Organic & Biomolecular Chemistry

RSC Publishing

PERSPECTIVE

Bioorthogonal labelling of biomolecules: new functional handles and ligation methods

Q1 Cite this: DOI: 10.1039/c3ob41329b

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Received 27th June 2013,
Accepted 6th August 2013

DOI: 10.1039/c3ob41329b

www.rsc.org/obc

This review provides a literature overview of bioorthogonal ligation methods for protein modification, which have largely evolved over the last 15 years. Since 1990, various new reactions have been developed that do not involve naturally occurring functional handles. Especially the development of such so-called bioorthogonal ligations has significantly contributed to our ability to selectively modify biomolecules not only in the test tube, but also in living systems.

1. Introduction

Bioconjugation is the process of linking two or more molecules, of which at least one is a biomolecule. In this way, the properties of individual components are combined.[1] Clearly, there are numerous examples of reactions ligating two or more molecules; however, biomolecule labelling poses important limitations on the reaction conditions that can be applied (solvent, concentration, temperature) and on the available reactive groups.

In general, biological systems flourish best in an aqueous environment meaning that reagents for bioconjugation should be stable in aqueous systems, and reactions need to proceed in water. In addition, biomolecules, and even more so their reactive groups, are present only in low concentrations. Therefore, reaction rates need to be high in order to obtain significant modifications within a convenient time span.

Many methods are known for modifying functional groups which are naturally present in biological systems,[2,3] but logically this poses significant challenges for achieving selectivity and in particular for *in vivo* applications. This realisation has

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Marjoke F. Debets

Dr Marjoke F. Debets (Rotterdam, 1986) received her MSc in chemistry at Radboud University Nijmegen in 2008. During her master's she did several internships, including one in the Synthetic Organic Chemistry group of Prof. Erik Sorensen (Princeton University, Princeton, USA). In June 2013 she received her PhD from Radboud University Nijmegen under the supervision of Prof. Floris Rutjes and Prof. Jan van Hest on the development and application of new bioorthogonal ligation methods. During her PhD she spent three months as a research scholar in the lab of Prof. Carolyn Bertozzi (University of California, Berkeley, USA). Currently, she is performing post-doctoral research in the group of Prof. Hermen Overkleeft (Leiden University, Leiden, NL) working on the synthesis of mannosidase inhibitors.

Jan C. M. van Hesta

Prof. Jan C. M. van Hest (Tilburg, 1968) conducted his doctoral research on molecular architectures based on dendrimers under the supervision of Prof. Bert Meijer (Eindhoven University of Technology, Eindhoven, NL), for which the PhD title was granted in 1996. As a postdoctoral researcher, he investigated the possibilities of protein engineering for the preparation of materials under the supervision of Prof. David Tirrell (University of Massachusetts, Amherst, USA). In 1997 he joined the chemical company DSM (Geleen, NL), where he worked as a group leader on the development of innovative material concepts. In 2000 he was appointed as full professor in Radboud University Nijmegen to set up a new group in bioorganic chemistry which focuses on bioinspired hybrid materials and processes.

led to the fast development of bioorthogonal reactions which, apart from the previously mentioned requirements for bioconjugation, need to be selective for their target, and should not give cross-reactivity with any of the naturally occurring functional groups.

Besides expanding the scope of biomolecule modification, bioorthogonal ligation allows for the selective and controlled introduction of labels, not only in single proteins, but also on cell-surfaces, in living cells and even in live animals. These features open up new opportunities for studying biological processes that do not exist when relying on, for example, more conventional lysine or cysteine labelling.

Although the complete genome of many organisms has been elucidated, many biological processes like post-translational modifications, protein–protein interactions, and protein translocation are not yet fully understood. With new bioorthogonal ligation reactions it has been possible to *e.g.* study protein formation during embryogenesis,[4] image tumours,[5] and track glycan formation and translocation.[6]

In the last decade, research on bioconjugation has grown at an incredible pace, and thousands of papers have been published on new ligation methods or applications thereof. This review offers an overview of the different bioorthogonal ligation strategies available for protein modification. We will thereby focus on azide-related ligation strategies, and more specifically on the Strain-Promoted Azide Alkyne Cycloaddition (SPAAC), since this is one of the most actively pursued ligation methods during the last decade. Furthermore, since the interest in dual labelling has significantly increased recently, we will devote the last section of this review to describing the mutual orthogonality of those bioorthogonal reactions.

Floris P. J. T. Rutjes

Prof. Floris P. J. T. Rutjes (Heiloo, 1966) obtained his MSc in chemistry at the University of Amsterdam (NL), where he also received his PhD under the supervision of Prof. Nico Speckamp in 1993. He then conducted post-doctoral research with Prof. K. C. Nicolaou (The Scripps Research Institute, La Jolla, USA), resulting in a total synthesis of the marine toxin brevetoxin B. In 1995, he was appointed as assistant professor at the University of Amsterdam and, four years later, became full professor in synthetic organic chemistry at Radboud University Nijmegen. His research interests include the application of catalysis (bio-, transition metal- and organocatalysis) in the synthesis of biologically relevant molecules and natural products, and the development of new molecular diagnostic tools for application in life sciences and flow chemistry.

2. Bioorthogonal reactions

Since the first modification of a biological system using an unnatural functional group in 1996,[7] various new bioorthogonal reactions have been published.[8–11] Handles which have been shown to be bioorthogonal and applicable in biological system modification include aldehydes, azides, nitrones, nitrile oxides, diazo compounds, tetrazines, tetrazoles, quadrocyclanes, alkenes, and iodobenzenes. All bioorthogonal reactions used in bioconjugations described in this review are summarized in Table 1.

2.1 Ligations with ketones and aldehydes

The first non-natural functional handle to be used for protein modification was the carbonyl group present in aldehydes and ketones. Although this moiety can react with amines forming imines, in physiological media the carbonyl lies in the favoured site of the equilibrium. On the other hand, ketones and aldehydes do form stable conjugation products with alkoxyamines (Table 1, entry 1) and hydrazines (Table 1, entry 2), forming oxime ethers and hydrazones, respectively.[12] These reactions have been used for the labelling of proteins,[7] including in living cells,[13] virus particles,[14] and cell surfaces.[15]

Recently, Bertozzi *et al.* showed that aldehyde-containing proteins can be modified with a Pictet–Spengler type reaction (Table 1, entry 3). This reaction showed good pH-dependent rate constants varying between 10.5 M^{-1} s^{-1} (pH 4.5) and 0.26 M^{-1} s^{-1} (pH 7). In addition, they compared the stability towards hydrolysis of the formed oxacarboline-linked product to the corresponding oxime-linked product. They found that the oxime-linked product is almost completely hydrolyzed within a week, while the oxacarboline-linkage is stable towards hydrolysis over this period.[16]

2.2 Ligations with azides

Currently, organic azides are the most commonly used reagents in the modification of biological systems. Their popularity can be explained by the inertness and small size of the azide moiety, which therefore generally has only minimal effect on the structure of the substrate in which it is introduced.[17] They are also readily introduced in biomolecules *via e.g.* diazo-transfer onto amines,[18] non-natural amino acid incorporation,[19] or expressed protein ligation.[20–22] Finally and most importantly, azides have a unique reactivity.

Reaction of azides in a Staudinger reduction and the Huisgen 1,3-dipolar cycloaddition were already well established in the previous century; however, these reactions either do not lead to bond formation between the two reaction partners, or require too harsh conditions for protein labelling.

2.2.1 Staudinger ligation. In 2000, Bertozzi *et al.* developed a Staudinger ligation reaction (Table 1, entry 5). This is a ligation between an azide and a triphenylphosphine derivative (PHOS, Table 2, entry 1) and is a modification of the long-known Staudinger reduction which was now applied for the formation of an amide bond *via* rearrangement of the aza-ylide

Table 1 Summary of bioorthogonal reactions used in the modification of biomolecules

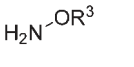
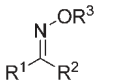
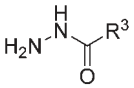
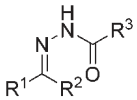
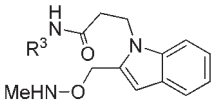
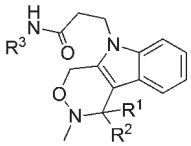
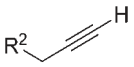
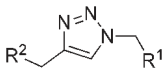
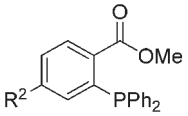
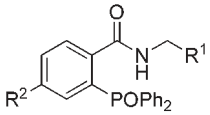
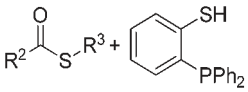
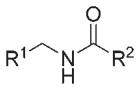
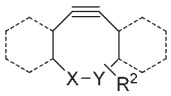
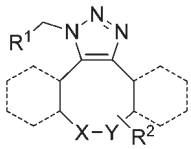
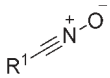
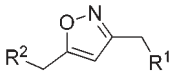
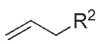
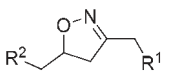
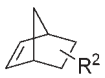
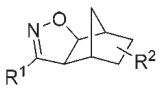
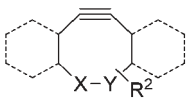
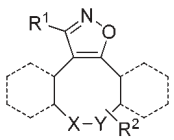
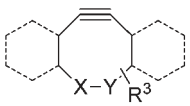
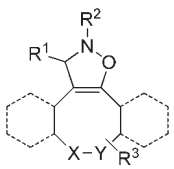
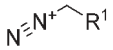
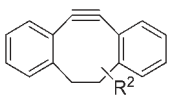
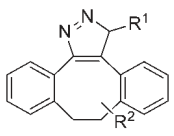
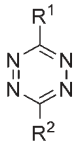
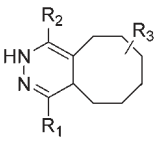
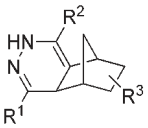
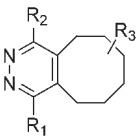
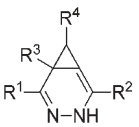
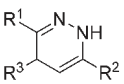
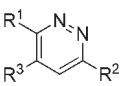
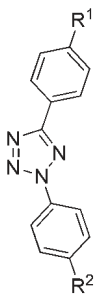
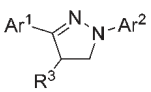
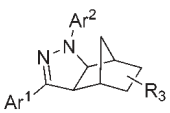
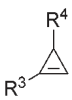
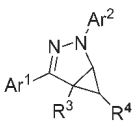
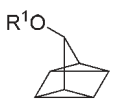
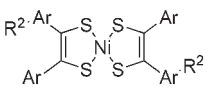
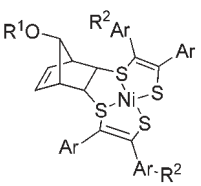
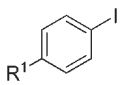
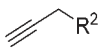
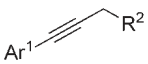
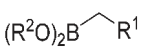
Entry	Functional handle	Reaction partner	Product	Catalyst
1				None
2				None
3				None
4				Cu(I) or Ru(II)[a]
5				None
6				None
7[b]				None
8				Cu(I) or Ru(II)[c]
9[b]				None
10[b]				None
11[b]				None
12[b]				None
13[b]				None

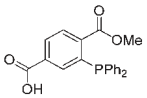
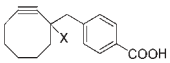
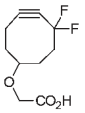
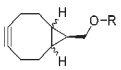
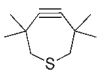
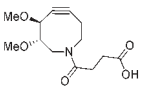
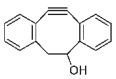
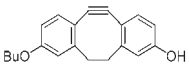
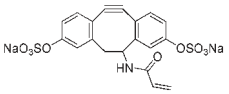
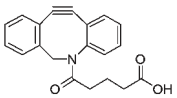
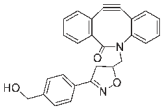
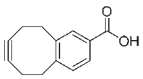
Table 1 *(Contd.)*

Entry	Functional handle	Reaction partner	Product	Catalyst
14[b]				None
15[b]				None
16[b]				None
17[b]				None
18[b]				None
19[b]				None
20[b]				UV-light
21[b]				UV-light
22[b]				UV-light
23[b]				None
24				Grubbs' catalyst
25				$Pd(OAc)_2(ligand)_2$
26				$Pd(OAc)_2(ligand)_2$

[a] Cu(I) catalysis yields the 1,4-isomer, and Ru(II) yields the 1,5-isomer. [b] Both regioisomers are obtained, although only one is shown in the table. [c] Catalyst is only required when regioselective control is desired, Cu(I) yielding the 1,4-isomer and Ru(II) yielding the 1,5-isomer.

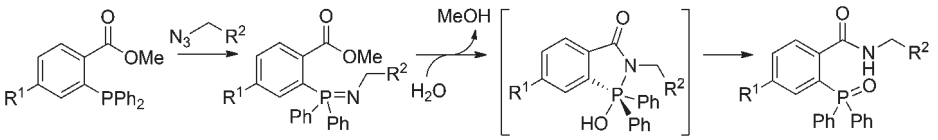
intermediate.[23] Key to the Staudinger ligation is the intramolecular trapping of a nucleophilic aza-ylide by an electrophilic ester, leading to a covalent amide bond *via* a putative pentacoordinate phosphine intermediate (Scheme 1).[24] This reaction proceeds with a rate constant of 2.4×10^{-3} M^{-1} s^{-1}. An alternative was developed by Raines and co-workers, who combined the Staudinger reduction with native chemical ligation (NCL) (Table 1, entry 6).[25] The power of the Staudinger ligation for *in vitro* applications was elegantly demonstrated in the cell-surface labelling of Jurkat and HeLa cells.[23] The Staudinger ligation has also been applied for cell surface remodelling,[26] live-cell imaging,[27] and the visualisation of *O*-linked

Table 2 Overview of azide-reactive probes, synthesis, rate constant and lipophilicity

Entry	Azidophile	Name	Synth. steps	Yield [%]	k^a [$\times 10^{-3}$ M^{-1} s^{-1}]	Solvent	log $P^{b,c}$	Ref.
1		PHOS	2	50	2.1	CD_3CN	4.74	23
2	 **OCT 1**: R = CH_2PhCO_2H **OCT 2**: R = CH_2CO_2H	OCT 1	4	52	2.4	CD_3CN	3.21	33
3		OCT 2	5	36	1.3	CD_3CN	1.65	52
4	 **OCT 3**: R = H **MOFO**: R = F	OCT 3	4	12	1.2	CD_3CN	3.77	52
5		MOFO	5	15	4.3	CD_3CN	3.76	52
6		DIFO 1	10	1.2	76	CD_3CN	1.28	6
7	 **DIFO 2**: R = CH_2PhCO_2H **DIFO 3**: R = CH_2CO_2H	DIFO 2	8	27	42	CD_3CN	4.14	53
8		DIFO 3	10	21	52	CD_3CN	1.71	53
9		BCN	4	15/35[c]	110/140[c]	CD_3CN–D_2O (3 : 1)	2.01	50
10		TMTH	6	0.8	4000	CD_3CN	4.00	61
11		DIMAC	11	5	3	CD_3CN	0.66	62
12		DIBO 1	5	10	57	CH_3OH	4.40	63
13		DIBO 2	5	9	76.3	CH_3OH	5.96	64
14		S-DIBO	6	18	112	CH_3OH	0.59	65
15		DIBAC	9	41	310	CD_3OD	3.51	67
16		BARAC	6	18	960	CD_3CN	4.20	51
17		COMBO	6	11	240	CD_3CN	2.19	74
18	 **OXA 1**: R = H **OXA 2**: R = Me	OXA 1	2	59	0.85	CD_3OD	0.27	80
19		OXA 2	2	58	0.41	CD_3OD	0.78	83

[a] 2nd order reaction rate constant determined with benzyl azide as the model, unless otherwise indicated. [b] Calculated by BioByte (embedded in ChemBioDraw 12.0) for the *N*-methylamide derivative of carboxylic acids and the *N*-methyl carbamate derivative of alcohols. [c] *Exo*-BCN/*endo*-BCN.

glycosylation in live mice.[28] Mice were labelled with azides *via* the metabolic incorporation of azide-functionalised sugars. After 8 days the mice were sacrificed and different organs were labelled with PHOS-FLAG followed by FITC-α-FLAG[29] followed by fluorescence imaging or the organs were harvested and homogenised followed by treatment with PHOS-FLAG and analysis by Western Blot using horseradish peroxidase-α-FLAG. Labelling was observed in different organs, *e.g.* liver, kidney,



Scheme 1 Staudinger ligation of a 2-(diphenylphosphoryl)benzoic ester with an alkyl azide.

heart, and serum. In addition, it was observed that when mice were treated with peracetylated *N*-azidoacetylmannosamine (Ac_4ManNAz) different glycans were labelled than when mice were treated with peracetylated *N*-azidoacetylgalactosamine (GalNAz). Later, it was shown that the Staudinger ligation can also be used to label the azido-sugars in live mice.[30] The Staudinger ligation has also been used for protein and DNA modification, and has been applied in activity-based profiling.[31] A nice addition to the Staudinger ligation potential was made by the development of a fluorogenic Staudinger probe, which turns fluorescent upon ligation.[27]

The alternative Staudinger ligation developed by Raines *et al.* requires a thioester as one of the ligating components, which significantly reduces the scope of the reaction. Nonetheless, this ligation found application in the attachment of proteins to a surface.[32]

Although the Staudinger ligation developed by Bertozzi *et al.* has proven to be a selective and efficient reaction, some specific disadvantages can be recognised. First of all, because phosphines are prone to oxidation by air, shelf-stability is limited and oxidation may take place before ligation. Secondly, the Staudinger ligation is relatively slow.[24] Unfortunately, improvement of the kinetics and water solubility proved troublesome.[33]

2.2.2 Cu-catalysed 1,3-dipolar cycloaddition (CuAAC). The discovery by Meldal[34] and Sharpless[35,36] that the 1,3-dipolar cycloaddition of azides and acetylenes can be catalysed by sources of copper(I) (Table 1, entry 4) has been of tremendous value for organic synthesis,[37] library synthesis of pharmacophores,[38] and bioorthogonal ligations. Over the last decade, the CuAAC has been used in protein modification,[39] DNA and RNA modification,[40] and glycan imaging.[41] Although cycloaddition has found wide application in chemical biology research, the required Cu(I)-catalyst has appeared to be toxic to living systems, causing cell death, preventing cell division, and killing zebrafish embryos.[2] Cu-ions are toxic mainly because they catalyse the production of reactive oxygen species from atmospheric oxygen.[42] It was recently shown that the addition of certain ligands can not only increase reaction speed but also significantly reduce the toxic effects.[43] Finn *et al.* showed that when THPTA (Fig. 1) was used as a ligand in a 5 : 1 ratio compared to Cu(I) all cell types tested survived and showed consistent labelling using CuAAC. In this manner, they labelled live cells and showed that labelling did not affect glycan trafficking.[42] Wu showed that a further improved ligand (BTTES, Fig. 1) even made CuAAC applicable for live zebrafish labelling. Alkyne functionalities were introduced by incorporating alkyne-containing sugars and zebrafish were subsequently

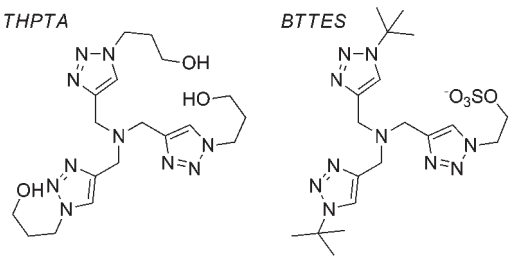


Fig. 1 Cu(I)-ligands used in CuAAC for *in vitro* and *in vivo* applications.

fluorescently labelled by the addition of an azide-dye and BTTES–Cu(I) complex.[44]

Although toxicity issues of Cu(I) can be solved by the addition of ligands, bioorthogonal labelling without the need of a metal catalyst is more straightforward. Especially in larger animals, colocalisation of the catalyst, azide, and alkyne reagents is probably hard to achieve. In addition, some applications, *e.g.* ligation of metal-chelators or modification of metal-binding enzymes, require metal-free conditions.

2.2.3 Strain-promoted azide alkyne cycloaddition (SPAAC). Already in 1953, Blomquist and Liu published the first synthesis of cyclooctyne and its suspected ring-strain, which became apparent from the reported *explosive* reaction of cyclooctyne and phenyl azide.[45] The structural identity of the resulting liquid was later corroborated as the product of a 1,3-dipolar cycloaddition.[46] Ever since, many different cycloalkynes have been reported. It was noted that a cyclooctyne is the smallest cycloalkyne which can be isolated. However, when bigger atoms are added (*e.g.* Si or S), also cycloheptynes can be isolated, especially when stabilised by the addition of methyl-groups adjacent to the alkyne.[47]

In spite of all this work, mainly performed in the 1960s and 1970s, it was not until 2004 that Bertozzi and co-workers realised the potential of these strained alkynes in the strain-promoted azide alkyne cycloaddition (SPAAC, Table 1, entry 7).[33] Ever since Bertozzi *et al.* demonstrated that cyclooctynes react spontaneously with azides in SPAAC and that this reaction could be used for cell-surface labelling, many new ring-strained systems have been published.[48,49] An overview of the published strained azide reactants is given in Table 2.

The following section will describe the development of the different azide-reactive strained alkynes. Considering (dibenzo)cyclooctynes, it must be noted that, although much progress has been made in the development of more reactive cyclooctynes, these improvements often came with a price. Some of the faster cyclooctynes are not shelf-stable (BCN)[50] or

show significant cross-reactivity with thiols (BARAC[51] and DIFO).[6] Also, most cyclooctynes are highly hydrophobic, and need to be prepared *via* lengthy and/or low yielding synthetic routes (Table 2). In fact, for many of these systems hydrophilicity and reaction kinetics are inversely related, as is shown in Fig. 3 where a plot is shown of all rate constants *versus* lipophilicity. Lastly, the crucial step in all cyclooctyne syntheses is the last, alkyne-forming step, which is the most troublesome step. This renders cyclooctyne synthesis a cumbersome and time-consuming process.

2.2.3.1 Cyclooctynes. The first cyclooctynes developed were OCT 1 (Table 2, entry 2) and OCT 2 (Table 2, entry 3) which could be synthesised in four steps (52 and 10% yield, respectively). Compared to the Staudinger ligation (Table 2, entry 1) both probes were significantly less prone to oxidation, but nevertheless not fully shelf-stable since prolonged storage of OCT 1 and OCT 2 at −20 °C led to decomposition. The developed 3-alkoxycyclooctynes showed rate constants for the cycloaddition with benzyl azide comparable to those of the Staudinger ligation ($k = 2.4 \times 10^{-3}$ M^{-1} s^{-1} for OCT 1 and $k = 1.3 \times 10^{-3}$ M^{-1} s^{-1} for OCT 2).[33]

To increase the stability of the 3-alkoxycyclooctyne derivatives, an alternative was developed with the alkyl-linked cyclooctyne conjugate OCT 3 (Table 2, entry 4).[52] Indeed, the latter was found to be more stable than OCT 1; however, the reactivity of OCT 3 towards azides ($k = 1.2 \times 10^{-3}$ M^{-1} s^{-1}) was also two-fold lower.

Since the alkyl-linked cyclooctyne was found to be more stable but less reactive than earlier cyclooctynes, a fluoride substituent was introduced adjacent to the acetylene.[52] It was assumed that lowering of the LUMO of the acetylene by a neighbouring electron-withdrawing group would increase the reactivity towards azides. Indeed MOFO (Table 2, entry 5) (synthesised in 15% yield over five steps) displayed a four-fold enhanced reactivity towards benzyl azide ($k = 4.3 \times 10^{-3}$ M^{-1} s^{-1}).[52] Surprisingly, when this probe was applied in cell-surface labelling and compared to OCT and Phos, the Staudinger ligation still outperformed the cyclooctynes, despite the better kinetics of MOFO. Possibly, the Staudinger ligation is more efficient with azides bearing electron-withdrawing or resonance-stabilising groups.[52]

The fact that monofluorination of the cyclooctyne system enhanced the reactivity was a stimulus for Bertozzi *et al.* to synthesise a difluorinated cyclooctyne (DIFO 1, Table 2, entry 6).[6] Although synthesis was troublesome and DIFO 1 was obtained in poor overall yield (1.2% over ten steps), this was fully compensated for by the observed rate constant. DIFO 1 reacted with benzyl azide with a rate constant of 7.6×10^{-2} M^{-1} s^{-1}, about 17-fold faster than the fastest previously reported Cu-free ligation. The high reactivity of DIFO 1 was also reflected in staining of Jurkat cells bearing SiaNAz with biotinylated DIFO 1, which proceeded with a 20-fold higher efficiency than with other cyclooctynes.

The great potential of DIFO 1 validated an alternative synthetic route. To avoid the cumbersome elimination step to the alkyne, Bertozzi *et al.* developed a synthesis with the ether linkage replaced by an alkyl linker.[53] Notably, elimination of the triflate now proceeded with excellent yield, and the total synthesis of DIFO 2 (Table 2, entry 7) was completed in eight steps with an overall yield of 27%.[53] Another DIFO-derivative, lacking the phenyl ring in the linker (DIFO 3, Table 2, entry 8), was also prepared (ten steps, 21% overall yield) to reduce non-specific protein and cell binding as a result of the lipophilic character of DIFO 2.

Kinetic experiments of DIFOs 2 and 3, termed 2nd generation DIFOs, with benzyl azide revealed rate constants of 4.2×10^{-2} M^{-1} s^{-1} for DIFO 2 and 5.2×10^{-2} M^{-1} s^{-1} for DIFO 3, slightly lower but similar to 1st generation DIFO. The relative rate constants were also reflected in the labelling of Jurkat cells that showed a 3-fold higher fluorescence for DIFO 1 than for DIFOs 2 and 3. As expected, with respect to DIFO 2, slightly less background labelling was observed for DIFO 3, lacking the hydrophobic phenyl group, in flow cytometry experiments. It must however be noted that DIFO was found to be less stable in serum compared to dibenzocyclooctynes, possibly due to its susceptibility towards Michael addition.[54,55]

A different way to attain a rate increase for cyclooctynes was published by van Delft *et al.*[50] They envisioned that installing a cyclopropane ring in the cyclooctyne opposite to the triple bond would result in additional ring strain and thereby increased kinetics. BCN (Table 2, entry 9) could be prepared in four steps, yielding both the *exo*- (15% yield) and *endo*-isomers (35% yield). The cyclopropane ring indeed increased ring-strain and thereby the rate constant. With rate constants of 0.11 M^{-1} s^{-1} for *exo*-BCN and 0.14 M^{-1} s^{-1} for *endo*-BCN, BCN is the fastest cyclooctyne known. Nonetheless, BCN was found to have limited shelf-stability, even at lower temperatures.[50,56]

Another alternative for increasing ring-strain is by decreasing the ring size. Benzyne-rings have been prepared and used in, among others, 1,3-dipolar cycloadditions. However, benzyne needs to be made *in situ* and cannot be isolated.[57–60] On the other hand, Krebs had already summarised in 1983 that some heteroatom-containing cycloheptynes can be prepared and isolated.[47] Only recently, Bertozzi *et al.* showed the synthesis of a thiacycloheptyne (TMTH, Table 2, entry 10) in 0.8% yield over six steps.[61] The rate constant of the reaction of TMTH with benzyl azide was 4 M^{-1} s^{-1}. For comparison purposes, also thiacyclooctyne was prepared, showing a rate constant of 3.2×10^{-4}, which is one order of magnitude lower than the rate observed for OCT 1. The effect of decreasing the size of the alkyne-containing ring with one atom was thus shown to be at least four orders of magnitude, although it is likely that the rate constant of thiacycloheptynes would even be higher without the adjacent methyl groups. Unfortunately, TMTH could not be equipped with a label; protein modification was however confirmed by mass spectrometry.

The cyclooctynes, as described above, all consist of only carbon atoms in the ring and have, without exception, negligible water solubility. Furthermore, background labelling was observed in biological experiments due to hydrophobic interactions with cell membranes and non-specific binding to serum proteins cannot be excluded. Therefore, Bertozzi *et al.*

set out to develop a more hydrophilic cyclooctyne by introduction of a nitrogen to break the hydrophobic character of the ring.[62] In addition, two methoxy groups were introduced to enhance polarity and water solubility. DIMAC (Table 2, entry 11) showed a kinetic constant of 3.0×10^{-3} M^{-1} s^{-1}, slightly faster than parent OCT 1, but significantly lower than DIFO 1. Nonetheless, DIMAC was found to be suitable for detection of metabolically incorporated ManNAz in Jurkat cells. As expected, background labelling was significantly lower than that for cyclooctyne OCT 2.

2.2.3.2 Dibenzocyclooctynes. Another interesting class of highly strained alkynes are the (di)benzocyclooctynes. Boons *et al.* envisioned that the introduction of fused aryl rings into the cyclooctyne system would enhance reaction kinetics by increased ring-strain and conjugation.[63] To this end, they designed DIBO 1 (Table 2, entry 12), and prepared it in five steps with an overall yield of 10%. In kinetic experiments, cycloaddition of DIBO with benzyl azide proceeded with a rate constant of $k = 5.67 \times 10^{-2}$ in methanol, about the same as that for DIFO 1.

In a follow-up paper by Popik *et al.*, a photo-triggerable cyclopropenone system was developed that formed the alkyne functionality upon UV-irradiation at 350 m.[64] Conveniently, the major product was isolated with one butyl group hydrolysed, thereby providing a handle for subsequent functionalisation.

While the cyclopropenone showed no reaction with azides, irradiation at 350 m resulted in the rapid formation (1 in) of a cycloalkyne (DIBO 2, Table 2, entry 13) that reacted with benzyl azide with a rate constant of 7.63×10^{-2} M^{-1} s^{-1}, as determined with a newly developed UV-based procedure.

In addition, recently a disulfated DIBO (s-DIBO, Table 2, entry 14) analogue was prepared, showing impressive water solubility compared to DIBO.[65] In addition Boons *et al.* found that s-DIBO did not cross the cell membrane whereas DIBO did. s-DIBO could thus be used for selective labelling of membrane glycoproteins.

Disadvantages of all the above described dibenzocyclooctynes are that, again, all ring-members are carbon atoms, and labelling of the hydroxyl *via* an ester- or carbamate linkage gave rise to problems with hydrolysis when labelling lipases.[66] Therefore, van Delft *et al.* envisioned that introduction of a nitrogen atom into the octyne-ring would break the hydrophobic character of the ring and allow for more stable amide-bond functionalization. This resulted in the preparation of dibenzoazacyclooctyne (DIBAC, Table 2, entry 15) in 41% yield over nine steps.[67] Kinetic experiments with benzyl azide showed an over 4-fold higher rate constant compared to DIBO 1 in reaction with benzyl azide in CD_3OD (k = 0.31 M^{-1} s^{-1}).

The rate of dibenzoazacyclooctynes could be further increased by the introduction of an amide bond into the eight-membered ring. This was pursued by Bertozzi *et al.* who prepared BARAC (Table 2, entry 16) in six steps and an overall yield of 18%.[51] BARAC reacted with benzyl azide with a rate constant of 0.96 M^{-1} s^{-1}.

However, BARAC is susceptible to Michael addition with thiols, though at a much lower rate than its cycloaddition with azides. Modelling studies by Houk *et al.* showed that the rate increase was caused by increased bond distortion of the alkyne bonds.[68]

The route towards BARAC was proven to be a highly versatile one, as several analogues with substituents on the benzene rings could be prepared by following the same synthetic pathway.[68] In this research, it was shown that electron-withdrawing and electron-donating groups on the aromatic rings have a positive effect on the rate constant. As expected, the electron-withdrawing groups showed a larger effect on the reaction speed than electron-donating groups. Remarkably, *ortho*-substitution with respect to the alkyne resulted in an impressive drop in cycloaddition reactivity.

A non-functionalisable dibenzocyclooctadiyne has long since been known and used in organic synthesis, for *e.g.* the preparation of extended aromatic systems, cyclophane synthesis and metal-complex formation.[69–71] This compound can react with azides twice, which theoretically would eliminate the need to introduce a functional handle as one of the two azides could be a functional handle. However, it was observed that the triazole-containing compound formed in the first cycloaddition is more reactive to a second cycloaddition to such an extent that always the bistriazole is formed. Nevertheless, Hosoya *et al.* showed that when used for protein modification the sterics of the protein avoid dimerization of the protein, leaving one highly strained alkyne intact.[72] This alkyne can subsequently be used for protein labelling in a second strain-promoted reaction.

Alternatively, some monobenzocyclooctynes have been prepared to decrease the hydrophobicity of the probes, but maintain the reactivity. Bertozzi *et al.* prepared MOBO and a difluorinated variant (DIFBO). MOBO showed a rate constant of 9.5×10^{-3} M^{-1} s^{-1}, about one order of magnitude slower than DIBO. On the other hand, as expected, introduction of two fluorides resulted in a 20-fold rate increase (0.22 M^{-1} s^{-1}). However, DIFBO could not be isolated unless stabilised by the addition of β-cyclodextrin.[73] Recently, Kele *et al.* synthesised COMBO (Table 2, entry 17), which has the benzene ring opposite to the alkyne. COMBO has a rate constant of 0.23 M^{-1} s^{-1}, almost identical to DIFBO and about 2-fold higher than the closely resembling BCN.[74]

2.2.3.3 Fluorogenic dibenzocyclooctynes. A separate class of dibenzocyclooctynes is formed by the fluorogenic probes. These molecules are not fluorescent in the alkyne state, but become fluorescent upon reaction with azides. This has several advantages over non-fluorogenic cyclooctynes. First of all, no fluorescent label needs to be introduced, thereby excluding the need of introducing a functionalisable handle which may compromise alkyne formation. In addition, a specific labelling, which is often a problem in biological experiments, is eliminated as no triazole formation is required for fluorescence to occur.

Fluorogenic azides, turning fluorescent upon cycloaddition, have been published. The first was based on coumarin, but

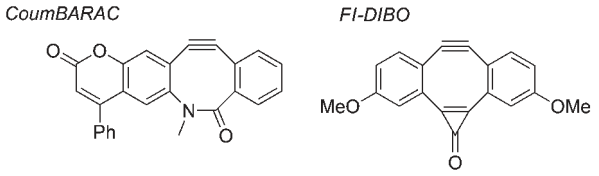


Fig. 2 Fluorogenic cyclooctynes.

such dyes are relatively unstable and the emission wavelength is not suitable for biological purposes.[75,76] Recently, Bertozzi *et al.* published a fluorogenic azide based on fluorescein, which proved suitable for cell-surface labelling.[77] In addition to fluorogenic azides, a fluorogenic coumarin-based alkyne was reported.[75] Inspired by this alkyne, Bertozzi *et al.* developed a fluorogenic cyclooctyne based on BARAC, coumBARAC (Fig. 2). This compound was prepared in 2% yield over six steps.[78] The dibenzocyclooctyne had, however, a similar excitation pattern to other coumarins and displayed only a moderate quantum yield. Another fluorogenic cyclooctyne (Fl-DIBO, Fig. 2) was published by Boons *et al.*[79] While the synthesis proceeded more smoothly (35% yield over five steps), the excitation pattern was similar to coumBARAC, still not overlapping with commonly used excitation wavelengths in biology research.

2.2.3.4 Oxanorbornadienes. The concept of cycloaddition of azides to strained bonds was also investigated for oxanorbornadienes by Rutjes *et al.*[80] It was envisioned that these oxanorbornadienes, apart from thiol-addition as described above,[81] could undergo cycloaddition with azides. Ju *et al.* already discovered that the spontaneous reaction of electron-deficient alkynes with azides proceeded in good yields under aqueous conditions.[82] Formation of an oxanorbornadiene from one of these electron-deficient alkynes yielded OXA 1. Treatment of OXA 1 (Table 2, entry 18) with an alkyl azide gave a triazoline intermediate that underwent retro-Diels–Alder reaction, leading to the triazole products and furan (Scheme 2). It was found that OXA 1 is partly susceptible to cycloaddition of the azide on the other double bond (Scheme 2). Introduction of a methyl-substituent (OXA 2, Table 2, entry 19), by initial reaction with 3-methylfuran, effectively eliminated the latter disadvantage.[83] While the rate constant of cycloaddition of OXA 1 with azide is relatively low

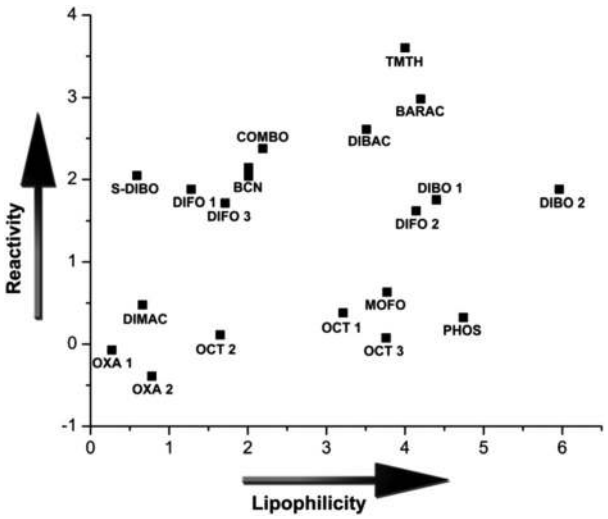
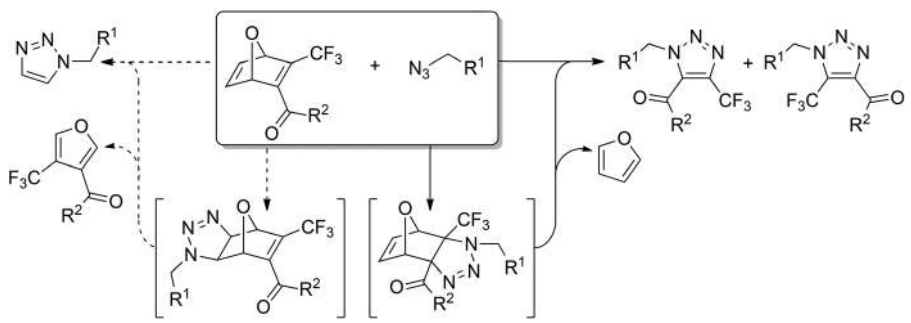


Fig. 3 Reactivity *versus* lipophilicity of functionalized cyclooctynes, based on Table 2.

($k = 8.5 \times 10^{-4}$ M^{-1} s^{-1}), and even slightly lower for OXA 2 ($k = 4.2 \times 10^{-4}$ M^{-1} s^{-1}), advantages of the oxanorbornadiene system are the easy preparation and high water solubility. The high hydrophilicity of OXA 1 and OXA 2 is clearly shown in Fig. 3. This figure shows the relation between the rate constant and lipophilicity of all azide-reactive probes, summarised in Table 2. It also shows that in general the more reactive a probe is, the more hydrophobic it will become. An outstanding example is s-DIBO, which shows a similar reactivity to BCN, DIFO and DIBO, but has a hydrophilicity compared to DIMAC and OXA, due to the introduced sulfate-groups.

2.2.4 Applications of SPAAC. The reaction between azides and highly strained probes has found wide application in many research areas. Probably, the numerous ways for azide introduction, combined with its stability and small size, render the azide a highly desirable handle for modification. In addition, due to the high research interest in cyclooctyne development, many fast cyclooctynes have been developed, allowing for fast labelling at low concentrations.

In the last decade numerous applications of SPAAC have been published;[84] describing all these examples would be too elaborate for this review. In short, SPAAC has been used for



Scheme 2 Cycloaddition of oxanorbornadiene with azide, followed by a retro-Diels–Alder reaction.

live cell labelling, protein labelling, lipid labelling, monitoring of non-natural amino acid incorporation, studying protein interactions, surface modification, preparation of radiolabelled substrates, polymer conjugation, proteomics, and *in vivo* imaging.[9,17,49,85]

An especially appealing field of applications is of course the *in vivo* applications of SPAAC, as these allow for direct visualisation of biological processes.

Bertozzi *et al.* showed that SPAAC could be used for the tracking of glycan synthesis in zebrafish.[4] Growing zebrafish in the presence of GalNAz, to incorporate azide handles in glycoproteins, followed by labelling resulted in observed glycan formation 24 hours post fertilisation. Especially between 60 and 72 hours after fertilisation a large increase in fluorescence was observed in the jaw region, pectoral fins, and olfactory organs. Further investigation was performed by labelling with different dyes at separate time points, thereby increasingly elucidating the synthesis of glycans in live zebrafish. Later it was shown that glycans could be imaged as soon as 7 hours post fertilisation, albeit that the azido-sugar needed to be micro-injected.[86]

It was also shown that SPAAC can be used for the labelling of mice splenocytes. To this end, mice were fed with azido-sugars for 7 days, whereupon different FLAG-labelled cyclooctynes were administered. After three hours, the mice were sacrificed and different splenocytes were analysed for labelling. Using different azide-reactive probes (PHOS, DIMAC, and DIFO) labelling of proteins in liver, heart and intestines was possible. Notably, when OCT 2 was used no protein labelling was observed, likely due to the low kinetics and limited absorption from the peritoneal cavity (where the cyclooctynes were injected) into the bloodstream.[30]

Recently, SPAAC was also used in mice to attach a *trans*-cyclooctene (TCO) to GalNAz-containing tumour cells. Subsequently the TCO was used for fluorescent labelling using an inverse-electron Diels–Alder reaction (described in Section 2.4).[87]

2.3 Ligations with other 1,3-dipoles

Compared to azides, nitrile oxides and nitrones are stronger 1,3-dipoles, making them faster partners in cycloadditions. Unfortunately, this also renders them less stable and therefore more troublesome in biochemical applications. For example, nitrile-oxides require *in situ* formation just before use[88] and nitrones can, due to their instability, not be introduced to cell surfaces.[89] An advantage of using nitrile oxides is that, because of their increased reactivity, they react with simple terminal alkynes without the need of a catalyst (Table 1, entry 8). This feature has mainly been applied by Heaney *et al.* for the modification of ribonucleosides.[90] In addition, Ravoo and co-workers showed that this reaction can be used for surface modification.[91] Carell *et al.* demonstrated that nitrile oxides can also be used for the reaction with alkenes (Table 1, entry 9) and norbornenes (Table 1, entry 10). Here, nucleotides were equipped with norbornenes or alkenes and were subsequently used in the synthesis of oligonucleotides, and lastly reacted with *in situ* formed nitrile oxides.[92,93] In the study involving alkene–nitrile oxide ligation, not only the alkene-functionality but also alkyne-groups were introduced. It was shown that the alkynes could first be labelled using CuAAC whereupon the alkenes were labelled with nitrile oxides.[93]

Most recently it was shown that nitrile oxides also react with cyclooctynes (so-called SPANOC, Table 1, entry 11).[88,94] This reaction showed almost a 100-fold rate increase compared to SPAAC, and was used for the modification of oligosaccharides,[94] small peptides,[88] and DNA immobilised in solid phase.[95]

A disadvantage of using nitrile oxides is that their reactivity also makes them unstable, and *in situ* preparation directly followed by cycloaddition is required. Nitrones, on the other hand, appear to be more stable and still react in a fast and selective manner with cyclooctynes in a strain-promoted alkyne nitrone cycloaddition (SPANC, Table 1, entry 12).[89,96]

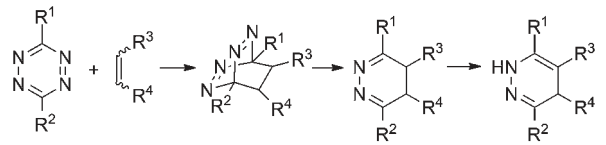
For this reaction, the reactivity largely depended on the nature of the nitrone, with rate constants ranging within 3 orders of magnitude. Cyclic nitrones were found to be most efficient.[97,98] SPANC has found multiple applications in cancer research. Pezacki *et al.* targeted human breast cancer cells with epidermal growth factor (EGF), which was functionalised with cyclic nitrones using NHS-based chemistry. The cells could subsequently be labelled using a biotin-functionalised cyclooctyne, and imaged with a fluorescent streptavidin.[97] Prosperi and co-workers showed that SPANC could be used for the attachment of nitrone-functionalised antibodies to magnetic nanoparticles. After modification, these particles still showed affinity for the tumour marker HER2.[99]

Raines *et al.* recently published that diazo compounds can also react in a 1,3-dipolar cycloaddition with a dibenzocyclooctyne (DIBONE,[100] Table 1, entry 13).[101] The diazo compounds were prepared by treating an azide with a phosphinoester. Depending on the electron delocalisation of the dipole, the reaction rate varied from 10-fold higher compared to the corresponding azide for a highly delocalised diazo compound, to over 10-fold lower for an electron-deficient diazo compound.

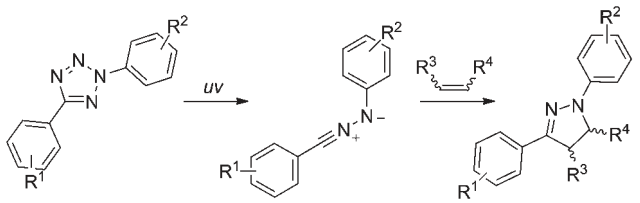
2.4 Ligations with tetrazines

An interesting alternative to the 1,3-dipolar cycloadditions described above is tetrazine-based ligations. These reactions are inverse electron demand Diels–Alder reactions, followed by a retro-Diels–Alder reaction, eliminating N_2, shown in Scheme 3. As for the reaction between cyclooctynes and azides, the literature already shows extensive investigations into cycloadditions between tetrazines and strained alkenes and alkynes. In these reports, the effect of ring strain, double bond stereochemistry, and the influence of heteroatoms in rings on the reaction rate have already been described.[102,103] In spite of this research, only in 2008 Hilderbrand and Fox almost simultaneously published the modification of proteins and cell surfaces using tetrazine-based ligations (Scheme 3).

Hilderbrand and co-workers demonstrated that norbornenes react with tetrazines (Table 1, entry 15) with similar rates (1.6–1.9 M^{-1} s^{-1})[104] as the fastest SPAAC reactions



Scheme 3 Inverse-electron demand Diels–Alder reaction, followed by a retro-Diels–Alder reaction.



Scheme 4 Formation of nitrile imines from tetrazoles, followed by a 1,3-dipolar cycloaddition with alkenes.

($1.5\ M^{-1}\ s^{-1}$ being the fastest rate reported).[68] This reaction was successfully applied in the labelling of tumour cells.

Fox *et al.* showed that tetrazines react extremely fast with different *trans*-cyclooctenes (Table 1, entry 14, rate constants of $1–22 \times 10^3\ M^{-1}\ s^{-1}$) compared to the SPAAC reaction.[105,106] This reaction has, for example, been applied for the intracellular labelling of the anti-cancer drug taxol, and the preparation of ^{18}F-PET tracers.[107] Interestingly, the incredible speed of these reactions also allowed for pretargeting. It was shown that sequential incubation of tumour cells with *trans*-cyclooctene-labelled antibodies, followed by tetrazine-functionalised nanoparticles, led to a higher degree of labelling as for the full antibody-nanoparticle construct.[108] Rossin *et al.* showed the potential of this reaction for *in vivo* labelling. They pretargeted tumors in mice with *trans*-cyclooctene functionalised antibodies, and then separately injected an In^{3+}-labelled tetrazine to show successful tumour labelling.[5] An advantage of this last method is that the small tetrazine is cleared within several hours from the blood, whereas the full antibody takes over 24 hours before clearance. This significantly decreases the time between injection and imaging, which especially might turn out to be useful for fast decaying radioisotopes. A similar observation was made by Brindle *et al.* who could increase the signal-to-noise ratio by using the TCO-tetrazine cycloaddition instead of SPAAC for label introduction.[87]

In addition, it was recently shown that tetrazines react with cyclooctynes (Table 1, entry 16),[109] which was applied for protein modification.[110,111] Interestingly, monosubstituted tetrazines do react at a much lower rate with dibenzocyclooctynes, compared to cyclooctynes. Disubstituted tetrazines give no cycloaddition product at all with dibenzocyclooctynes. Another interesting, newly arisen probe for tetrazine-based ligation is the cyclopropene (Table 1, entry 17),[113] which already found applications in cell surface labelling.[113–115] Most recently, it was also shown that tetrazines can react with alkenes (Table 1, entry 18)[116] and isonitriles (Table 1, entry 19),[117] and both reactions were successfully used in cell surface labelling.

Notably, not all tetrazines are stable in serum, some degrading in water and/or by the addition of thiols. Hilderbrand *et al.* prepared a wide range of tetrazines and tested them for reactivity and serum stability.[118] In addition, they prepared tetrazines which become fluorescent upon reaction.[119]

2.5 Ligations with tetrazoles

Tetrazoles themselves are no reactive partners in cycloaddition reactions. However, it was shown that upon irradiation with UV-light, nitrogen is excluded and a nitrile imine 1,3-dipole is formed.[120] In the absence of a reactive partner for this dipole, formation of a water-adduct was observed; however, in the presence of acrylamides (Table 1, entry 20),[121] norbornenes (Table 1, entry 21)[122] and cyclopropenes (Table 1, entry 22)[123] the nitrile imines rapidly form pyrazolines, as shown in Scheme 4. The reactivity of the 1,3-dipole depends on the substituents on the nitrile imine.[124] It was shown that this reaction can be used for the labelling of proteins, also inside cells,[125] for example towards studying perturbing protein localisation.[126] Notably, the reaction only occurs upon UV irradiation, thereby allowing temporal and spatial control over the ligation.

2.6 Other bioorthogonal ligation reactions

In the last couple of years, also metal-catalysed reactions have found applications in the modification of proteins. For example, Davis *et al.* showed that cross-metathesis can be used for protein modification (Table 1, entry 24). For this modification, an *S*-allyl cysteine or Se-allyl functionality is required which reacts with allyl-functionalized saccharides or small ethylene glycol tails.[127,128] It was also demonstrated that the Suzuki–Miyaura coupling (Table 1, entry 26)[129] and the Sonogashira reaction (Table 1, entry 25)[130] can be applied for the labelling of proteins.

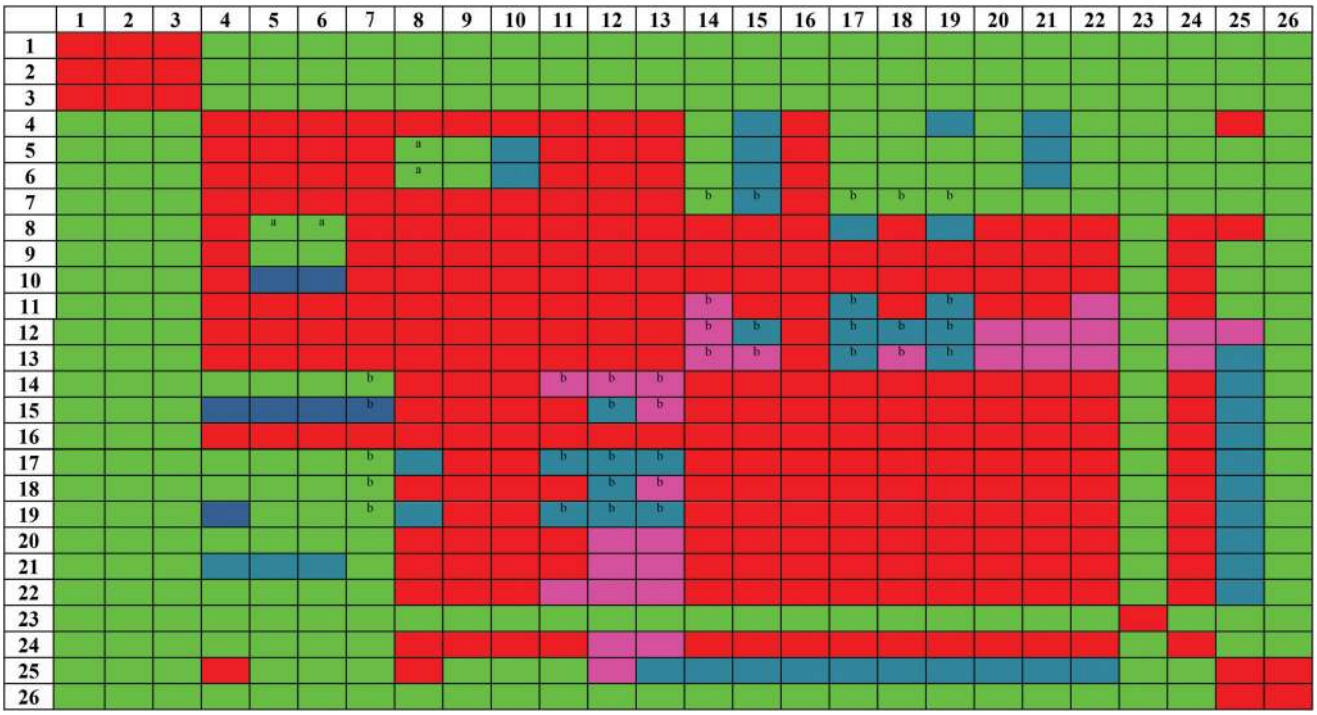
A completely new bioorthogonal reaction was recently developed by Sletten and Bertozzi, who sought to develop a new reaction, orthogonal to the known cohort of bioconjugations.[131] They encountered that quadrocyclanes have a large ring strain which makes them likely to react fast with a suitable reaction partner, which was found in the Ni(dithiolene)$_2$ complex (Table 1, entry 23). The obtained cycloaddition product however slowly decomposed, unless a metal-chelator was added. This reaction could be used for protein modification and proved orthogonal to SPAAC and oxime-formation.

3. Orthogonality

In addition to the search for bioorthogonal reactions, interest has also grown in bioorthogonal reactions which are also mutually orthogonal. The latter class of reactions can be useful in, for example, dual labelling or for the introduction of multiple functional handles.

The abovementioned reaction between a quadrocyclane and an Ni(dithiolene)$_2$ complex was specifically developed for this

Table 3 Overview of mutual orthogonality of bioorthogonal reactions when performed in a "one-pot procedure". ■ means the reactions are orthogonal, ■ means they are not, ■ implies the reactions are likely to be orthogonal, and ■ implies the reactions are unlikely to be orthogonal.



[a] If no catalyst is used to control the regiochemistry of nitrile-oxide alkyne cycloaddition. [b] Only for DBCO.

purpose, and was found to be orthogonal to SPAAC and oxime ligation.[131] However, also other bioorthogonal reactions are found to be mutually orthogonal. In Table 3 an overview is provided of the bioorthogonal reactions depicted in Table 1 and their compatibility in reactivity when performed simultaneously. Apart from adding all reagents at once in one pot, reactions can also be performed consecutively. Performing bioorthogonal reactions in this manner, possibly with an intermediate purification step, allows for significantly more bioorthogonal reactions to be used on the same biological system. An overview of these possibilities is depicted in Table 4. Obviously, reactions using one or more identical reagents cannot be used simultaneously. Besides, reactions requiring either a catalyst or activation by UV-light form orthogonal pairs when performed consecutively, as long as both the reactions do not require the same type of activation.

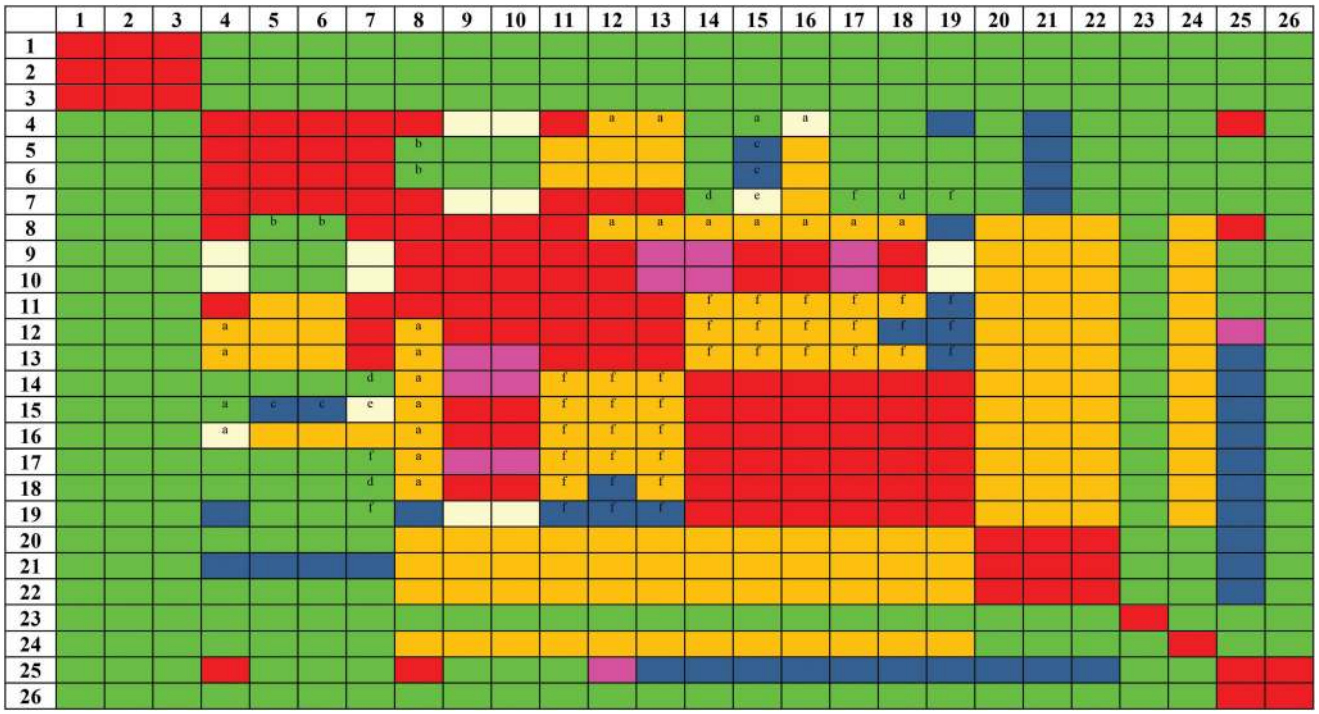
Entries 1–3, which involve different reactions with aldehydes and/or ketones, are probably orthogonal towards all other bioorthogonal reactions. Nonetheless, one might argue the real bioorthogonality of the carbonyl moiety, especially in biological media containing high concentrations of amines.

In the case of the azide moiety (entries 4–7) orthogonality is harder to achieve. The Staudinger-type reactions (entries 5 and 6) involve such azide-specific chemistry that these are likely orthogonal to all reactions not involving azides or azide-reactive groups.

However, for entries 4 and 7, due to the reactivity of the azide towards terminal alkynes (when catalysed by Cu(I) or Ru(II)) and (dibenzo)cyclooctynes, in combination with the reactivity of other functional handles (nitrile-oxides, nitrones, diazo compounds, tetrazines, and tetrazoles) towards these moieties, only a few of these reactions are likely to be mutually orthogonal. Nonetheless, there are some examples of using multiple bioorthogonal reactions in one pot, of which one involved an azide. Hilderbrand *et al.* demonstrated that SPAAC with dibenzocyclooctynes and tetrazines with TCOs can be used in a single pot.[112] No cross reaction between tetrazines and dibenzocyclooctynes was observed, and the rate constant of TCOs with azides was almost three orders of magnitude slower than SPAAC. To demonstrate the orthogonality, two different cell types were equipped with either a dibenzocyclooctyne or a TCO and subsequently mixed, and selectively labelled with two different dyes. It was also demonstrated that cells grown with both azide- and cyclopropene-containing sialic acids could be labelled orthogonally.[114] Most recently, it was shown that alkene-containing Ac_4ManNAc and Ac_4GalNAz can be incorporated in the same cell and subsequently labelled selectively using a tetrazine-dye for the alkene-sugar and a dibenzocyclooctyne for the azido-sugar.[116]

The lack of reactivity between tetrazine and dibenzoazacyclooctyne compared to tetrazine and cyclooctyne and the low reactivity between azide and TCO compared to tetrazine and TCO has been explored by Houk *et al.*[132] Based on DFT

Table 4 Overview of mutual orthogonality of bioorthogonal reactions when performed in a consecutive manner. ■ means the reactions are orthogonal, ■ means they are not, ■ implies the reactions are likely to be orthogonal, ■ implies the reactions are unlikely to be orthogonal, ■ means the reaction corresponding to the lowest entry number is performed first, and ■ means the reaction corresponding to the highest entry number is performed first.



[a] Only if no reaction of nitrone/diazo compound/tetrazine with terminal alkyne. [b] If no catalyst is used to control regiochemistry of nitrile-oxide alkyne cycloaddition. [c] S goes anyway, T probably, assuming that the reaction between azide and norbornene is significantly slower. [d] Both ways for dibenzocyclooctynes, only T for cyclooctynes. [e] Also S in the case of DBCOs. [f] Only for DBCO.

calculations, they reasoned that the activation energy for the reaction between TCO and azide is significantly higher than that for the inverse electron demand Diels–Alder reaction, causing high selectivity of TCO for the tetrazine. The low reactivity of dibenzocyclooctynes (as opposed to cyclooctynes) with tetrazines is caused by steric effects, resulting in a high distortion energy.

The orthogonality of these two reactions was also used by Brindle *et al.* who demonstrated that a double-click protocol can be performed for tumour-labelling in live mice.[87] Tumour cells were grown in mice in the presence of $Ac_4GalNAz$, thereby equipping the tumour cells with azides *in vivo*. Hereupon, the azides were reacted with a bifunctional probe, containing a dibenzocyclooctyne and a TCO. The azides now selectively reacted with the dibenzocyclooctyne, and upon clearance of the bifunctional probe, the TCO could be used for an inverse electron demand Diels–Alder reaction. An advantage of this approach compared to one step labelling with an imaging probe containing dibenzocyclooctyne is that due to its high reaction kinetics, the tetrazine probe needs to be administered in a much lower concentration compared to the dibenzocyclooctyne, thereby increasing the signal-to-noise ratio. However, one might argue that a one-step approach involving one fast ligation method would be more straightforward. It was also demonstrated by Jäschke *et al.* that the CuAAC and TCO-tetrazine ligation can be used simultaneously, to achieve dual labelling of DNA.[133]

Nitrile-oxides (entries 8–11) react with similar moieties as tetrazoles and tetrazines, making it hard to find orthogonal pairs. Nonetheless, it was shown that dual-labelling of DNA can be achieved by performing CuAAC, followed by a cycloaddition between a nitrile-oxide and an alkene.[93]

Reactivity of nitrones and diazo compounds (entries 12 and 13) with other reactive groups than cyclooctynes has not been widely studied and therefore it is hard to estimate the compatibility with other reactions. However, as the reactivity of nitrones and diazo compounds with strained alkynes is comparable to or higher than the reactivity of nitrile-oxides with strained alkynes, the reactivity of nitrones and diazo compounds likely resembles the reactivity of nitrile-oxides.

Looking at the moieties that react in a rapid manner with tetrazines (entries 14–19), orthogonality towards other reactive groups is hard to achieve. As mentioned earlier, the only surprising lack of reactivity is observed when mixing tetrazines with dibenzocyclooctynes. Especially for the consecutive reactions this opens up some possibilities for orthogonal reactivity.[112] Consecutive reactions of azide-related chemistry followed by most tetrazine-involving cycloadditions (except

entry 16) can be performed, due to the low reactivity of azides with TCOs,[134] norbornenes, cyclopropenones,[114] and alkenes.[116]

For tetrazoles (entries 20–22), cycloaddition has been observed with similar reactive groups as for tetrazines and nitrile-oxides; however, as mentioned earlier, no reactivity of alkenes, norbornenes, and cyclopropenes has been observed for azides, allowing for some orthogonality in these cases. For consecutive reactions, the big advantage of tetrazoles is that they require activation. Therefore, the first reaction (*e.g.* a tetrazine with an alkene) can be performed whereupon the tetrazole is activated and reacted with another functional handle. This only works under the assumption that all tetrazines have reacted and that the first reagent can be washed away.

The quadrocyclane (entry 23), which was developed by Bertozzi *et al.* keeping orthogonality in mind, was shown to be orthogonal to SPAAC and the reaction between an aldehyde and an alkoxyamine.[131] Although further research on orthogonality has not been performed, likely due to a lack of double bonds, the reaction is orthogonal to the full cohort of reactions described in Table 1.

The last category is formed by the metal-catalysed reactions (entries 24–26). Although the cross-reactivity of alkenes and alkynes with several functional handles excludes some reactions to be performed simultaneously, the catalyst requirements do allow for performing consecutive reactions.

4. Conclusions and outlook

This literature overview makes clear that the field of bioorthogonal ligation reactions is still expanding. In recent years, various highly promising reactions have been developed, and a wide range of applications have been reported.

For reactions to be applicable in biological systems, they need to fulfil several requirements. Hydrophilicity, reaction kinetics, and selectivity are probably the most important factors for a successful ligation. With these requirements in mind, the tetrazine-based ligations with *trans*-cyclooctenes or cyclopropenes seem the most suitable reactions. These ligations show amazing rate constants (10^3–10^4 M^{-1} s^{-1}) and high selectivity. However, as the tetrazine is a relatively large residue and hard to selectively introduce compared to the azide, protein functions are more likely to be disturbed. Azides, on the other hand, are easily introducible small residues, forming a peptide bond isostere upon cycloaddition.

Overall, it can be concluded that for each application a different ligation might be preferred. For example, bioorthogonal ligation is not at all required if the aim is to prepare a labelled protein irrespective of the modification site or number of labels introduced.[3] With that aim in mind probably lysine or cysteine modification is the easiest choice.

On the other hand, introduction of azides into multiple proteins can relatively easily be achieved *in vitro* and *in vivo*. Subsequent labelling with CuAAC or SPAAC allows for proteomics and *in vivo* imaging of glycan synthesis,[4] respectively. Tetrazine-based ligations are most suitable when a fast reaction is required, *e.g.* for *in vivo* radiolabelling[5] or in single protein labelling.[110] Nonetheless, there is still room for improvement. One of the challenges in the coming years is to develop a fluorogenic probe which after reaction shows an emission–excitation pattern that is more suitable for biological research than coumarin-based dyes, as for FI-DIBO[79] and CoumBARAC.[78] Very recently, two fluorogenic probes, *i.e.* an azide[77] and a tetrazine,[135] were reported which display more preferable excitation wavelengths (around 500 m) upon cycloaddition. Also, the development of tunable probes is likely to prove valuable in biological research, allowing for spatial and temporal control. The tetrazole-based ligation[121] and cyclopropene-protected dibenzocyclooctyne[64] are already examples in this direction. However, both require irradiation with UV-light, which makes them less suitable for biological studies. Possibly, different types of probes can be developed which become reactive upon irradiation with for example α-particles, X-rays, or by drug interaction. Lastly, the fastest bioorthogonal ligations involve relatively large reactive groups. The larger the molecules, the bigger the chances that the biological processes, epitope affinity, or enzyme functioning are influenced. To circumvent this, a challenge lies in the development of two small reactive partners which react in a fast and selective manner with each other. A promising advance in this field was recently made by the development of a tetrazine-cyclopropene[113] and tetrazole-cyclopropene[123] cycloaddition.

As bioconjugation has proven its value more and more in the last decade, it is likely that additional research areas will pick up on bioorthogonal ligation reactions. Currently, mainly biology-oriented chemists and macromolecular chemists have discovered this chemistry. However, with a growing number of reagents, *e.g.* cyclooctynes and tetrazines, becoming commercially available, also biologists will have access to these types of chemistries and will be able to apply it in their respective research fields.

In addition, interest in the development of new bioorthogonal ligation strategies certainly has not decreased over the years; instead the opposite appears to be true. Reactivity which can be achieved with SPAAC seems to have reached its limit; however, other, new reactions with superior kinetics have been developed and have not been optimized yet. Therefore, one may expect that in the years to come, even better and more selective ligation strategies will be discovered.

Notes and references

1 G. T. Hermanson, *Bioconjugate Techniques*, Elsevier Inc., London, 2nd edn, 2008.
2 E. M. Sletten and C. R. Bertozzi, *Angew. Chem., Int. Ed.*, 2009, **48**, 6974–6998.
3 N. Stephanopoulos and M. B. Francis, *Nat. Chem. Biol.*, 2011, **7**, 876–884.
4 S. T. Laughlin, J. M. Baskin, S. L. Amacher and C. R. Bertozzi, *Science*, 2008, **320**, 664–667.

5 R. Rossin, P. R. Verkerk, S. M. van den Bosch, R. C. M. Vulders, I. Verel, J. Lub and M. S. Robillard, *Angew. Chem., Int. Ed.*, 2010, **49**, 3375–3378.

6 J. M. Baskin, J. A. Prescher, S. T. Laughlin, N. J. Agard, P. V. Chang, I. A. Miller, A. Lo, J. A. Codelli and C. R. Bertozzi, *Proc. Natl. Acad. Sci. U. S. A.*, 2007, **104**, 16793–16797.

7 V. W. Cornish, K. M. Hahn and P. G. Schultz, *J. Am. Chem. Soc.*, 1996, **118**, 8150–8151.

8 R. K. Lim and Q. Lin, *Chem. Commun.*, 2010, **46**, 1589–1600.

9 E. M. Sletten and C. R. Bertozzi, *Acc. Chem. Res.*, 2011, **44**, 666–676.

10 S. Chattopadhaya, F. B. Abu Bakar and S. Q. Yao, *Curr. Med. Chem.*, 2010, **16**, 4527–4543.

11 Y. Takaoka, A. Ojida and I. Hamachi, *Angew. Chem., Int. Ed.*, 2013, **52**, 4088–4106.

12 W. P. Jencks, *J. Am. Chem. Soc.*, 1959, **81**, 475–481.

13 Z. Zhang, B. A. Smith, L. Wang, A. Brock, C. Cho and P. G. Schultz, *Biochemistry*, 2003, **42**, 6735–6746.

14 T. L. Schlick, Z. B. Ding, E. W. Kovacs and M. B. Francis, *J. Am. Chem. Soc.*, 2005, **127**, 3718–3723.

15 L. K. Mahal, K. J. Yarema and C. R. Bertozzi, *Science*, 1997, **276**, 1125–1128.

16 P. Agarwal, J. van der Weijden, E. M. Sletten, D. Rabuka and C. R. Bertozzi, *Proc. Natl. Acad. Sci. U. S. A.*, 2013, **110**, 46–51.

17 M. F. Debets, C. W. J. van der Doelen, F. P. J. T. Rutjes and F. L. van Delft, *ChemBioChem*, 2010, **11**, 1168–1184.

18 S. F. M. van Dongen, R. L. M. Teeuwen, M. Nallani, S. S. van Berkel, J. J. L. M. Cornelissen, R. J. M. Nolte and J. C. M. van Hest, *Bioconjugate Chem.*, 2009, **20**, 20–23.

19 K. L. Kiick, E. Saxon, D. A. Tirrell and C. R. Bertozzi, *Proc. Natl. Acad. Sci. U. S. A.*, 2002, **99**, 19–24.

20 J. Xiao and T. J. Tolbert, *Org. Lett.*, 2009, **11**, 4144–4147.

21 P.-C. Lin, S.-H. Ueng, M.-C. Tseng, J.-L. Ko, K.-T. Huang, S.-C. Yu, A. K. Adak, Y.-J. Chen and C.-C. Lin, *Angew. Chem., Int. Ed.*, 2006, **45**, 4286–4290.

22 M. F. Debets, W. P. Leenders, K. Verrijp, M. Zonjee, S. A. Meeuwissen, I. Otte-Holler and J. C. van Hest, *Macromol. Biosci.*, 2013, DOI: 10.1002/mabi.201300039. Q3

23 E. Saxon and C. R. Bertozzi, *Science*, 2000, **287**, 2007–2010.

24 F. L. Lin, H. M. Hoyt, H. van Halbeek, R. G. Bergman and C. R. Bertozzi, *J. Am. Chem. Soc.*, 2005, **127**, 2686–2695.

25 B. L. Nilsson, L. L. Kiessling and R. T. Raines, *Org. Lett.*, 2000, **2**, 1939–1941.

26 J. A. Prescher, D. H. Dube and C. R. Bertozzi, *Nature*, 2004, **430**, 873–877.

27 M. J. Hangauer and C. R. Bertozzi, *Angew. Chem., Int. Ed.*, 2008, **47**, 2394–2397.

28 D. H. Dube, J. A. Prescher, C. N. Quang and C. R. Bertozzi, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, **103**, 4819–4824.

29 FLAG refers to the so-called FLAG-tag. An octapeptide which can be used for affinity purification and imaging. FITC-α-FLAG refers to an antibody which recognizes the FLAG-tag which is conjugated to a fluorescein isothiocyanate (FITC).

30 P. V. Chang, J. A. Prescher, E. M. Sletten, J. M. Baskin, I. A. Miller, N. J. Agard, A. Lo and C. R. Bertozzi, *Proc. Natl. Acad. Sci. U. S. A.*, 2010, **107**, 1821–1826.

31 S. S. van Berkel, M. B. van Eldijk and J. C. M. van Hest, *Angew. Chem., Int. Ed.*, 2011, **50**, 8806–8827.

32 J. Kalia, N. L. Abbott and R. T. Raines, *Bioconjugate Chem.*, 2007, **18**, 1064–1069.

33 N. J. Agard, J. A. Prescher and C. R. Bertozzi, *J. Am. Chem. Soc.*, 2004, **126**, 15046–15047.

34 C. W. Tornøe, C. Christensen and M. Meldal, *J. Org. Chem.*, 2002, **67**, 3057–3064.

35 V. V. Rostovtsev, L. G. Green, V. V. Fokin and K. B. Sharpless, *Angew. Chem., Int. Ed.*, 2002, **41**, 2596–2599.

36 H. D. Agnew, R. D. Rohde, S. W. Millward, A. Nag, W. S. Yeo, J. E. Hein, S. M. Pitram, A. A. Tariq, V. M. Burns, R. J. Krom, V. V. Fokin, K. B. Sharpless and J. R. Heath, *Angew. Chem., Int. Ed.*, 2009, **48**, 4944–4948.

37 M. Meldal and C. W. Tornøe, *Chem. Rev.*, 2008, **108**, 2952–3015.

38 S. G. Agalave, S. R. Maujan and V. S. Pore, *Chem.–Asian J.*, 2011, **6**, 2696–2718.

39 A. J. Dirks, J. J. L. M. Cornelissen, F. L. van Delft, J. C. M. van Hest, R. J. M. Nolte, A. E. Rowan and F. P. J. T. Rutjes, *QSAR Comb. Sci.*, 2007, **26**, 1200–1210.

40 A. H. El-Sagheer and T. Brown, *Acc. Chem. Res.*, 2012, **45**, 1258–1267.

41 S. R. Hanson, W. A. Greenberg and C.-H. Wong, *QSAR Comb. Sci.*, 2007, **26**, 1243–1252.

42 V. Hong, N. F. Steinmetz, M. Manchester and M. G. Finn, *Bioconjugate Chem.*, 2010, **21**, 1912–1916.

43 V. Hong, S. I. Presolski, C. Ma and M. G. Finn, *Angew. Chem., Int. Ed.*, 2009, **48**, 9879–9883.

44 D. Soriano Del Amo, W. Wang, H. Jiang, C. Besanceney, A. C. Yan, M. Levy, Y. Liu, F. L. Marlow and P. Wu, *J. Am. Chem. Soc.*, 2010, **132**, 16893–16899.

45 A. T. Blomquist and L. H. Liu, *J. Am. Chem. Soc.*, 1953, **75**, 2153–2154.

46 G. Wittig and A. Krebs, *Chem. Ber.*, 1961, **94**, 3260–3275.

47 A. Krebs and J. Wilke, *Top. Curr. Chem.*, 1983, **109**, 189–233.

48 J. C. Jewett and C. R. Bertozzi, *Chem. Soc. Rev.*, 2010, **39**, 1272.

49 M. F. Debets, S. S. van Berkel, J. Dommerholt, A. J. Dirks, F. P. J. T. Rutjes and F. L. van Delft, *Acc. Chem. Res.*, 2011, **44**, 805–815.

50 J. Dommerholt, S. Schmidt, R. P. Temming, L. J. A. Hendriks, F. P. J. T. Rutjes, J. C. M. van Hest, D. J. Lefeber, P. Friedl and F. L. van Delft, *Angew. Chem., Int. Ed.*, 2010, **49**, 9422–9425.

51 J. C. Jewett, E. M. Sletten and C. R. Bertozzi, *J. Am. Chem. Soc.*, 2010, **132**, 3688–3690.

52 N. J. Agard, J. M. Baskin, J. A. Prescher, A. Lo and C. R. Bertozzi, *ACS Chem. Biol.*, 2006, **1**, 644–648.

53 J. A. Codelli, J. M. Baskin, N. J. Agard and C. R. Bertozzi, *J. Am. Chem. Soc.*, 2008, **130**, 11486–11493.
54 S. M. van den Bosch, R. Rossin, P. Renart Verkerk, W. Ten Hoeve, H. M. Janssen, J. Lub and M. S. Robillard, *Nucl. Med. Biol.*, 2013, **40**, 415–423.
55 H. L. Evans, R. L. Slade, L. Carroll, G. Smith, Q.-D. Nguyen, L. Iddon, N. Kamaly, H. Stöckmann, F. J. Leeper, E. O. Aboagye and A. C. Spivey, *Chem. Commun.*, 2012, 3–5.
56 H. Stöckmann, A. a. Neves, S. Stairs, H. Ireland-Zecchini, K. M. Brindle and F. J. Leeper, *Chem. Sci.*, 2011, **2**, 932.
57 L. Campbell-Verduyn, P. H. Elsinga, L. Mirfeizi, R. A. Dierckx and B. L. Feringa, *Org. Biomol. Chem.*, 2008, **6**, 3461–3463.
58 F. Zhang and J. E. Moses, *Org. Lett.*, 2009, **11**, 1587–1590.
59 F. Shi, J. P. Waldo, Y. Chen and R. C. Larock, *Org. Lett.*, 2008, **10**, 2409–2412.
60 S. Chandrasekhar, M. Seenaiah, C. L. Rao and C. R. Reddy, *Tetrahedron*, 2008, **64**, 11325–11327.
61 G. de Almeida, E. M. Sletten, H. Nakamura, K. K. Palaniappan and C. R. Bertozzi, *Angew. Chem., Int. Ed.*, 2012, **51**, 2443–2447.
62 E. M. Sletten and C. R. Bertozzi, *Org. Lett.*, 2008, **10**, 3097–3099.
63 X. Ning, J. Guo, M. A. Wolfert and G.-J. Boons, *Angew. Chem., Int. Ed.*, 2008, **47**, 2253–2255.
64 A. A. Poloukhtine, N. E. Mbua, M. A. Wolfert, G.-J. Boons and V. V. Popik, *J. Am. Chem. Soc.*, 2009, **131**, 15769–15776.
65 F. Friscourt, P. A. Ledin, N. E. Mbua, H. R. Flanagan-Steet, M. A. Wolfert, R. Steet and G.-J. Boons, *J. Am. Chem. Soc.*, 2012, **134**, 5381–5389.
66 Unpublished results of van Hest *et al*.
67 M. F. Debets, S. S. van Berkel, S. Schoffelen, F. P. J. T. Rutjes, J. C. M. van Hest and F. L. van Delft, *Chem. Commun.*, 2010, **46**, 97–99.
68 C. G. Gordon, J. L. Mackey, J. C. Jewett, E. M. Sletten, K. N. Houk and C. R. Bertozzi, *J. Am. Chem. Soc.*, 2012, **134**, 9199–9208.
69 M. Brettreich, M. Bendikov, S. Chaffins, D. F. Perepichka, O. Dautel, H. Duong, R. Helgeson and F. Wudl, *Angew. Chem., Int. Ed.*, 2002, **41**, 3688–3691.
70 H. N. C. Wong, *Acc. Chem. Res.*, 1989, **22**, 145–152.
71 S. Shimada, M. Tanaka and K. Honda, *Inorg. Chim. Acta*, 1997, **265**, 1–8.
72 I. Kii, A. Shiraishi, T. Hiramatsu, T. Matsushita, H. Uekusa, S. Yoshida, M. Yamamoto, A. Kudo, M. Hagiwara and T. Hosoya, *Org. Biomol. Chem.*, 2010, 8.
73 E. M. Sletten, H. Nakamura, J. C. Jewett and C. R. Bertozzi, *J. Am. Chem. Soc.*, 2010, **132**, 3065–3068.
74 B. R. Varga, M. Kállay, K. Hegyi, S. Béni and P. Kele, *Chem.–Eur. J.*, 2012, **18**, 822–828.
75 Z. Zhou and C. J. Fahrni, *J. Am. Chem. Soc.*, 2004, **126**, 8862–8863.
76 K. Sivakumar, F. Xie, B. M. Cash, S. Long, H. N. Barnhill and Q. Wang, *Org. Lett.*, 2004, **6**, 4603–4606.
77 P. Shieh, M. J. Hangauer and C. R. Bertozzi, *J. Am. Chem. Soc.*, 2012, **134**, 17428–17431.
78 J. C. Jewett and C. R. Bertozzi, *Org. Lett.*, 2011, **13**, 3097–3099.
79 F. Friscourt, C. J. Fahrni and G.-J. Boons, *J. Am. Chem. Soc.*, 2012, **134**, 18809–18815.
80 S. S. van Berkel, A. J. Dirks, M. F. Debets, F. L. van Delft, J. J. L. M. Cornelissen, R. J. M. Nolte and F. P. J. T. Rutjes, *ChemBioChem*, 2007, **8**, 1504–1508.
81 V. Hong, A. A. Kislukhin and M. G. Finn, *J. Am. Chem. Soc.*, 2009, **131**, 9986–9994.
82 Z. Li, T. S. Seo and J. Ju, *Tetrahedron Lett.*, 2004, **45**, 3143–3146.
83 S. S. van Berkel, A. J. Dirks, S. A. Meeuwissen, D. L. L. Pingen, O. C. Boerman, P. Laverman, F. L. van Delft, J. J. L. M. Cornelissen and F. P. J. T. Rutjes, *ChemBioChem*, 2008, **9**, 1805–1815.
84 The first paper on SPAAC has been cited in 276 separate (non-review) papers.
85 Y. Manabe, *J. Synth. Org. Chem., Jpn.*, 2012, **70**, 754–755.
86 J. M. Baskin, K. W. Dehnert, S. T. Laughlin, S. L. Amacher and C. R. Bertozzi, *Proc. Natl. Acad. Sci. U. S. A.*, 2010, **107**, 10360–10365.
87 A. A. Neves, H. Stockmann, Y. A. Wainman, J. C. Kuo, S. Fawcett, F. J. Leeper and K. M. Brindle, *Bioconjugate Chem.*, 2013, DOI: 10.1021/bc300621n.
88 A. M. Jawalekar, E. Reubsaet, F. P. J. T. Rutjes and F. L. van Delft, *Chem. Commun.*, 2011, **47**, 3198–3200.
89 X. Ning, R. P. Temming, J. Dommerholt, J. Guo, D. B. Ania, M. F. Debets, M. A. Wolfert, G.-J. Boons and F. L. van Delft, *Angew. Chem., Int. Ed.*, 2010, **49**, 3065–3068.
90 F. Heaney, *Eur. J. Org. Chem.*, 2012, 3043–3058.
91 C. Wendeln, I. Singh, S. Rinnen, C. Schulz, H. F. Arlinghaus, G. A. Burley and B. J. Ravoo, *Chem. Sci.*, 2012, **3**, 2479–2484.
92 K. Gutsmiedl, C. T. Wirges, V. Ehmke and T. Carell, *Org. Lett.*, 2009, **11**, 2405–2408.
93 K. Gutsmiedl, D. Fazio and T. Carell, *Chem.–Eur. J.*, 2010, **16**, 6877–6883.
94 B. C. Sanders, F. Friscourt, P. A. Ledin, N. E. Mbua, S. Arumugam, J. Guo, T. J. Boltje, V. V. Popik and G.-J. Boons, *J. Am. Chem. Soc.*, 2011, **133**, 949–957.
95 I. Singh and F. Heaney, *Chem. Commun.*, 2011, **47**, 2706–2708.
96 C. S. McKay, J. Moran and J. P. Pezacki, *Chem. Commun.*, 2010, **46**, 931–933.
97 C. S. McKay, J. A. Blake, J. Cheng, D. C. Danielson and J. P. Pezacki, *Chem. Commun.*, 2011, **47**, 10040–10042.
98 C. S. McKay, M. Chigrinova, J. A. Blake and J. P. Pezacki, *Org. Biomol. Chem.*, 2012, **10**, 3066–3070.
99 M. Colombo, S. Sommaruga, S. Mazzucchelli, L. Polito, P. Verderio, P. Galeffi, F. Corsi, P. Tortora and D. Prosperi, *Angew. Chem., Int. Ed.*, 2012, **51**, 496–499.
100 N. E. Mbua, J. Guo, M. a. Wolfert, R. Steet and G.-J. Boons, *ChemBioChem*, 2011, 1–11.

101 N. A. McGrath and R. T. Raines, *Chem. Sci.*, 2012, **3**, 3237–3240.
102 F. Talhammer, U. Wallfahrer and J. Sauer, *Tetrahedron Lett.*, 1990, **31**, 6851–6854.
103 J. Sauer, D. K. Heldmann, J. Hetzenegger, J. Krauthan, H. Sichert and J. Schuster, *Eur. J. Org. Chem.*, 1998, 2885–2896.
104 N. K. Devaraj, R. Weissleder and S. A. Hilderbrand, *Bioconjugate Chem.*, 2008, **19**, 2297–2299.
105 M. L. Blackman, M. Royzen and J. M. Fox, *J. Am. Chem. Soc.*, 2008, **130**, 13518–13519.
106 M. T. Taylor, M. L. Blackman, O. Dmitrenko and J. M. Fox, *J. Am. Chem. Soc.*, 2011, **133**, 9646–9649.
107 N. K. Devaraj and R. Weissleder, *Acc. Chem. Res.*, 2011, **44**, 816–827.
108 J. B. Haun, N. K. Devaraj, S. A. Hilderbrand, H. Lee and R. Weissleder, *Nat. Nanotechnol.*, 2010, **5**, 660–665.
109 W. Chen, D. Wang, C. Dai, D. Hamelberg and B. Wang, *Chem. Commun.*, 2011, **48**, 7–10.
110 K. Lang, L. Davis, S. Wallace, M. Mahesh, D. J. Cox, M. L. Blackman, J. M. Fox and J. W. Chin, *J. Am. Chem. Soc.*, 2012, **134**, 10317–10320.
111 A. Borrmann, S. Milles, T. Plass, J. Dommerholt, J. M. M. Verkade, M. Wiessler, C. Schultz, J. C. M. van Hest, F. L. van Delft and E. A. Lemke, *ChemBioChem*, 2012, **13**, 2094–2099.
112 M. R. Karver, R. Weissleder and S. A. Hilderbrand, *Angew. Chem., Int. Ed.*, 2012, **51**, 920–922.
113 J. Yang, J. Sečkutė, C. M. Cole and N. K. Devaraj, *Angew. Chem., Int. Ed.*, 2012, **51**, 7476–7479.
114 D. M. Patterson, L. A. Nazarova, B. Xie, D. N. Kamber and J. A. Prescher, *J. Am. Chem. Soc.*, 2012, **134**, 18638–18643.
115 C. M. Cole, J. Yang, J. Seckute and N. K. Devaraj, *ChemBioChem*, 2013, **14**, 205–208.
116 A. Niederwieser, A. K. Spate, L. D. Nguyen, C. Jungst, W. Reutter and V. Wittmann, *Angew. Chem., Int. Ed. Engl.*, 2013, **52**, 4265–4268.
117 S. Stairs, A. A. Neves, H. Stockmann, Y. A. Wainman, H. Ireland-Zecchini, K. M. Brindle and F. J. Leeper, *ChemBioChem*, 2013, DOI: 10.1002/cbic.201300130.
118 M. R. Karver, R. Weissleder and S. A. Hilderbrand, *Bioconjugate Chem.*, 2011, **22**, 2263–2270.
119 N. K. Devaraj, S. A. Hilderbrand, R. Upadhyay, R. Mazitschek and R. Weissleder, *Angew. Chem., Int. Ed.*, 2010, **49**, 2869–2872.
120 J. S. Clovis, A. Eckell, R. Huisgen and R. Sustmann, *Chem. Ber./Recl.*, 1967, **100**, 60–70.
121 W. Song, Y. Wang, J. Qu, M. M. Madden and Q. Lin, *Angew. Chem., Int. Ed.*, 2008, **47**, 2832–2835.
122 E. Kaya, M. Vrabel, C. Deiml, S. Prill, V. S. Fluxa and T. Carell, *Angew. Chem., Int. Ed.*, 2012, **51**, 4466–4469.
123 Z. Yu, Y. Pan, Z. Wang, J. Wang and Q. Lin, *Angew. Chem., Int. Ed.*, 2012, **51**, 10600–10604.
124 Y. Wang, W. Song, W. J. Hu and Q. Lin, *Angew. Chem., Int. Ed.*, 2009, **48**, 5330–5333.
125 R. K. V. Lim and Q. Lin, *Acc. Chem. Res.*, 2011, **44**, 828–839.
126 W. Song, Z. Yu, M. M. Madden and Q. Lin, *Mol. BioSyst.*, 2010, **6**, 1576–1578.
127 Y. A. Lin, J. M. Chalker and B. G. Davis, *J. Am. Chem. Soc.*, 2010, **132**, 16805–16811.
128 Y. A. Lin, J. M. Chalker, N. Floyd, G. J. L. Bernardes and B. G. Davis, *J. Am. Chem. Soc.*, 2008, **130**, 9642–9643.
129 C. D. Spicer and B. G. Davis, *Chem. Commun.*, 2011, **47**, 1698–1700.
130 N. Li, R. K. V. Lim, S. Edwardraja and Q. Lin, *J. Am. Chem. Soc.*, 2011, **133**, 15316–15319.
131 E. M. Sletten and C. R. Bertozzi, *J. Am. Chem. Soc.*, 2011, **133**, 17570–17573.
132 Y. Liang, J. L. Mackey, S. A. Lopez, F. Liu and K. N. Houk, *J. Am. Chem. Soc.*, 2012, **134**, 17904–17907.
133 J. Schoch, M. Staudt, A. Samanta, M. Wiessler and A. Jaschke, *Bioconjugate Chem.*, 2012, **23**, 1382–1386.
134 M. R. Karver, R. Weissleder and S. a. Hilderbrand, *Angew. Chem., Int. Ed.*, 2011, **51**, 920–922.
135 J. C. T. Carlson, L. G. Meimetis, S. A. Hilderbrand and R. Weissleder, *Angew. Chem., Int. Ed.*, 2013, **52**, 6917–6920.