, by Paulson et al. on a hyposialylated human B-cell line.[23]

Very recently, a new addition has been made to the bioorthogonal reactions utilizing aldehyde tags, a modification of the Pictet-Spengler reaction (Scheme 3).[24] Even though this

Scheme 3. Modified Pictet-Spengler Reaction on Glyoxyl Horse Heart Myoglobin (pdb: 3RJ6) Generated by *N*-Terminal Transamination with Pyridoxal-5-Phosphate Described by Bertozzi et al.[24]



reaction occurs with a rather moderate kinetic constant of 1 × 10^{-4} M^{-1} s^{-1}, its product was proven to be very stable under physiological conditions. It remains to be proven whether this reaction is applicable not only on purified proteins but *in vitro* as well.

To summarize, carbonyl based ligation reactions are of significant interest for their selectivity and the unobtrusiveness of their functional groups. On the other hand, they appear to be limited to cell surface reaction to date, as their catalysts or acidic conditions are only applicable outside living cells.

Native Chemical Ligation. About 10 years before the definition of the "bioorthogonal" concept, another very proficient reaction was developed by Kent et al. in 1994, the native chemical ligation (NCL).[25] The basis for this technique is the S,N-acyl shift discovered in 1953 by Wieland et al.[26] which occurred when a thioester found itself in close proximity to a primary amine function. This thioester is then effectively attacked by the amine and a natural amide bond is formed (Scheme 4). Similar to carbonyl ligation, where the aldehyde is predominantly generated inside the biomolecule, NCL mainly targets thiols in biomolecules while the thioester acts as the artificial probe.

Early applications of the NCL were formation of cyclic peptides[27] as well as the breach of peptide length restraint previously posed by SPPS.[25,28] When considering the mechanism of NCL, one might recognize it to be quite akin to the mechanism by which self-splicing proteins eliminate their intein in living organisms (Scheme 5).[29] Following this chain of thought, two major techniques have been developed in order to acquire custom-made proteins from recombinant origin. The

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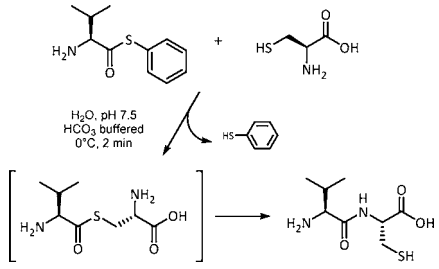
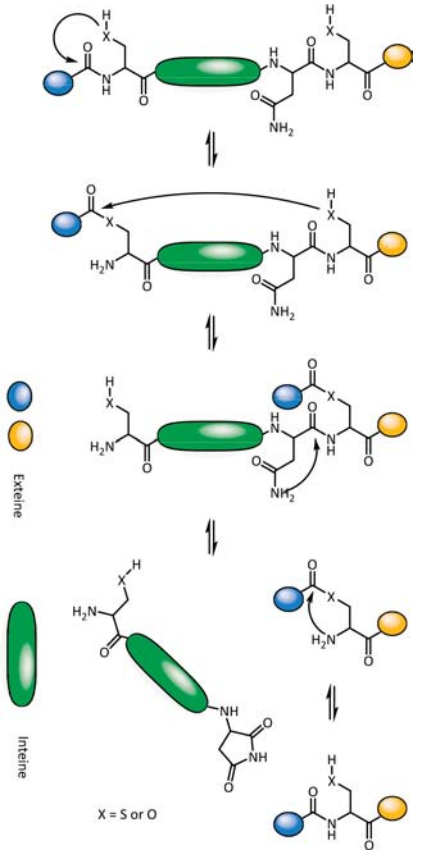
Scheme 4. NCL between Valyl Thiophenol and Cysteine as First Described by Wieland et al.[26]



Scheme 5. Protein Splicing Mechanism Involving 2 Exteins and 1 Intein Which Has Been the Role Model for NCL and Derived Techniques



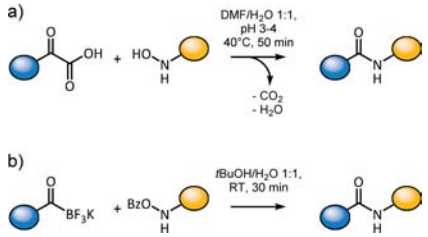
first, the expressed protein ligation (EPL), retains the thioester located on a modified intein, which is then cleaved by a N-terminal cysteine.[30] The second, the protein-*trans* splicing (PTS) technique, works with the *in situ* assembly of a complete intein that, after splicing with the cysteine of the extein, releases a natural peptide bond.[31] Despite their application in protein assembly, the EPL especially offers huge opportunities to introduce a variety of probes into proteins.

Moving from the subject of protein assembly, there have been several occasions on which the NCL was used more like a bioorthogonal ligation reaction rather than a mean to build peptide bonds. For instance, Yao et al. used thioester containing fluorophore probes to detect overexpressed GST proteins bearing a terminal cysteine moiety.[32] In a similar approach, Wu et al.[33] labeled a protein in an orthogonal fashion with two fluorophores in order to monitor the resulting FRET effect between both dyes. They were able to achieve this feature in a one-pot process, as the functional groups of the applied oxime ligation (see above) did not interfere with those for classical NCL. A recent example totally leaving the field of protein chemistry is the DNA templated NCL for signal amplification during PCR by Roloff and Seitz.[34]

Despite the successes reported by Yao et al. at labeling one protein specifically, it remains uncertain whether NCL bears further potential for *in cell* ligation. The main hampering reasons would most certainly be the necessity of an excess of the thiol leaving group to prevent ligation to other endogenous thiols and the presence of other N-terminal cysteine carrying proteins. Nonetheless, the NCL does represent a very interesting approach for labeling outside living cells as well as for protein modification.

Alternative methods (Scheme 6) to the classical NCL methods have been developed by Bode et al. that, albeit resulting in the same native amide bond, originate from different functional groups.

Scheme 6. Alternative Methods[a]



[a](a) KAHA and (b) ATHA generating native amide bonds to the classical NCL approach.

Although the second order rate constant of the 2006[35] published method of ketoacid-hydroxylamine ligation (KAHA) was not yet reported, experimental procedures subsequently reported indicate it to be rather mediocre.[36] Nonetheless it was successfully applied to protein modification[37] and to cyclization of unprotected linear proteins.[38] Furthermore, protected variants for the necessary ketoacids and hydroxylamines were developed.[38] In contrast to that, the recently developed acyltrifluoroborate-hydroxylamine (ATHA)[39] has not yet found widespread application. Although significant improvements such as increased reaction speed and nonacidic conditions have been reported.

Staudinger Ligation. One of the most suited groups for application as a bioorthogonal reporter tag is the azide function. The reason is the fact that there are hardly any azides occurring in generic biology, they possess a high intrinsic energy but no naturally available reaction partner, and finally they are small in size and have a neutral overall charge.[40] Therefore, the reaction with the likewise bioorthogonal phosphine function described by Staudinger and Meyer in 1919[41] appeared to be a valuable bioorthogonal ligation candidate. Even though the original Staudinger reaction is a method to reduce azides to primary amines,[42] in 2000 Bertozzi et al. demonstrated in a very elegant fashion how it could be set to use as a coupling reaction, now known as Staudinger ligation (Scheme 7).[43]

Later that year, the two groups of Bertozzi[44] and Raines[45] advanced the "classic" Staudinger ligation (Scheme 8a) by forming a natural peptide bond as a product of this "Traceless Staudinger" ligation. This was done by inversing the orientation of the ester[44] or thioester[45] on the phosphine resulting in

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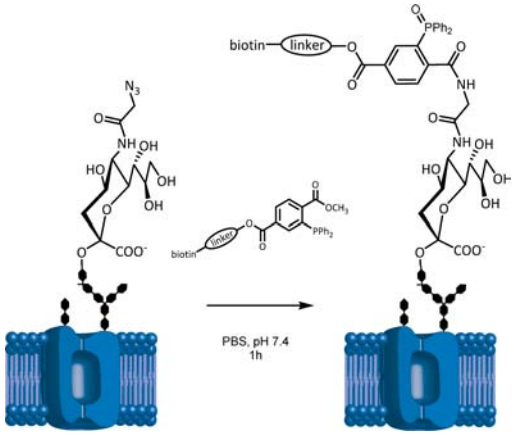
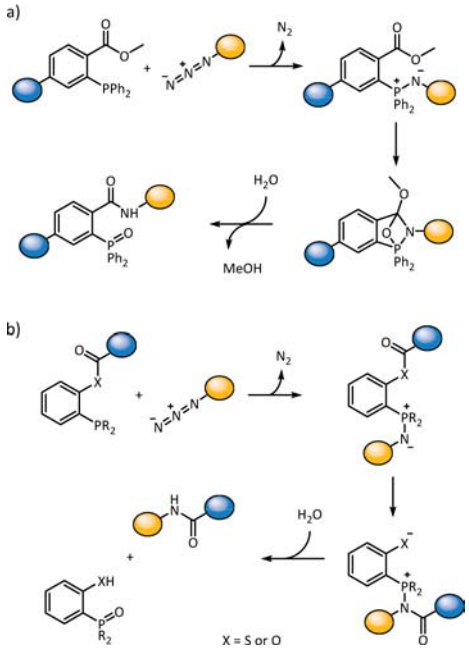
Scheme 7. First *in Vitro* Application of Staudinger Ligation for Cell Surface Labeling of Azido Sialic Acid with Biotin Reported by Bertozzi et al.[43]



Scheme 8. Mechanism of the (a) "Classical" and (b) "Traceless" Staudinger Ligation by Bertozzi et al. and Raines et al.[43−45]



elimination of the phosphine oxide moiety in the final hydrolysis step (Scheme 8b).

Mechanistic studies of the Staudinger ligation identified the initial formation of the phosphazide as the rate limiting step and measured the rate constant at $(2.5 \pm 0.2) \times 10^{-3}$ M^{-1} s^{-1}.[46] The Staudinger ligation soon found a wide variety of applications. For example, Raines et al. applied it as cysteine free alternative to NCL for peptide ligation[47−49] and in combination with NCL for on bead partial, and ultimately in solution complete, assembly of artificial RNAase A.[50] Other applications were protein immobilization on solid support,[51,52] *in vitro* and *in vivo* imaging,[43,53,54] protein enrichment[55] and detection,[56] as well as execution[57] of protein modification.

Main drawbacks of the Staudinger ligation are the oxidation lability of its phosphine compounds, their cross-reactivity toward disulfide bonds, and the fact that there is evidence for a slow reduction of azides by glutathione at physiological conditions,[58] even though this last fact is true for a variety of bioorthogonal ligation reactions.

■ ALKYNES IN BIOORTHOGONAL CHEMISTRY

Probably one of the most important functional groups in bioorthogonal chemistry, next to the azide, is the alkyne. The reasons for the alkyne's suitability are its small size, its high intrinsic energy, its stability, its absence from generic organisms, and that there are virtually no reaction partners present in biological organisms that it can react with, without prior activation. The activation of alkynes necessary in bioorthogonal ligation reactions is in general achieved by one of two mechanisms, metal catalysis or ring strain in cycloalkynes.

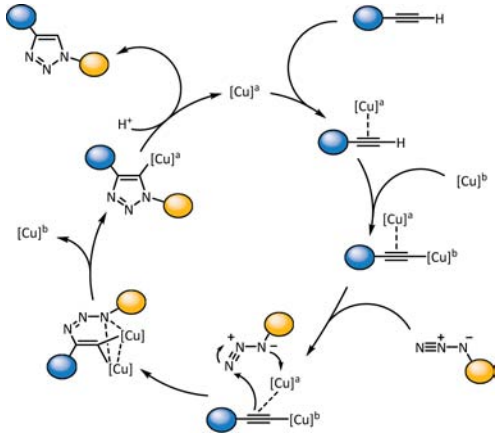
The bioorthogonal reactions of alkynes have been the focus of a number of recently published reviews.[59−61] Therefore, we will only give a short survey of the reactions covered elsewhere to allow a comprehensive overview of the bioorthogonal toolbox.

Copper Catalyzed Azide Alkyne Cycloaddition. The second very influential reaction in which azide features a prominent role is the 1,3 dipolar [3 + 2] cycloaddition to alkynes which has been first reported 120 years ago.[62] Independently, the groups of Sharpless[63] and Meldal[64] reported the regioselective Cu(I) catalyzed variant (CuAAC) that could be carried out at room temperature, in various solvents and at high yields.

The mechanism proposed by Sharpless et al. describes the first step as an introduction of the terminal alkyne into a copper acetylide and a subsequent attack of the azide. Sharpless et al. proved that the reaction proceeds via the sequential pathway rather than the concerted pathway by DFT calculations and found the transition state between copper acetylide and azide to be higher in energy than even the uncatalyzed transition state.[66] Recently, the mechanism of CuAAC was further refined, when Fokin et al. presented their evidence for a dicopper intermediate (Scheme 9).[65] Thus, the mechanism explains not only the stereochemistry of the products and the huge rate increase, but also the incapability of internal alkynes to participate in CuAAC.

With regard to bioorthogonal labeling in living organisms, CuAAC suffers mainly from the cytotoxicity of its Cu(I) catalyst[67,68] and therefore has predominantly been applied to labeling in the extracellular space. A recent development is the

Scheme 9. Refined CuAAC Mechanism Featuring a Dinuclear Copper Complex Published by Fokin et al.[65]



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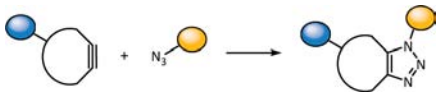
application of various ligands, serving two purposes, protecting the living organisms from the cytotoxicity and the copper from oxidation.[69,70] Accordingly CuAAC was applied to phospholipid imaging *in vivo*[71] in mice with propagylcholine and *in vitro*[72] on living RAW macrophages with fluorogenic coumarin azide. Furthermore, it was applied to virus surface remodeling,[73] protein labeling,[74] nucleic acid labeling,[75] as well as affinity based probe profiling.[76]

Nonetheless, Cu(I) still poses a significant threat to living organism by generating reactive oxygen species.[77] Thus, mammalian cells survive Cu(I) concentrations up to 500 μM for 1 h, but once exceeding 1 mM, copper proved fatal to both cell culture cells as well as living zebrafish embryos.[78] This effect can be moderated and the time of ligation can be reduced by carefully choosing ligands for the copper species.[77] This makes it clear that bioorthogonality is not an absolute characteristic, but on the contrary, there exist several degrees of bioorthogonality. Nonetheless, CuAAC remains an interesting example of bioorthogonal ligation reactions especially in regard to orthogonal multitarget applications. As the review by Lin and Ramil[60] describes the development of the mechanism, recent new developments, and various ligands for CuAAC more fully, the reader is redirected there for more details.

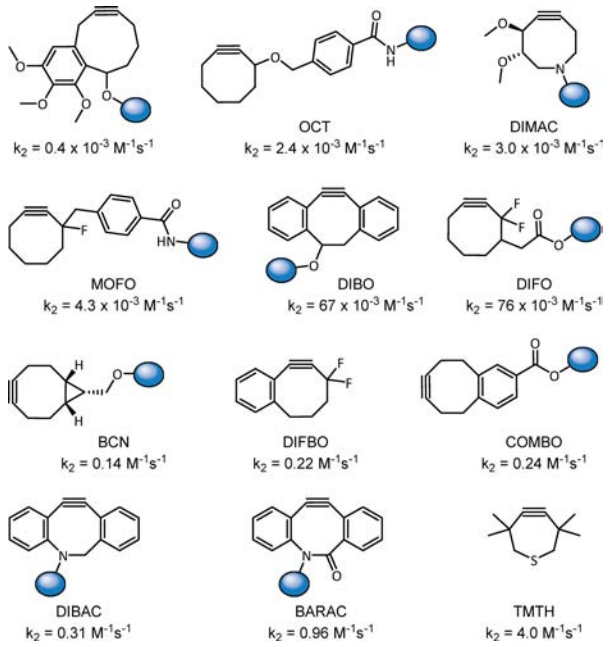
Strain Promoted Azide Alkyne Cycloaddition. In order to circumvent the cytotoxicity of copper, Bertozzi et al.[79] in 2004 salvaged a phenomenon that was first described by Blomquist et al.[80] in 1953 and was more completely studied by Krebs and co-workers in 1961,[81,82] the cycloaddition between azides and strained cyclic alkynes. As a result, Bertozzi et al. were able to develop a copper free and strain-promoted azide–alkyne cycloaddition (SPAAC) reaction with enhanced biocompatibility but reduced reaction kinetics yielding isomeric mixtures (Scheme 10).[79]

Scheme 10. Reaction Schematic of SPAAC



As a result of the successful demonstration of SPAAC a huge variety of derivatives have been developed (Scheme 11). Starting from the original OCT[79] in 2004, Bertozzi et al. proceeded to the monofluorinated MOFO[83] in 2006 and the difluorinated DIFO[84] in 2008. In the course of this progress, they were able to increase the rate of the reaction almost 40-fold. Furthermore, Bertozzi et al. published the derivative DIMAC[85] that exhibited a slightly increased reactivity compared to OCT but a highly enhanced hydrophilicity. As well in 2008, Boons et al. introduced a new way of activation by fusing benzyl rings to their DIBO[86] reagent. In 2010, several new reagents for SPAAC were presented. Van Delft et al. and Popik et al. both picked up the dibenzyl strategy and introduced an exocyclic amide into DIBAC[87,88] which resulted in a 5-fold rate increase. The same year, Bertozzi et al. moved the amide function from the exocyclic to endocyclic orientation in BARAC[89] and thus reached almost 1 M^{-1} s^{-1}. In addition, they tried to combine both ways of activation by applying one fused benzyl ring and two geminal fluorides. Unfortunately, this approach proved to be one step too far in activation and the resulting DIFBO[90] was not stable in neat conditions and had to be stabilized in a cyclodextrin complex. A contribution by van Delft et al. containing a new structural motif was the BCN[91]

Scheme 11. Cycloalkynes Developed for SPAAC and Their Respective Second-Order Rate Constants Determined in AcCN or MeOH



that featured a fused cyclopropyl ring. Even though its reactivity dropped 5-fold compared to BARAC, BCN especially excelled with a facile 4 step synthesis. In 2012, Kele et al. published a monobenzyl-activated COMBO[92] that reacted slightly faster than BCN but did not reach BARAC. A complete new approach was chosen by Dudley et al. when they presented their cyclononyne derivative,[93] which offers only a moderate reaction rate but was especially designed for water solubility. In the opposite direction searched Bertozzi et al.[94] in the same year, as they reintroduced cycloheptynes studied by Krebs and Kimling[95,81] in the early 1970s like TMTH for bioorthogonal ligation reactions. Regrettably, efforts to introduce anchor points into the simple TMTH turned the resulting molecules either unreactive or unstable; nonetheless, the fastest so far reported SPAAC of TMTH with benzyl azides will ensure further efforts in this direction.[94]

As it had been originally designed for biological applications, SPAAC has found a wide variety of applications. In their initial publication,[79] Bertozzi et al. proved its applicability on modification of purified proteins. Later on, the method was extended to *on cell* surface labeling of Jurkat cells after feeding with azido glycans.[96] In further experiments, the reaction was subsequently successfully applied *in vitro* to fibroblast cells[97] after incorporation of azidohomoalanine by the biosynthetic pathway and to *Mycobacterium smegmatis*[98] after doting with azido trehalose. Additional *in vivo* experiments were conducted on zebrafish,[99] *Caenorhabditis elegans*,[100] and mice[101] specimens, each after incorporation of azido mannosamine into cell surface glycans. Furthermore, SPAAC was used to image tumors in living mice with the help of nanoparticles[102] and ^{18}F PET where the fluorine was attached to both azide[103] and cycloalkyne.[104] Other fields of application were found in virus modification[105] and DNA labeling.[106]

Despite these applications, a new problem emerged with increasing cycloalkyne activation,[107] the side reaction with naturally occurring thiols, which might also be the source of

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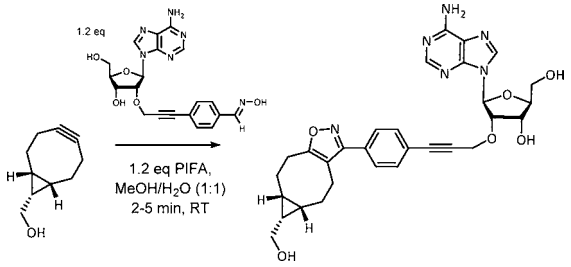
complications in *in vivo* experiments.[101] Although recent experiments indicate that terminal[108,109] alkynes are more prone to react with thiols than internal,[110] this still remains an issue to be solved. First attempts in this direction been made by Boelens et al. by scavenging thiols with iodoacetamide.[111]

Strain Promoted Alkyne Nitrile-Oxide Cycloaddition. Concurrent with the tremendous efforts put into optimization of reactive cycloalkyne reagents for SPAAC, the dipole reagent has recently drawn an increasing amount of attention. Among others, nitrones, diazocarbonyl, and nitrile oxides have been reported to be capable of strain promoted cycloaddition to cycloalkynes (SPANOC).[112−115] Especially, nitrile oxide has received considerable attention, due to a calculated rate increase of about 12-fold compared to azide.[66] Kinetic studies were able to experimentally confirm the calculation results, and for instance, van Delft et al. have reported the reaction between BCN and benzonitrile-*N*-oxide to be 10-fold faster than with benzylazide (Scheme 12).[112]

Scheme 12. Application of SPANOC Reported by van Delft et al.[112] in 2011



A major drawback of nitrile oxides for strain promoted cycloaddition remains their tendency to dimerize readily.[116] This problem is usually circumvented by generating the nitrile oxide *in situ*, and thus several bioorthogonal applications have been reported.

Early hints of compatibility with unprotected nucleosides and peptides[112] were successfully proven,[115] and polymer supported DNA[117] and RNA[118] ligation experiments have been published. Another application was found in the sequential buildup of bifunctional molecules by subsequent SPAAC and, after nitrile-oxide generation, SPANOC.[113]

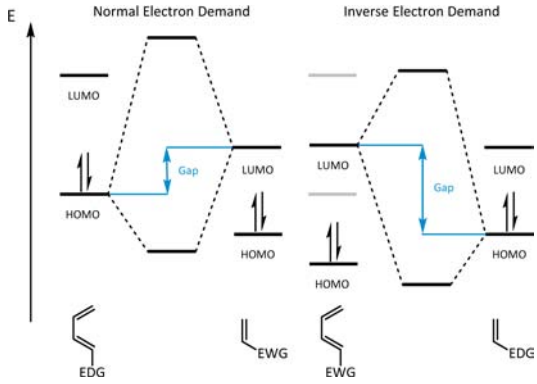
■ ALKENES FOR BIOORTHOGONAL LIGATION

Alkenes have been applied in bioorthogonal reactions very early in the course of their development. Nonetheless, their potential has only been recently realized.

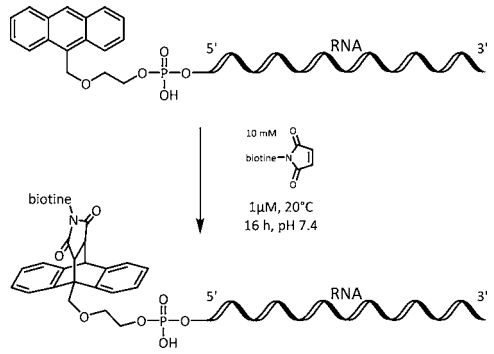
Diels−Alder Reaction. When this highly renowned reaction yielded the Nobel Prize to its discoverers who also gave it their names in 1950,[119] it was mainly for its application in natural product synthesis.[120] Nonetheless, it brought with it a series of characteristics mandatory for bioorthogonal applications, such as no naturally occurring functional groups, good compatibility with water, and high selectivity.[121] Diels−Alder reactions as [4 + 2] cycloadditions fall into two major categories (Scheme 13) with respect to the electronic situation, normal electron demand and inverse electron demand.

Normal Electron Demand Diels−Alder. The normal electron demand (NED) Diels−Alder reaction has been put to use as a bioorthogonal reaction for about 15 years now and has been applied to oligonucleotide (Scheme 14), protein, and oligosaccharide labeling.[122−125] Additionally, Waldmann et al.

Scheme 13. Distinction between NED and IED According to their FMO Configuration



Scheme 14. NED Diels−Alder Modification of RNA Reported by Jäschke et al.[124]



were able to use the Diels−Alder reaction in order to immobilize streptavidin on glass plates and detected it by the fluorescence of a biotin cyanine 5 probe.[123] Despite these accomplishments and the fact that the Diels−Alder reaction experiences a significant rate increase when it is performed in water,[126] it still was utilized only scarcely in biological applications.[127,128] The reason for this neglect might have been the sluggish kinetics and side reactions of its primary dienophile maleimide.

Inverse Electron Demand Diels−Alder. Similar to the NED Diels−Alder reaction, the inverse electron demand (IED) Diels−Alder reaction was discovered several decades ago and has mainly been applied to synthetic problems.[129] With the first being only a curiosity of reagents, which did not follow the then-valid Alder rule, they were ultimately termed "inverse" and categorized by Sauer[130] and Sustmann.[131] In 2008 finally, the IED Diels−Alder was introduced to the bioorthogonal ligation pool, when Fox et al. (Scheme 15)[132] and Hilderbrand et al.[133] published the reaction of a tetrazine with *trans*-cyclooctene and norbornene, respectively, over the course of a few months.

In order to bolster the suitability of their reaction for protein modification, Fox and co-workers[132] functionalized thioredoxin with a *trans*-cyclooctene and followed the reaction with mass spectrometric analysis. They also calculated the second order rate constant between tetrazine and *trans*-cyclooctene to be 2000 ± 400 M^{-1} s^{-1}, which places it several orders of magnitude higher than any other bioorthogonal ligation reaction reported to date. Hilderbrand et al.[133] in their paper took the bioorthogonality one step further and proved that cell surface labeling of living cells is possible. They exposed overexpressing cancer cells to an antibody for Her2/neu

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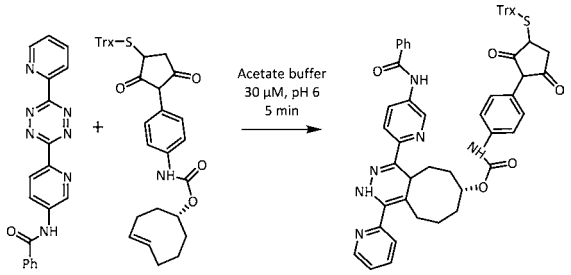
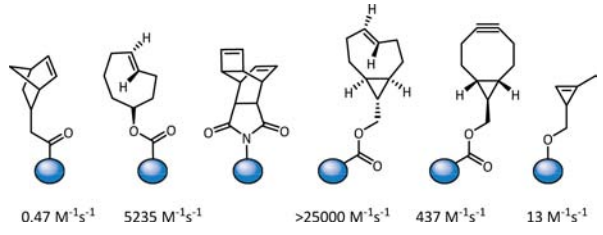
Scheme 15. Thioredoxin Modification Reported by Fox et al.[132]



receptors which contained norbornene and rhodamine. After labeling with a tetrazine-VT680, they could prove selective labeling by the occurring colocalization of fluorescence of both dyes.

Last but not least, a norbornene amino acid was developed by two groups in parallel, which they applied to selectively label isolated proteins[134] and cell surface proteins[135] on living mammalian cells. Despite the obvious potential of norbornene as a dienophile, soon the advantages of *trans*-cyclooctene started to become clear, mainly because the kinetics of norbornene at 1.6 M^{-1} s^{-1} was found lacking, and most recent studies have been conducted featuring *trans*-cyclooctene.[136] In further experiments, it was established that the tetrazine click is suited not only for cell surface labeling, but also for *in cell* imaging of the microtubule after poisoning with paclitaxel tetrazine.[137] Furthermore, it was applied to enhance the sensitivity of cancer cell detection by ligation of the magneto-fluorescent nanoparticles to the detection antibodies compared to direct covalent linkage.[138] Also, radiolabeling with ^{111}In was carried out in living mice with the help of a *trans*-cyclooctene carrying antibody and a metal chelating tetrazine.[139] In ^{18}F radiolabeling, the NED Diels–Alder reaction was used to quickly incorporate the hot fluorine moiety into molecules of interest.[140,141] In addition, both reaction partners have been successfully genetically engineered into proteins *in vivo*.[142,143] Very recently, the possibility to utilize tetrazine click to release an active drug from an inactive prodrug is being explored.[144] In addition, several dienophiles have been screened in order to improve the reaction characteristics, among others, cyclooctynes, cyclobutenes, and cyclopropenes (Scheme 16).[143,145,146]

Scheme 16. Dienophiles Reported for Their Reactivity Towards Tetrazines

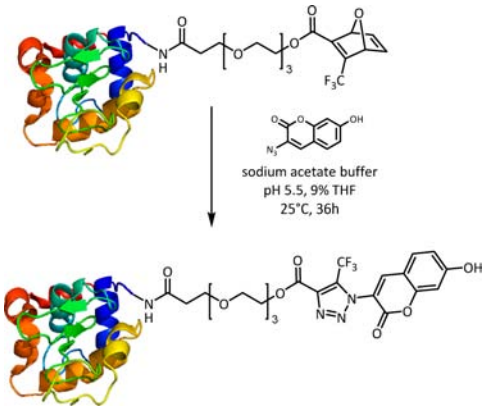


A comparative study of dienophiles resulted in a wide range of constants under comparable conditions,[143] while the cyclopropene constant was determined with a different tetrazine and is therefore only of limited significance.[146] By the combination of *trans*-cyclooctene strain and cyclopropyl fusing, the best results were obtained, de facto faster than detectable in a stop flow setup monitoring the disappearance of the tetrazine absorbance at 320 nm and were estimated to be 50 times higher than unmodified *trans*-cyclooctene.[143] Therefore, tetrazine click appears to be particularly suited for the detection of fast biological processes, as drawbacks of the reaction such as the bulky size of its reactive functions come to mind. Furthermore, the orthogonality of tetrazine click toward SPAAC or CuAAC has been proven, making it a valuable tool for multitarget approaches.[147,148]

[3 + 2] Cycloadditions. As shown above, alkenes and alkynes share similar reactivity patterns in Diels–Alder reactions. Therefore, the reactivity of alkenes versus dipoles, which was so successfully exploited for alkynes, was of major interest. This question was raised about 40 years[149,150] after alkynes were subjected to [3 + 2] reactions, and finally specific alkenes were actually found to be highly reactive toward dipoles.[151,152]

With Azides. The idea of cycloaddition between strained alkenes and azides was taken up for bioorthogonal ligation by Rutjes et al. in 2007. They were able to selectively label oxanorbornadiene residues artificially incorporated into hen egg white lysozyme with a fluorogenic azidocoumarine (Scheme 17).[153] The arising problem was the azide addition to the

Scheme 17. Modification of HEWL (pdb: 4AXT) by Classical 1,3 Dipolar Cycloaddition Reported by Rutjes et al.[153]



second double bond present in oxanorbonadiene, which would lead to unligated substituted furan. This was solved by steric protection of this bond.[154] Unfortunately, this caused the kinetics to drop from the already moderate 8.7 × 10^{-4} M^{-1} s^{-1} by about half.

Nonetheless, Boerman et al. used a synthetic cyclic RGD protein containing an azide moiety to prove the bioorthogonality of the reaction. Therefore, they incubated an oxanorboradiene containing a metal chelating moiety with ^{111}In and labeled the RGD protein in human serum and blood. In the further course of the experiment, the preligated protein was used to introducing ^{111}I into tumor cells to prove the affinity of the adduct, as the concentrations needed for cycloaddition could not be applied in living mice.[155] Another successful example was published in 2010 by van Hest et al., when they applied this method to assemble functional artificial organelles with the help of oxanorboradiene and introduced it into living cells.[156]

With Nitrile Oxides. Along similar lines were the thoughts of Carell et al. in 2009,[157] when they introduced the 1,3 dipolar

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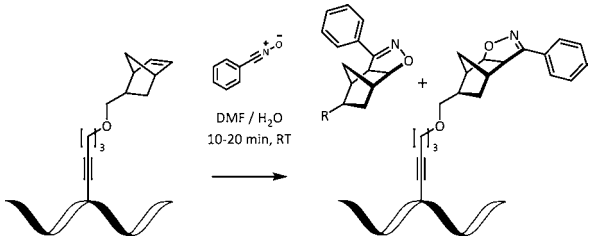
cycloaddition between norbornene and nitrile oxides to the diversity of bioorthogonal ligation reactions (Scheme 18).

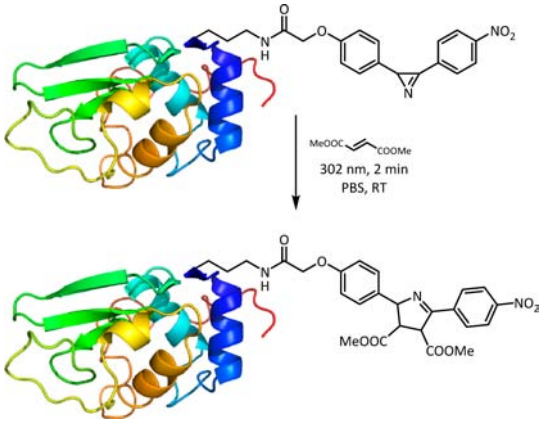
Scheme 18. 1,3 Dipolar Cycloaddition with Nitrile Oxides as Reported by Carell et al.[157]



They were able to selectively label artificially incorporated norbornene moieties in oligonucleotides in solution and on solid support with *in situ* generated nitrile oxide probes. Experiments with comparable substrates and under application of traditional CuAAC conditions were proven to require up to 3 times longer to reach the same degree of completion. Probably due to the large number of new bioorthogonal ligation reaction being published at the same time, it did not receive its due attention, and to date nitrile oxides are mainly used with strained alkynes for 1,3 dipolar cycloaddition.

With Nitrile Ylides. In 2010, a new aspect was introduced by Lin et al. when they used photoactivation for the first time to generate reactive nitrile ylides *in situ* from azirine derivatives (Scheme 19).[158]

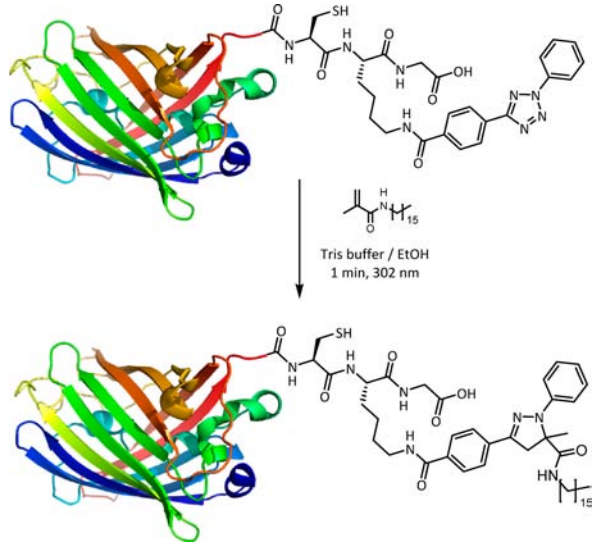
Scheme 19. Photoactivated Labeling of Lysozyme (pdb: 2LYZ) via Azirine Ligation Reported by Lin et al.



With the help of this technique, they were able to introduce a PEG chain under physiological conditions into lysozyme at a yield of 41% after only 2 min of irradiation at 302 nm. Despite this example for the potential of photoactivation, little else was published on this matter probably due to the immense popularity of the second photoinducible bioorthogonal ligation reaction, the "tetrazole click".

With Nitrile-Imines. The most commonly known alkene 1,3 dipolar cycloaddition in bioorthogonal ligation is the reaction with nitrile imines as used in the most remarkable example, the "tetrazole click". As with most examples of the bioorthogonal ligation reactions, this reaction has been described in the past[159,160] and was rediscovered for this new application only recently.[161] Curiously, from the very beginning there have been two ways described in order to receive the activated nitrile imine, by basic treatment of halohydrazones[159] and photodegradation (Scheme 20)[160] or thermolysis of

Scheme 20. Lipidation of EGFP (pdb: 2Y0G) by Tetrazole Ligation Published by Lin et al.[163]



tetrazoles. However, photoactivation has received wider attention due its noninvasive nature and pH-independence. Both precursors, upon activation eliminate a small molecule, nitrogen and hydrochloric acid, respectively, before they can be reacted with a variety of cyclic, in-chain, and terminal alkenes[161] at rates up to 58 ± 16 M^{-1} s^{-1}, what makes them 10 times faster than the fastest reported SPAAC.[162]

Serendipitously, the pyrazoline product did in itself display fluorescence, which could be utilized to monitor the reaction progress by measuring the appearance of fluorescence and to localize adducts by imaging the fluorescent bands of the labeled proteins in the resulting gel.[164] In order to minimize biohazardous irradiation at low wavelength, extensive studies have been conducted to enhance reactivity by HOMO energy tuning[165] and shifting of the absorbed light into longer wavelength ranges by addition of highly absorbing scaffolds.[166]

In order to establish the applicability for bioorthogonal ligation reactions, first the application for protein modification was further tested. To this end, a tetrazole tag was introduced by NCL into a GFP and after ligation with an alkene, in gel fluorescence was recorded to detect occurred labeling, while control experiments without tetrazole or irradiation ensured the selectivity of the reaction.[163] Further efforts were made to test the compatibility with living organisms and thus both alkene[164,134] and tetrazole[167] amino acids have been genetically encoded into proteins and labeled with their respective partner. One step further was the approach to manipulate peptide distribution in living cells and force GFP into lipid vesicles by "tetrazole clicking" with *N*-palmityl fumaric acid.[168]

Thiol−Ene Coupling. Thiol−ene coupling (TEC) was described long before the introduction of the bioorthogonal concept in 2003, in this special case almost 100 years before, in 1905.[169] The radical mechanism (Scheme 21) was described about 30 years later, by Kharasch et al.,[170] and ever since it has found widespread application in synthesis to this day.[171]

Though TEC was soon after the introduction of the "click" philosophy considered part of its spectrum,[172] TEC applications with biological substrates were mainly restricted to sugars.

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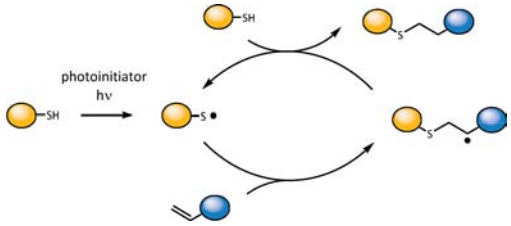
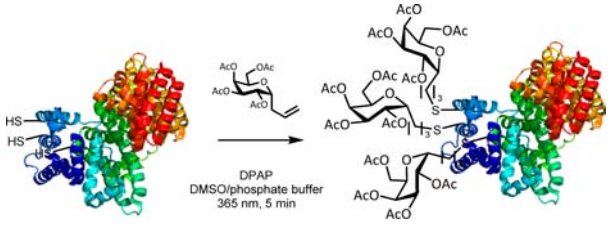
Scheme 21. Radical Mechanism of the Thiol−Ene Coupling Reaction



First studies on sugars were reported as early as 1988[173,174] and a wide variety of applications have been reported ever since.[175] The first biopolymers to be analyzed with the help of TEC were proteins in 2008 by Waldmann et al., though first TEC was only applied to immobilize biotin in specific light activated pattern on a solid support before a pull-down experiment was conducted.[176] One step further went similar experiments (Scheme 22), which were conducted by Dondoni et al. one

Scheme 22. Glycosylation of Cysteine Residues in Native BSA (pdb: 4F5S) Reported by Dondoni et al.[177]



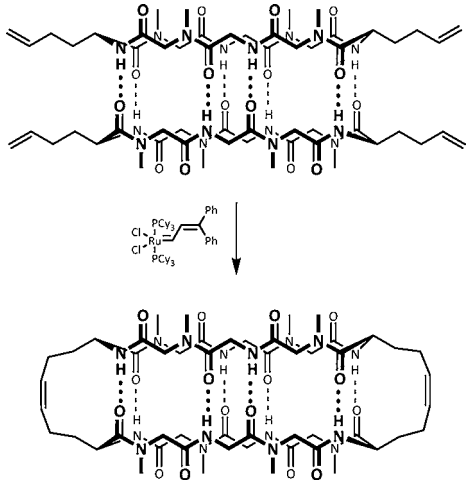
year later, when they directly targeted the singular cysteine moiety of glutathione, a synthetic nonapeptide and three thiol residues in native BSA with an allyl galactoside.[177] Instead of targeting natural or genetically encoded cysteine after specific treatment, Chen et al. genetically incorporated alkene bearing pyrrolysine into HedA and AsnII in *E. coli* and applied TEC to label them with dansyl or a PEG residue.[178]

Olefin Metathesis. The olefin metathesis is, like the Diels−Alder reaction, most well-known for its expansive application in a wide variety of fields in classical organic synthesis[179−182] and received the Nobel Prize for it in 2005.[183] Nonetheless, metathesis features certain characteristics well suited for bioorthogonal ligation, e.g., small robust functional groups and stable product scaffolds.

The scene was set when the first protein modification via metathesis (Scheme 23)[184] and the *in vitro* introduction of alkene moieties into *E. coli*[185] were reported. Another important step was done by Davis et al., who published their work on aqueous metathesis[186] for protein modification effectively moving from organic solvents. They also proposed an alternative reaction mechanism in order to account for the apparent contradiction to "a quite general incompatibility of the ruthenium based metathesis catalysts with substrates containing sulfur(II) donor sites"[187] as they have found selenium, sulfur, and oxygen based allyl compounds to be especially suited for cross-metathesis.

Beside this, only a scarce number of applications[188−190] have been reported, including the initial utilization of Davis et al. to label surface *S*-allylcysteines of a mutant subtilisin S156C out of *Bacillus lentus* with the help of second generation Hoveyda-Grubbs catalyst.[186] Major drawback of these applications is

Scheme 23. Early Application of RCM on Peptides by Ghadiri et al.[184]



their use of a minimum of 30% *tert*-butanol restricting their use in bioorthogonal applications. The variety of substrates in these experiments ranged from glycoside derivatives to capped and uncapped allyl-PEG. One of the few other examples is the application of a streptavidin immobilized ruthenium complex as an artificial metalloenzyme for ring closure metathesis reported by Ward et al. in 2011.[191]

MISCELLANEOUS LIGATION REACTIONS

Aside from the aforementioned reactions, which form the bulk of bioorthogonal ligation reaction and account for the majority of applications, there are several reactions which have proven their applicability but have yet to receive the attention that is their due. Many of these variants are based on very successful synthetic reaction and make up the cream of the crop in their respective fields.

Palladium Catalyzed Coupling Reactions. Palladium catalyzed coupling reactions have become of tremendous importance in synthetic organic chemistry, which resulted in three outstanding examples to receive jointly the Nobel Prize in 2010.[192] Aside from their growing importance in large scale production,[193] palladium catalyzed coupling reaction are of special interest in chemical biology ever since the introduction of water-soluble catalyst.[194] They can be counted as a part of the bioorthogonal ligation reaction pool suited for *in vivo* imaging, since 2011, when Bradley et al. presented their carrier system to transport Pd-catalysts into living cells.[195]

Heck Reaction. The first reports on palladium catalyzed chemistry applied to biomolecules were filed in 2006 by Yokoyama et al., who utilized the water-soluble catalyst Pd-triphenylphosphine-3,3′,3″-trisulfonate (Pd-TPPTS) for protein modification.[196] They successfully implemented the reaction, also known as Mizoroki-Heck, on labeling a genetically engineered protein (iF32-Ras-His) bearing an aryl iodide moiety with a biotin alkene probe in basic buffer. However, this approach was far from being ideal, as it was low in yield (∼2%) and required long reaction time (50 h), high concentration of DMSO (12%), and argon atmosphere.

A Heck-type variant, circumventing the problem of catalysis at high dilution, was developed by Myers et al. by generating a stable and storable arylpalladium(II) reagents and applying them to the modification of a styryl moiety in a modified lysozyme.[197] Inspired by this development, Lin et al. designed

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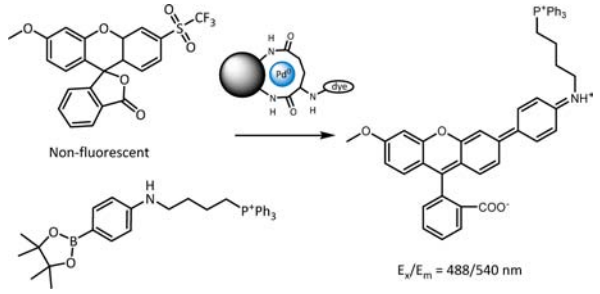
palladacyclic reagents and applied them in a Heck type fashion to label the terminal alkyne moiety in homopropargylglycine ubiquitin.[198] Nonetheless, this marked the first application of Pd-catalysis on biomolecules and therefore opened up interesting new approaches.

Sonogashira Reaction. First applications of the Sonogashira reaction for peptide modification have been reported as early as the turn of the millennium,[199,200] but it took almost another decade until Yokoyama et al. made it more widely known in 2007.[201] They exploited the fact that their previous studies on Heck reaction utilized the same functional group, aryl iodide, and therefore the iF32-Ras-His protein was an attractive test substrate. These experiments proved successful, and after extensive condition screening, the yield was improved to 25%.[202] The low yield of the palladium catalyzed reaction remained an issue until Davis et al. reported a new water-soluble catalyst system for Suzuki-Miyaura coupling that achieved almost quantitative yields.[203] This system was soon adapted to Sonogashira reactions and so Lin et al., in addition to utilizing the new catalyst system, also reduced the complexity of reagents used and formulated a copper free approach.[204] In this facilitated setup, they were able to label genetically modified ubiquitin containing a propagyl function in *E. coli* with PEG or fluorescent tag in up to 93% yield. Finally *in vivo* application was achieved by Chen et al. two years later applying a copper and ligand free variant using $Pd(NO_3)_2$ as an alternate palladium source.[205]

Suzuki Reaction. The latest addition to the Pd-catalyzed bioorthogonal pool, but probably the most influential, is the Suzuki coupling. The first report of a protein applied Suzuki reaction was done by Hamachi et al. in 2005, when they generated fluorescent biosensors by ligating fluorophores to the aryl iodide moiety of an WWiF domain of a Pin1 protein.[206] In 2008, Schultz et al. presented their work on incorporating a boronate into the genetic code of *E. coli* followed by imaging with a BODIPY iodide.[207] The inverse strategy was followed by Liu et al. in 2011, when they genetically encoded a iodphenylalanine and labeled it with a boronic acid.[208] Probably the most influential report in regard to protein modification by palladium catalyzed reaction was done by Davis et al. in 2009[203] and redeemed them from the formerly deficient yields. They proved their point by modifying the single cysteine of SBL S156C (see above)[186] with a covalently binding boronic acid and subsequently labeling the resulting aryl iodide with a variety of aromatic systems and sugars under atmospheric conditions and at 37 °C. In order to enhance their method and to avoid unspecific binding of palladium to protein surfaces, Davis and co-worker developed a scavenger that could remove the unspecifically bound catalyst in 2011.[209] Another interesting application was reported in cooperation by Gouverneur and Davis in 2013, when they used Suzuki coupling to site-specifically introduce ^{18}F into SBL-156ArI.[210] Recently further examples of protein and DNA modification by Suzuki-Miyaura reaction have been reported.[211,212]

The first reports of Suzuki ligation in living cells were published in 2011 by Bradley et al.,[195] who were able to introduce palladium-containing microspheres into HeLa cells (Scheme 24). With the help of these microspheres, they were able to ligate nonfluorescent triflate and boronate inside the cytoplasm of living cells generating a fluorescent ligation product. In order to avoid application of heterogeneous catalysts, Davis et al. incorporated aryl halides into outer membrane proteins of *E. coli* by genetic modification and

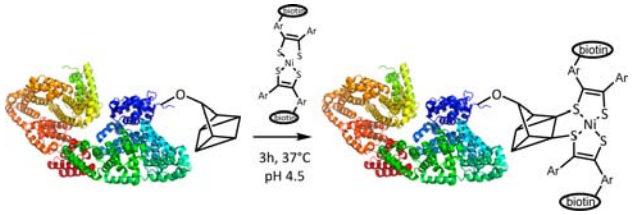
Scheme 24. Suzuki Coupling Performed by Bradley et al. Inside the Cytoplasm of Living HeLa Cells



labeled them with the help of a classic palladium complex,[213] ultimately modifying their glycosylation pattern.[214] In addition, they investigated the toxicity of their catalyst on living cells and found it to be toxic from concentration of 1 mM[213] contrary to Lin et al., who did not report any cytotoxicity of their preactivated catalyst;[204] yet the toxicity mechanism remains to be investigated.[202]

Quadricyclane. In 2011, Bertozzi et al.[215] reported this very unusual bioorthogonal reaction, unusual as it contains an actual metal complex at the center of its ligation product (Scheme 25). This is formed by a [2 + 2 + 2] cycloaddition of the

Scheme 25. Quardicyclane Ligation on BSA (pdb: 4F5S) Reported by Bertozzi et al.[215]



strained tetracycle to a nickel bis(dithiolene) at a rate constant of 0.25 M^{-1} s^{-1}, that is on par with most SPAAC reagents. A promising feature of the ligation product is its photolability as it might be utilized in a label and release application approach.

In addition to this, Bertozzi and her co-worker were able to show the orthogonality of the quadricyclane ligation to SPAAC and oxime ligation while labeling selectively 3 distinct proteins in a complex mixture. All in all, this technique shows a lot of promise, although no more applications have yet been reported.

Multitarget Approaches. This growing variety of bioorthogonal ligation reactions make it gradually more feasible to investigate and manipulate more and more complex mixtures under biological conditions.

A very early experiment in this direction was undertaken by Davis et al. in 2007,[216] when they combined CuAAC and the thiol−disulfide exchange reaction to glycosylate Tamm-Horsfall protein and SSbG-Aha43-Cys439 respectively. In addition, the compatibility of the modifications with biological functionality was shown by the retained activity of the modified proteins.

Another combination of bioorthogonal and bioselective reaction was published by Deniz et al. a year later,[217] when they genetically engineered *E. coli* to express a T4 lysozyme containing carbonyl functions as well as a cysteine. After subsequent labeling with fluorescent alkoxyamine and maleimide, an SDS-page reveiled the specific labeling.

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Under relatively controlled conditions, Bertozzi et al. managed to distinguish a mixture of three proteins bearing an aldehyde, an azide, and a quadricyclane function, respectively, by oxime ligation, SPAAC, and quadricyclane ligation with probes bearing a FLAG tag, a biotin, or fluorescein.[215]

Overkleeft et al. went further and performed a similar experiment with various functionalized proteasome ABP and labeled them selectively by Staudinger ligation, CuAAC, and tetrazine click in cell lysate and living cells.[148]

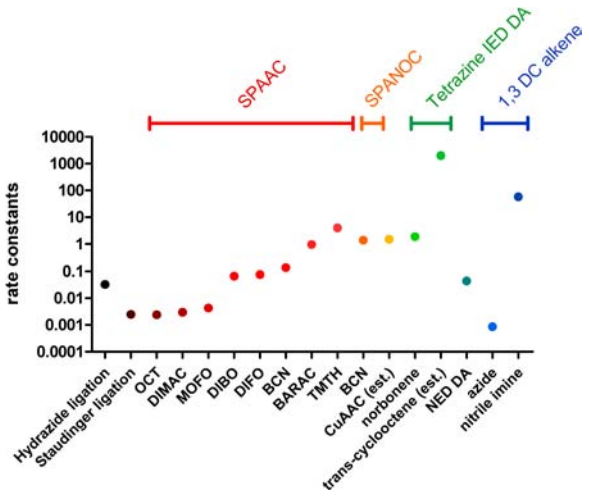
Away from isolated biopolymers moved Hilderbrand et al.,[147] when they treated SKBR-3 cells with the antibodies Herceptin-AF568-TCO and Cetuximab-AF488-DBCO containing *trans*-cyclooctene for tetrazine click and DIBAC for SPAAC. The subsequent labeling with AF647-azide and AF750-tetrazine was then proven by fluorescent colocalization of both dye pairs, constituting the first multitarget bioorthogonal experiment on living cells.

Akin were the intentions of Wittmann et al. when they recently published their work on two colored glycan imaging.[218] To this end, they employed azido- and alkene-saccharides which were incorporated into the cell membrane of HeLa cells by the biosynthetic pathway within 3 days. After labeling with tetrazine and cyclooctyne containing fluorescent probes, the colocalization of fluorescence on the living cells validated the orthogonality of SPAAC and the Diels–Alder reaction.

CONCLUSIONS

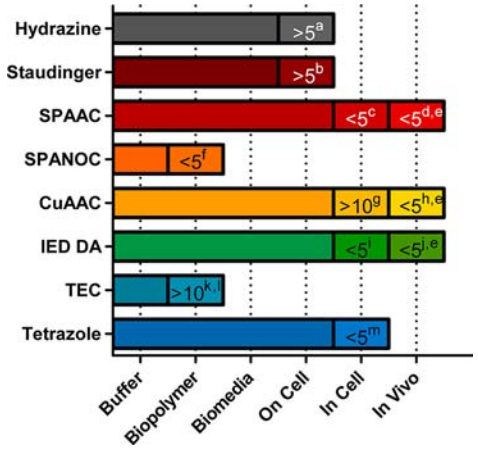
Ever since the definition of the field in 2003, bioorthogonal reactions have been a highly dynamic research field. In recent years, tremendous progress has been made and a wide variety of applications and techniques have been published. The progress of these passed years exhibited growth in mainly three directions. The primary focus thereby lay on kinetic improvement (Scheme 26), in order to complete reaction in ever higher dilution and ever shorter time scale. Furthermore, the selectivity of the reactions has been stressed, to reduce the background labeling and enhance the signal-to-noise ratio. And finally, almost as a byproduct of the continuous improvement of speed and precision, a diversification of reaction mechanisms utilized in bioorthogonal ligation reaction has been occurring.

Scheme 26. Second Order Rate Constant of Several Bioorthogonal Reactions and Reagents



Despite these very promising and encouraging developments, still great challenges need to be met to propagate bioorthogonal chemistry. One of these challenges is the fact that, to date, a great many of reactions have only been demonstrated on selected, isolated biopolymers, thus providing only restricted amounts of evidence on their true degree of bioorthogonality. Furthermore, the main focus until now has been lying on the development of bioorthogonal ligation reactions as a tool for chemical biology, while applications and investigations of biological queries were not yet explored to their full potential (Scheme 27).

Scheme 27. Degree of Bioorthogonal Application of Various Reactions:[a]



[a] (a) Refs 16,18,19,23,54,219,220; (b) Refs 5,43,53,54,221,222; (c) Refs 91,96,107; (d) Refs 99–102; (e) *in vivo* labeling on cell surface; (f) Refs 112,113,117,118; (g) Refs 223–228; (h) Refs 69,70,229; (i) Refs 133,142,143,148; (j) Refs 139; (k) Refs 174,177,178,225; (l) Reviews; refs 172,175; (m) Refs 162,164,165,168.

Therefore, major breakthroughs and trendsetting applications can still be anticipated even after more than 10 years of development of bioorthogonal chemistry. For one, there is a great variety of reactions and reagents described in the literature that are still unexplored and unevaluated for their bioorthogonal properties. For another, there have been only a limited amount of applications of more than one bioorthogonal reaction at a time. Therefore, multitarget investigations with several compatible bioorthogonal reactions will receive increased attention in the years to come.

In summary, it can be said that bioorthogonal reactions have come a long way in the last 10 years and a wide field of applications and improvements lie ahead of them.

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Notes

The authors declare no competing financial interest.

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