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Bioorthogonal chemistry for pre-targeted molecular imaging – progress and prospects

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The aim of this perspective is to critically review the three most prominent bioorthogonal reactions that are used presently, on both a purely chemical level and in the context of biological systems. This includes the uses both for synthesis of therapeutic molecules, modification of large biomolecules or antibodies, and in particular, the exciting use in the field of 'pre-targeting', for both possible treatment and imaging technologies. We will compare the validity of each reaction when compared to others, and their usefulness in biological systems, as each methodology has clear advantages over the others in differing environments.

1. Introduction

'Bioorthogonal' is a term used to broadly define any chemical reaction that can occur in a living system without interfering with any of the system's native biochemical processes.[1] Historically, the concept is new, being first discussed by Bertozzi in 2000.[2] It is however an extension of the 'click chemistry' concept developed by Sharpless, whereby a series of reactions were grouped together as a result of meeting several compatibility criteria, some of which are shared with bioorthogonal reactions.[3,4] Bioorthogonal reactions must produce a chemically and biologically inert linkage/product *via* a reaction that displays high selectivity between the two coupling partners, is kinetically fast and is biocompatible in terms of operating at physiological pH, temperature and in a physiologically relevant solvent milieu. Because of this, these reactions are highly desirable for use in chemical biology.

One significant advantage of deploying bioorthogonal reactions as opposed to conventional alternatives is the possibilities this opens up for pre-targeting. Pre-targeting refers to scenarios where a living system is treated with a biologically active molecule, such as a peptide or antibody, which is allowed to accumulate at a designed target area as the result of a non-covalent binding interaction with target tissue, before a second compound is added which selectively reacts with the first molecule. This reaction can be used to generate a reactive species (*e.g.* a therapeutic agent) or to allow for a specific imaging modality (*e.g.* a fluorescent probe) with high spatial precision inside a living system as the result of targeted accumulation of the initial biologically active molecule. By using a bioorthogonal reaction in this manner, the incidence of off-target side reactions leading to damage in the wrong tissues during therapy or poor contrast during imaging can be minimised relative to conventional approaches. Additionally, biomolecules with slower pharmacokinetics, such as antibodies, which would traditionally be difficult to image using short-lived isotopes directly, are able to be looked at in this way, providing a safer method of molecular imaging for subjects. As such, applications in imaging in particular are the focus of this current perspective.

There have been a number of bioorthogonal reactions published over the past ten years, but this review will focus on three of the main reactions – Staudinger–Bertozzi ligation, strain-promoted alkyne–azide cycloadditions (SPAAC, more commonly known as the copper-free click reaction) and the inverse-electron demand Diels–Alder reaction (IDDA, sometimes referred to as the tetrazine ligation reaction).[5a–e] Whilst the last of these reactions has so far received less attention than the other two due to its more recent discovery, its potential is already apparent from recent publications. We discuss what we consider to be the key factors in determining each reaction's utility within bioorthogonal chemistry with a focus on prospects for their use in pre-targeting. We provide critical analysis of the field rather than comprehensive coverage, with the intention to enable a prospective practitioner to determine when deployment of one of these reactions might be expected to be advantageous.

2. The Staudinger–Bertozzi ligation

The Staudinger–Bertozzi ligation, which is sometimes simply referred to as Staudinger ligation, was first described in 2000 by Saxon and Bertozzi in a seminal publication that has come

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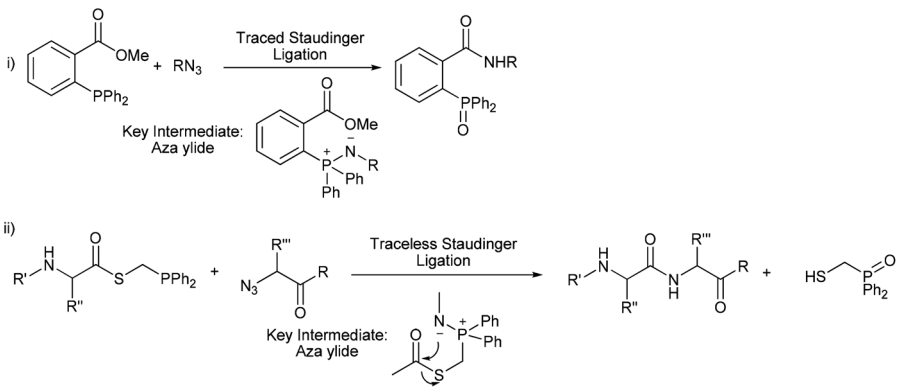
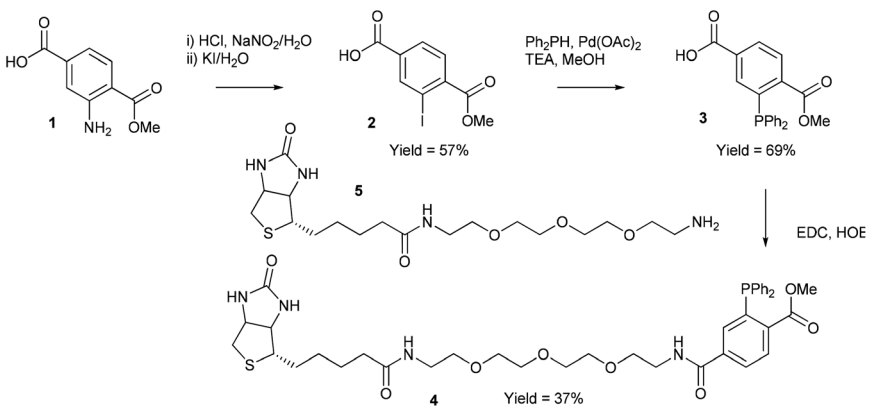
to mark the birth of bioorthogonal chemistry.[2] Staudinger–Bertozzi ligation is a modification of the ninety-year old Staudinger reaction between an azide and a phosphine giving an azaphosphorane (which is traditionally hydrolysed to give the corresponding amine and phosphine oxide). The specific modification introduced by Bertozzi involves carrying out this reaction such that the generated azaphosphorane (also sometimes referred to as an aza ylide) is trapped out by an electrophilic carbonyl containing functional group (*e.g.* an ester or thioester) in an intramolecular fashion to give a secondary amide linkage with a pendent phosphine oxide (Scheme 1, i). More recently, a traceless variant of the Staudinger ligation has been developed wherein by judicious initial phosphine design the phosphine oxide function is not part of the final ligated product but rather extruded as a separate by-product (Scheme 1, ii).

Both these reactions are highly selective; neither azides or phosphines are present in living systems and both are highly tolerant of physiological fluids making them ideal for use as bioorthogonal reaction partners. Additionally, the synthesis of both reactive partners for the Staudinger–Bertozzi ligation (both traced and traceless) is straightforward. By virtue of their small size, azides can be readily incorporated into a wide-range of biomolecules such as antibodies without significantly perturbing their pharmacokinetics. Moreover, azides generally do not elicit significant toxicity as evidenced by the number of azide-containing drug molecules that have gained FDA-approval as pharmaceuticals. Introduction of the α-phosphinylbenzoate reaction partner, as required for a traced Staudinger ligation, is usually accomplished by standard amide coupling techniques on a benzoic acid derivative (Scheme 2). Although phosphines are bulkier than azides, and the product formed contains a phosphine oxide, the perturbations caused to the biomolecule by these groups appear in many cases to be well tolerated. If the α-phosphinylbenzoate is part of the molecule added second into the system (*e.g.* imaging group), then some allowance should be made to maximise the biodistribution of this fragment *e.g.* by addition of a hydrophilic group such as a polyethyleneglycol (PEG) chain. In the absence of such a modification, the reagent will likely be taken up without specificity in all cells and provide a high level of background noise during any attempt to image the pre-targeted area of interest.

Initially, the synthesis of thiophosphine esters as required for the traceless variant of this reaction, was synthetically challenging due to the number of steps involved and the ease by which the diphenylphosphine can be oxidised to a phosphine oxide thereby rendering it inert to the Staudinger conditions. However, thanks to the commercialisation of (diphenylphosphino)methanethiol **10**, just three steps are now required to



Scheme 1 The Staudinger ligation, "traced" and "traceless" variants.



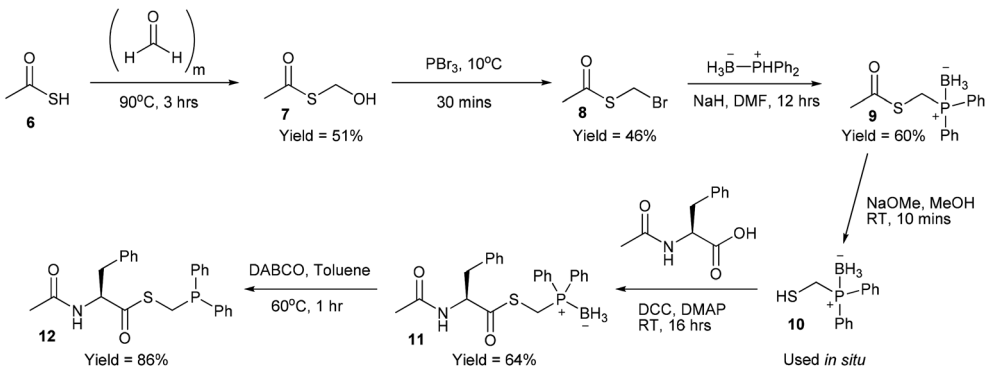
Scheme 2 Synthesis of phosphine-ester **3** and subsequent coupling to a biotin residue **5** to give **4**.

give the coupled deprotected thiophosphine compounds.[6] Moreover, protection of the phosphine as a borane adduct prior to unmasking with 1,4-diazabicyclo[2.2.2]octane (DABCO), can minimise unwanted oxidation prior to the Staudinger–Bertozzi ligation reaction, improving the synthesis further. Consequently, both forms of the Staudinger–Bertozzi ligation can now be accessed without a large investment in organic synthesis (Scheme 3).

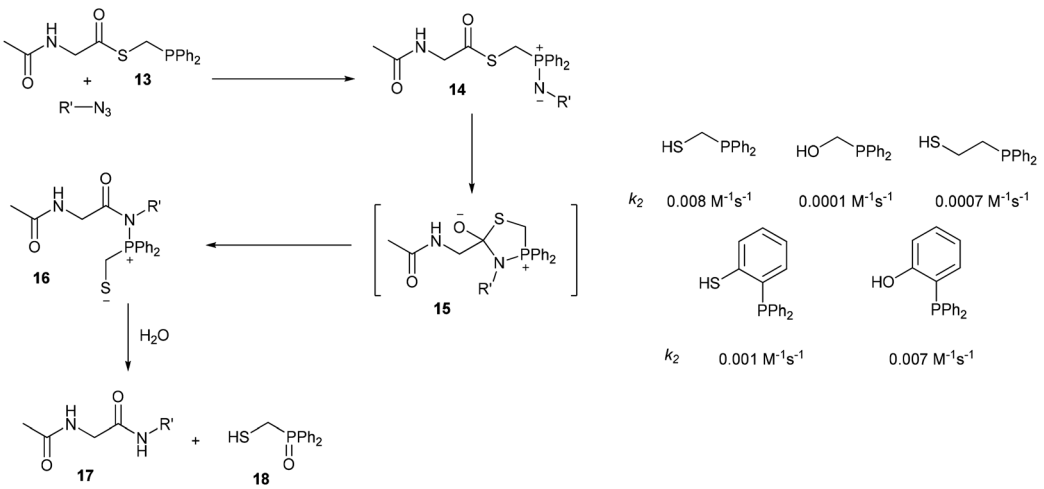
Whilst both the synthetic availability of the reactive components and the slight perturbation they impart on the pharmacokinetic properties of the derivatised biomolecules are favourable attributes of Staudinger–Bertozzi ligation, the modest rates of reaction displayed by these coupling processes is a significant drawback, particularly in the context of pre-targeting (Scheme 4).[7,8] Work carried out by Raines *et al.* on the mechanism of the traceless Staudinger–Bertozzi ligation using ^{13}C-labelling demonstrated that the reaction displays 2nd order kinetics: the ligation rate is controlled by the formation of the tetrahedral intermediate **15** and its subsequent breakdown give the desired amide product **17**. Whilst there are some caveats as to the relevance of these findings to biological situations, because the studies were carried out in 6 : 1 DMF–H_2O, the rate constants are small. This means that long reaction times are required to reach full conversion and that this type of ligation will be unsuitable for measuring rapid biological processes. In practice, any species modified for Staudinger–Bertozzi ligation must be present in high enough concentrations to allow for moderate conversion within a short timescale for visualisation purposes.

Additionally, as mentioned previously in this discussion, the reactive thiophosphine (or phosphine-ester in the context of the traced variant) is susceptible to being oxidised by air, making it inert to the ligation conditions. There is a reasonable possibility that this oxidation can occur *in vivo*, either simply by oxygen present in blood as part of the circulation system of the subject, or by enzymes such as cytochrome P450 that catalyse a large number of oxidation reactions, notably causing the oxidation (and subsequent metabolism) of a wide variety of drug compounds. This would decrease the active phosphine present at the desired site *in vivo*, to the detriment of the final pre-targeting reaction.

The Staudinger–Bertozzi ligation, as the first bioorthogonal reaction designed, has clear advantages over traditional coupling reactions, because it displays high regio- and



Scheme 3 Synthesis of (diphenylphosphino)methanethiol **10** and subsequent coupling to acetylphenylalanine and deprotection to give **12**.

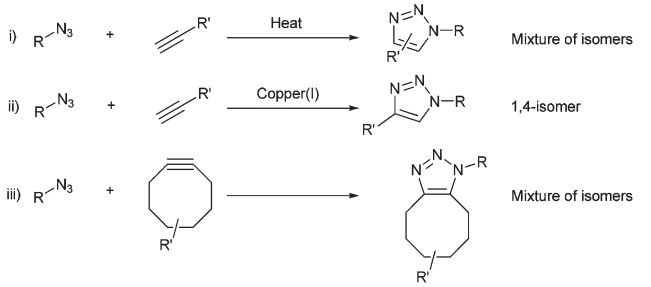


Scheme 4 Mechanism of the Staudinger–Bertozzi ligation and example 2nd order rate constants of several phosphines.

chemoselectivity as well as forming an amide bond in aqueous media whilst being highly biocompatible. As such, it has great utility for modifying molecules such as antibodies and peptides in a highly selective fashion whilst minimally altering their pharmacokinetic properties. However, it suffers from slow reaction kinetics, meaning the Staudinger ligation is probably restricted to pre-targeting with fluorescent compounds (where isotopic decay is not an issue) and is unlikely to be useful for pre-targeting *in vivo*.

3. Strain-promoted alkyne–azide cycloaddition

The SPAAC reaction was discovered serendipitously by Krebs and Wittig in 1961. While examining the properties of cyclooctyne, they noted that its reaction with phenyl azide gave a single triazole product.[9] However, the reaction remained a synthetic curiosity until 2004, when Bertozzi and co-workers used it as a bioorthogonal reaction as an alternative to Staudinger–Bertozzi ligation (Scheme 5).[10] The reaction is analogous to the original Huisgen [3 + 2] azide–alkyne cycloaddition, and the more recent copper-catalysed alkyne–azide cycloaddition, or 'click' reaction, which proceeds in the presence of a copper(I) catalyst under physiological conditions. Unfortunately, the use of copper salts has drawbacks in the context of bioorthogonal chemistry: copper salts are toxic to living tissue as they coordinate/chelate to many biomolecules, the catalytically active copper(I) salts, which are generally formed *in vitro* by mixing copper(II) sulphate and sodium ascorbate, can be difficult to generate *in vivo*, and most critically in the context of pre-targeting, there is insufficient endogenous copper(I) present in living systems to sustain catalysis. Consequently, Bertozzi and co-workers investigated the SPAAC reaction, which relies primarily on the ring-strain of cyclooctyne, the smallest ring system that can contain an internal alkyne whilst remaining stable for long periods of time. Molecular strain in the alkyne leads to the [3 + 2] cycloaddition occurring without the need for either elevated temperatures or any catalysis. This enables the reaction to be used as a bioorthogonal reaction, as it can occur under physiological conditions, with complete chemoselectivity and most significantly, very rapidly.



Scheme 5 Current variations of [3 + 2] azide–alkyne cycloaddition; (i) [3 + 2] Huisgen cycloaddition, (ii) copper-catalysed azide–alkyne cycloaddition and (iii) strain-promoted azide–alkyne cycloaddition.

The chemical synthesis of cyclooctynes is perhaps one of the most challenging aspects of the SPAAC reaction.[1] Although simple cyclooctynes, or modified cyclooctynes that include functionality for attachment to biomolecules, are commercially available, second generation structures with increased reactivity towards azides need to be prepared by multistep synthesis. Several examples are shown in Fig. 1, which shows both the number of synthetic steps required for the synthesis of each cyclooctyne derivative and also the bimolecular rate constants for their cycloaddition reaction with an azide. In general, more synthetic steps are required in order to prepare the cyclooctynes with the highest rate constants. Pleasingly, as the result of significant synthetic optimisation, the overall synthetic yields for the multi-step pathways to these cyclooctynes are generally good. However, the time required to access most is still greater than that for the corresponding Staudinger–Bertozzi ligation substrates. For example, the synthesis of **24** (4 steps) takes ~2 weeks. In all cases a pendant functional group is incorporated to allow conjugation of the cyclooctyne to a biomolecule, except for the case of cyclooctadiyne **24**

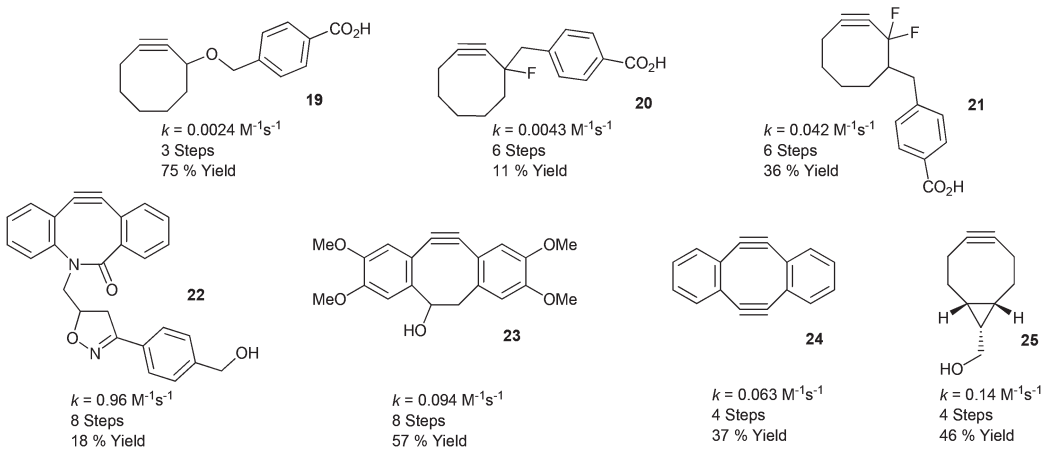


Fig. 1 Series of modified cyclooctynes, with the number of synthetic steps, overall chemical yield and 2nd order rate constants shown.

which relies on two consecutive SPAAC reactions for biomolecule conjugation and then cycloaddition.

By incorporating electron-withdrawing groups such as the *gem*-difluoromethylene unit in DIFO (**21**), the rate constants for cycloaddition can be increased progressively from a value around that for the best Staudinger–Bertozzi ligation reaction (*ca.* 2×10^{-3} M^{-1} s^{-1}) up to about 20 times greater (*i.e. ca.* 4×10^{-2} M^{-1} s^{-1}). This effect reflects a lowering in the activation energy of the SPAAC as a consequence of polarising the alkyne which in turn lowers the energy of its LUMO. The rate can be further modulated by inducing greater ring strain in the core cyclooctyne. An unstrained alkyne is of course linear whereas the C–C–C angle in cyclooctyne itself is ~160°. By reducing this angle still further, towards that required for the transition state for concerted cycloaddition, further increases in reactivity can be achieved. For example cyclooctynes **22–23**, which are highly strained but apparently stable for long periods when stored in the absence of air and light, react up to 20 times faster than DIFO **21** (*i.e. ca.* 9×10^{-1} M^{-1} s^{-1}). As a result, perhaps the most balanced cyclooctyne in terms of reaction kinetics and synthetic availability is BCN **25**, which has a rate constant around 1×10^{-1} M^{-1} s^{-1}, a synthesis of 4 steps (comprising of approximately 4–5 days laboratory work) in a global yield of 46%.[11] This means that certain optimised SPAAC reactions proceed up to 100 times faster than the fastest Staudinger–Bertozzi ligations, allowing for their use for studying more rapid biological reactions in living systems.

In 2007, Bertozzi and co-workers were the first to demonstrate successful pre-targeting: various fluorescent residues were derivatised with modified DIFO **26** and reacted with an azido-glycan **25** inside Jurkat cells (Fig. 2).[12] This was arguably a watershed moment in the field of pre-targeting, because although the Staudinger–Bertozzi ligation was well established, this work was the first to demonstrate that pre-targeting on the surface of live cells was more than just an abstract theoretical concept. Subsequent work within the Bertozzi group has gone on to demonstrate pre-targeting inside a series of different systems, including successful fluorescence imaging in mice and zebrafish.[13,14] In all cases, it has been shown that accumulation of the fluorescent tag only occurs when a pre-targeting group is present within the tissue of interest.

This work on pre-targeting, whilst ground-breaking in its design and execution, is not without drawbacks. All the work carried out to this point *in vivo* has involved fluorescence imaging. However, fluorescence imaging requires analysis of the tissue to be carried out *ex vivo*, which is not practical for human subjects. Accounting for the background fluorescence seen in all tissues is also problematic unless a full *ex vivo* biodistribution of the subject is carried out. This is a time consuming and inexact corrective technique. A further issue seen in some of the experiments in mice, is the apparent binding of cyclooctynes to albumin in red blood cells.[1] Most cyclooctyne scaffolds contain a large number of carbons and are lipophilic as well as being somewhat insoluble in aqueous media. This generally leads to derivatives containing them displaying rather poor pharmacokinetic profiles *in vivo* as manifested by indiscriminate background take up in all tissues. Depending on the pre-treatment model used, and time prior to the imaging being carried out post addition of the cyclooctyne, the background noise resulting from this unspecific uptake will limit clear imaging of the target tissue. It must be noted that several groups have been working to synthesise more biocompatible cyclooctynes, by attaching more hydrophilic groups such as pegylated chains.[15] This was demonstrated on zebrafish embryos, and the improved pharmacokinetics, whilst maintaining the reaction kinetics, allowed for visualisation of the pre-targeted area within 5 minutes which is much faster than previous examples. However, currently there are few examples of this, and it has yet to be tested in a more complex biological system. As a final point, although better than Staudinger–Bertozzi ligation, the 2nd order rate constant for the SPAAC is still somewhat lower than that of the copper-catalysed click reaction (which has a rate constant of *ca.* 1–10 M^{-1} s^{-1}).

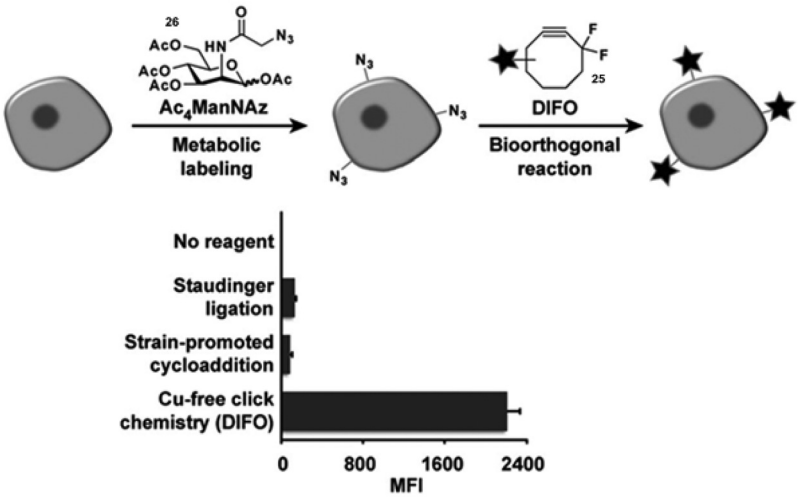
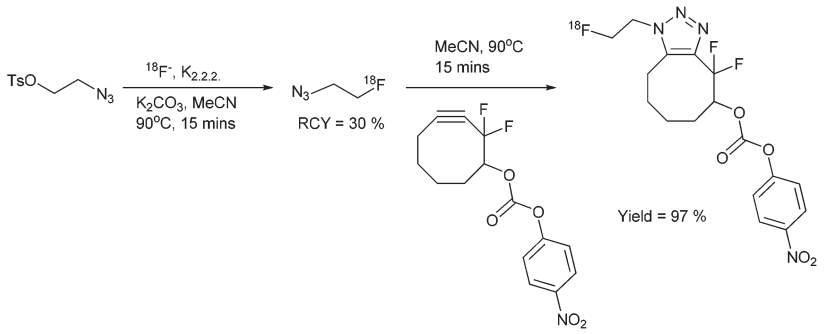


Fig. 2 First example of pre-targeting using a modified cyclooctyne **26** and an azido-glycan **25**.



Scheme 6 Example of an ^{18}F-labelled SPAAC reaction.

This means that this method is still probably not useful for pre-targeting using short-lived radioisotopes because they will not have long enough to accumulate in the desired area before decaying.[16]

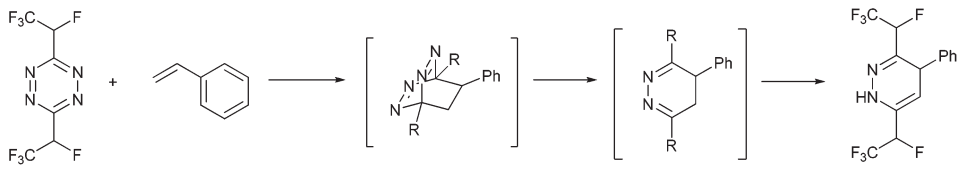
Despite these caveats, a number of groups have attached short-lived isotopes to biomolecules using the SPAAC reaction (Scheme 6).[17–21] To date, most examples have involved the attachment of a molecule containing a fluorine-18 label to the cyclooctyne partner, although due to the pharmacological profile mentioned previously, this will most likely lead to significant amounts of unspecific binding *in vivo*. In each case, both radiochemical yields for the incorporation of the fluorine-18 label and conversion to the product 1,2,3-triazoles are good, but require high temperatures for both steps and solvents such as DMF or MeCN; conditions which are clearly incompatible with living systems. These methods can however be used for labelling compounds within a vial before administering to a subject.

The wide availability of cyclooctynes from commercial sources has enabled a large number of biological chemists access to a difficult synthetic scaffold, and examples of antibodies and peptides being pre-targeted in this way are starting to appear in the literature. Although improved over those of the Staudinger–Bertozzi ligation, the pharmacokinetic profile of SPAAC-modified reaction precursors still leaves much to be desired due to the high lipophilicity of cyclooctynes. Moreover, the still moderate rate constants mean that the prospects for the use of SPAAC to monitor rapid biological processes *via* pre-targeting using short-lived radioisotopes are not good, particularly in light of newer bioorthogonal reactions being developed.

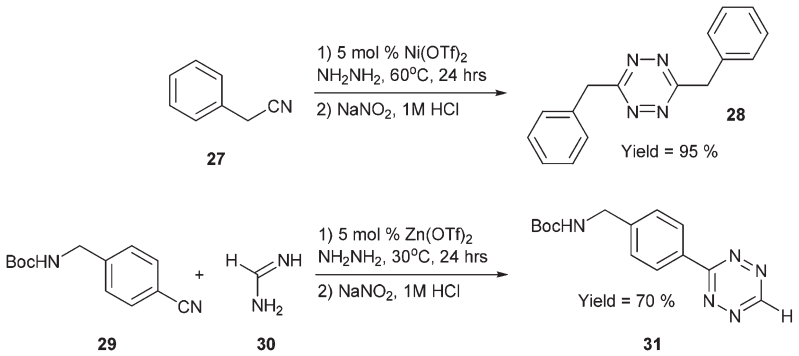
4. Inverse-demand Diels–Alder reaction

The inverse-demand Diels–Alder reaction (IDDA) was first discovered by Carboni and Lindsey in 1959, who reported that the reaction of phenylstyrene with a fluorinated tetrazine was highly exothermic and rapid at room temperature (Scheme 7).[22] Subsequently, this [4 + 2]-cycloaddition reaction has been used sporadically, but it was only in 2008 that Fox and co-workers first described the reaction as being bioorthogonal. They demonstrated that reactions of tetrazines with a range of strained alkenes displayed orthogonality towards biological processes, compatibility with aqueous media and fast reaction rates. (*E*)-Cyclooctenes were found to be particularly advantageous dienophile partners in terms of reaction rate due to the high relief of ring-strain associated with their cycloaddition reactions.[23,24]

Many strained alkenes can act as successful dienophiles for the IDDA reaction whilst maintaining good stability *in vivo*, and in general they are not difficult to synthesise. Synthesis of the tetrazine partners however is more complex, requiring a condensation reaction between hydrazine and a nitrile **27**, catalysed by either zinc or nickel salts, followed by oxidation of the resulting 1,2-dihydrotetrazine to the 1,2,4,5-tetrazine product **28** (Scheme 8).[25,26] Modification of one of the nitrile partners allows access to wide structural variations in the products (*e.g.* compound **31**) in moderate to good isolated yields using essentially constant reaction conditions and allowing for incorporation of functional groups such as pendant amines for convenient biomolecule attachment. Consequently, synthesis and structural modification of these reaction partners is easier and quicker than with either the Staudinger–Bertozzi



Scheme 7 First example of the IDDA reaction.



Scheme 8 Synthesis of 1,2,4,5-tetrazines using transition metal catalysis.

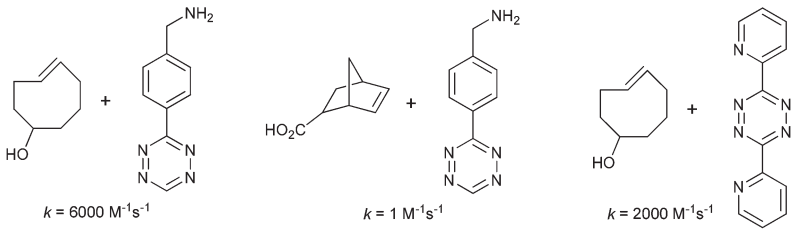
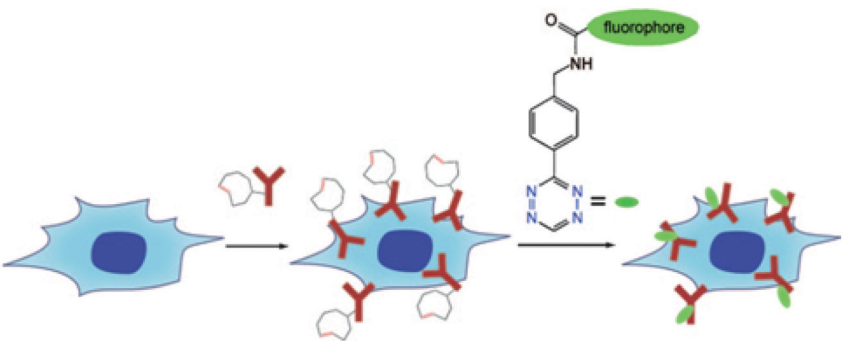


Fig. 3 Rate constants for representative tetrazine ligation reactions.

ligation or the SPAAC reaction, with the tetrazine product additionally benefiting from being more stable for long-term storage.

Whilst a more straightforward synthesis is an advantage when compared to other bioorthogonal reactions, the tetrazine ligation comes into its own when the rate constants are compared (Fig. 3).[23,27] The 2nd order rate constants for these reactions are up to 5 orders of magnitude higher than those of the SPAAC, and some 7 orders compared to the Staudinger–Bertozzi ligation. This brings the rate of reaction into the range displayed by fast biological processes, which cannot be observed by other bioorthogonal reactions, as well as opening the door for possible reactions using short-lived isotopes for imaging purposes *in vivo*. Even when using simple dienophile scaffolds such as norbornene in conjunction with non-optimal tetrazine structures, the 2nd order rate constants are still superior to those of other bioorthogonal reactions, making the IDDA reaction a prime candidate for deployment in pre-targeting. The stability of most strained alkenes *in vivo* has not been measured, but in the case of *trans*-cyclooctene, isomerisation to the unreactive *cis*-isomer has been seen, thought to be caused by the presence of thiols.[28] In the example where this phenomenon was noted, use of the cyclooctyne BCN **25** instead of a strained alkene gave improved results, although the isomerisation of *trans*-cyclooctene was believed to only occur in a fraction of the sample.

To date, there have been few examples demonstrated using a tetrazine ligation for pre-targeted reactions.[27,29] One of the first such examples was the use of a fluorescence-linked tetrazine reacting with the antibody cetuximab (which targets the EGF receptor, over expressed in cancer cells), which had been modified to contain a *trans*-cyclooctene (Scheme 9, Fig. 4). Despite a small amount of internalisation of the antibody (see Fig. 4B), the fluorescent tag accumulates in the same areas as



Scheme 9 Synthesis and fluorescence labelling of cetuximab, attached to cancer cells.

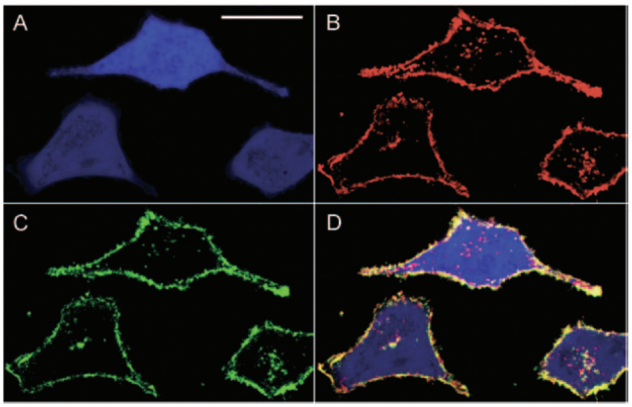


Fig. 4 Confocal microscopy of cetuximab-pretargeted GFP-positive A549 lung cancer cells after tetrazine–fluorophore labeling. (A) GFP channel. Scale bar: 30 mm. (B) Red channel: Cetuximab–*trans*-cyclooctene antibodies were also labeled with AF555 directly and imaged in the rhodamine channel. Some of the antibody has been internalized, as indicated by the signal inside the cells. (C) Near-IR channel showing the location of bound tetrazine–VT680 probe (500 nm, 10 min, 100% FBS, 378C). (D) Merging of GFP, red, and near-IR channels.

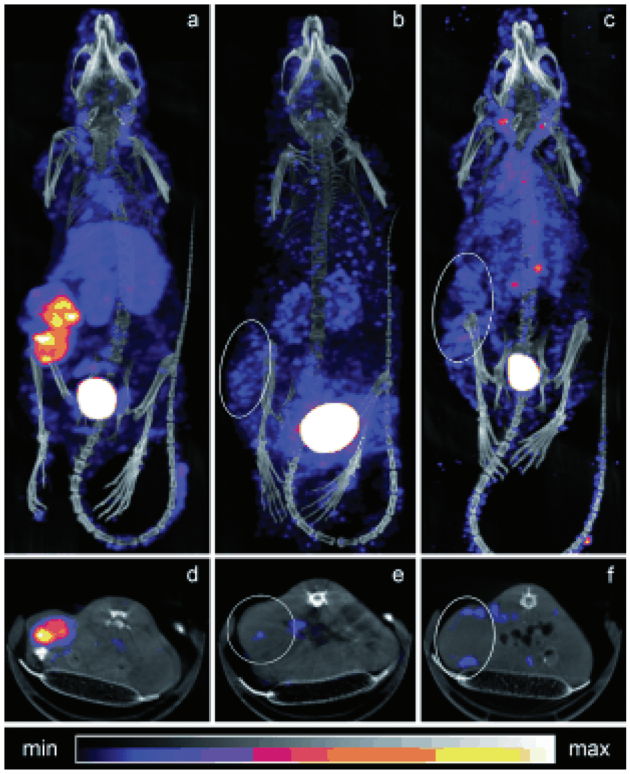


Fig. 5 Small-animal SPECT/CT imaging of live mice bearing colon carcinoma xenografts: posterior projections of mice preinjected with (a) CC49-TCO (100 mg) followed one day later by [^{111}In]-1 (25 equiv. to CC49; 3.4 equiv. to TCO, 42 MBq), (b) CC49 (100 mg) followed one day later by [^{111}In]-1 (same amount as in (a), 20 MBq), and (c) Rtx-TCO (100 mg) followed one day later by [^{111}In]-1 (same amount as in (a), 50 MBq); (d)–(f) single transverse slices (2 mm) passing through the tumors in (a)–(c).

that of the antibody within 60 minutes without passing into the cell (see Fig. 4C and 4D). This fluorescence work has since been extended using other biological interactions and employing alternative strained alkenes designed to give higher reaction rates.[27]

A subsequent example of the tetrazine ligation *in vivo*, was the pre-targeted ^{111}In labelling of tumours using monoclonal antibodies (mAb) (Fig. 5).[30]

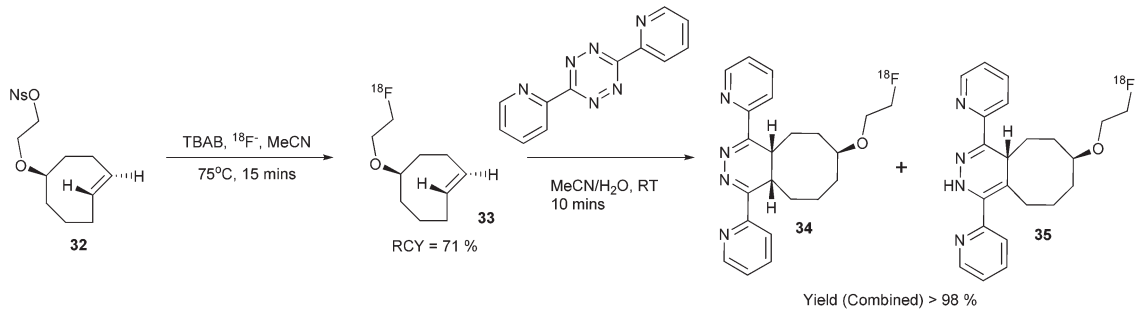
Indium-111 is a SPECT isotope with a long half-life (2.8 days), which allows for the isotope to be present in the circulation of a subject for a significant duration before decay. *trans*-Cyclooctene was conjugated to the mAb CC49, and injected into a set of mice containing tumours sensitive to the mAb. After allowing 24 hours for the mAb CC49 conjugate to accumulate, ^{111}In-labelled tetrazine was injected, and the mouse imaged 3 hours later, giving pre-targeted uptake in the tumour of 4% i.d. g^{-1}, in comparison to the minimal tumour uptake seen in the control experiments [Fig. 5, (a) *versus* (b) and (c)]. These experiments showed clearly that the tetrazine ligation can be used for pre-targeted molecular imaging, and whilst there are some drawbacks associated with the use of SPECT compared to PET imaging, and with the long half-life of the radioisotope used, the importance of this work cannot be underestimated.

Due to its superior rate of reaction and the possibility of its use for pre-targeting, the tetrazine ligation reaction has also been used several times recently with the short-lived isotopes fluorine-18 and carbon-11 (*e.g.* Scheme 10).[31,32] Interestingly, in the ^{18}F work the immediate cycloaddition product, diimine **34** undergoes a subsequent slow isomerisation to give enamino imine **35**. Incorporation of the fluoride into the (*E*)-cyclooctene derivative proceeds in good radiochemical yield. Attempts to alternatively label the tetrazine with ^{18}F in order to invert the reactive partners were unsuccessful, with only a 1% radiochemical yield of fluorine-18 labelled tetrazine obtained.

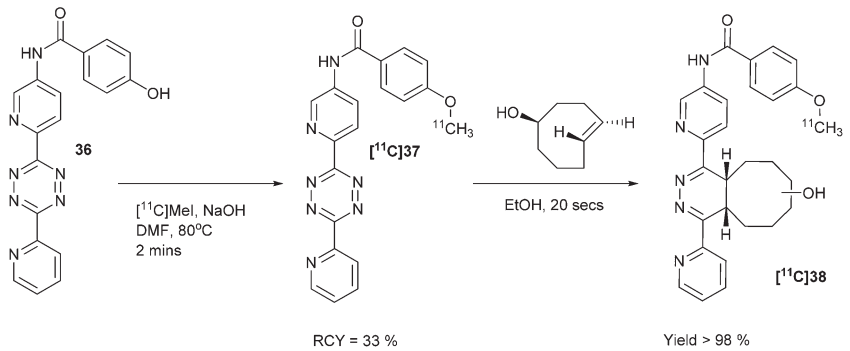
A subsequent example, by Herth and co-workers, showed that even in the hands of a shorter-lived isotope such as carbon-11 (half-life = 20.3 minutes), the tetrazine ligation can be carried out, giving high radiochemical yields (Scheme 11).[32]

Tetrazine **36** was methylated using [^{11}C]MeI–NaOH to give a good radiochemical conversion to **[^{11}C]37** within 2 minutes, followed by complete conversion to the various isomers of **[^{11}C]38** after a further 20 seconds at room temperature. Even inclusive of semi-preparative HPLC purification, the whole synthesis could be carried out within two half-lives (~40 minutes), which makes it a very promising method for the development of new radiotracers using carbon-11 with high specific activity.

Overall, tetrazine ligation is a valuable addition to the field of bioorthogonal chemistry. Improved reaction kinetics combined with a simplified synthesis of both partners for the reaction suggest that further exploration is warranted, especially in the area of pre-targeting. Of course, extensive *in vivo* testing has yet to be reported, so such variables as binding to red blood cells and stability to decomposition inside a living system have yet to be evaluated. However, as this reaction enables the use of shorter-lived isotopes, which in turn allows



Scheme 10 Example of the tetrazine ligation using fluorine-18.



Scheme 11 Example of the tetrazine ligation using carbon-11.

for the possible *in vivo* imaging of subjects *via* pre-targeting without the need for *ex vivo* analysis (*cf.* fluorescence), the tetrazine ligation appears poised to prove an extremely useful bioorthogonal reaction.

5. Conclusions

Bioorthogonal reactions have attracted significant interest from the biological and medicinal chemistry communities for a number of years now, and the hope is that this article has been able to highlight advantages and disadvantages of three of the main reactions in this field. Over the past seven years, there have been in the region of 700 publications describing the application and/or development of these reactions and the criteria for selection of one ligation reaction over another are not always clear. What is clear, however, is that the three ligation reactions focused on here are the preferred bioorthogonal reactions; fewer than 20 papers in total have been published over the same time period which describe alternative ligation reactions such as the quadricyclane reaction or the tetrazole photo click reaction (Fig. 6).[33,34]

In particular, each reaction's long term prospects for use in the field of pre-targeting have been evaluated. The Staudinger–Bertozzi ligation provides the most biocompatible resultant linkage, *i.e.* an amide bond!, but it has a number of drawbacks, including slow rates of reaction and low substrate stability under biological conditions. The SPAAC is currently the most popular bioorthogonal reaction and gives faster reaction

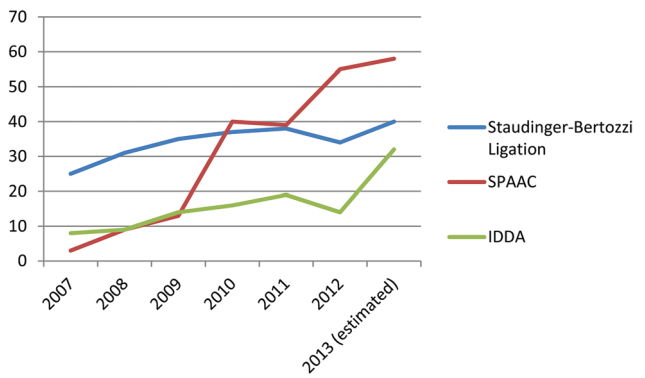


Fig. 6 Graph showing the number of papers using each bioorthogonal reaction per year (2013 an estimate based on the first 6 months).

rates and greater substrate stability *in vivo* relative to the Staudinger–Bertozzi ligation but it usually requires more sophisticated and time-consuming chemical synthesis to generate the required strained cyclooctyne substrates and still does not provide fast enough reaction rates to allow work with the short-lived isotopes required for some imaging applications. The IDDA appears to provide an attractive combination of straightforward synthetic access to the required substrates which are stable *in vivo* and reaction rates that are fast enough to enable work with even short-lived radioisotopes. Whilst clearly a perfect system has yet to emerge, the hope is that efficient protocols for pre-targeting with short-lived isotopes, magnetic resonance and other imaging modalities will soon

be developed and translated to allow use in the clinic in the near future. The field will undoubtedly continue to grow as researchers strive to find faster and more robust reactions capable of operating in complex living systems, and we hope that this perspective will inspire more researchers to contribute to this development and to contemplate using these methods in their own work.

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