Inverse electron demand Diels–Alder reactions in chemical biology

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The emerging inverse electron demand Diels–Alder (IEDDA) reaction stands out from other bioorthogonal reactions by virtue of its unmatchable kinetics, excellent orthogonality and biocompatibility. With the recent discovery of novel dienophiles and optimal tetrazine coupling partners, attention has now been turned to the use of IEDDA approaches in basic biology, imaging and therapeutics. Here we review this bioorthogonal reaction and its promising applications for live cell and animal studies. We first discuss the key factors that contribute to the fast IEDDA kinetics and describe the most recent advances in the synthesis of tetrazine and dienophile coupling partners. Both coupling partners have been incorporated into proteins for tracking and imaging by use of fluorogenic tetrazines that become strongly fluorescent upon reaction. Selected notable examples of such applications are presented. The exceptional fast kinetics of this catalyst-free reaction, even using low concentrations of coupling partners, make it amenable for in vivo radiolabelling using pretargeting methodologies, which are also discussed. Finally, IEDDA reactions have recently found use in bioorthogonal decaging to activate proteins or drugs in gain-of-function strategies. We conclude by showing applications of the IEDDA reaction in the construction of biomaterials that are used for drug delivery and multimodal imaging, among others. The use and utility of the IEDDA reaction is interdisciplinary and promises to revolutionize chemical biology, radiochemistry and materials science.

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Introduction

Chemical site-selective protein modification has become increasingly popular for probing and controlling protein functions in vitro and in living systems. The progressive developments of the last decade in genetic encoding and aqueous chemoselective reactions for protein modification have provided tools to “decorate” biomolecules with the desired functionality (affinity probes, fluorophores, reactive tags, post-translational protein modifications – PTMs, etc.) without significantly perturbing their native functions (Fig. 1).

These transformations have been used, for example, for the spatial and temporal control of biomolecules in vivo, for super-resolution imaging, and for the elucidation of the role of post-translational modifications, among others. Ideally, such bioorthogonal reactions must be (1) selective over other potential reactive functional groups present on biomolecules, (2) proceed in aqueous media at (3) near physiological pH and (4) have fast reaction rates at room temperature (or up to 37 °C) using low reactant concentrations, all to ensure high modification efficiency. However, the existing bioorthogonal reactions are not yet able to meet all the bioorthogonal requirements simultaneously, and thus, the specific labelling of biomolecules in their native environment using chemical reactions is still a very challenging task.

For instance, the requirement of Cu(I) to catalyse the 1,3 dipolar cycloaddition between azides and alkynes raises toxicity issues for its direct utilization in living cells due to Cu(I)-mediated generation of reactive oxygen species (ROS) from O₂. To circumvent this issue, a strain-promoted azide–alkyne cycloaddition version that does not require the use of copper was developed. However, the bioorthogonality of this approach remains an issue, since the cyclooctyne reagents may undergo side reactions with cellular and plasma nucleophiles (e.g. the sulphydril side chain of free Cys).

Bioorthogonal reporter chemistry (BORGChem) is a tool for probing the mechanism and function of bioactive molecules in living systems. For such applications, the rate constant is of critical importance as very fast kinetics are required for labelling cellular processes that occur on biological time scales. In this regard, the exceptional speed of the IEDDA reaction has triggered the development of new tetrazine and dienophile precursors to create more rapid and robust bioorthogonal reaction and highlight its recent applications in the abovementioned fields. We begin in Section 1 by presenting a general overview of the most representative bioorthogonal reactions with a focus on their general utilities and challenges. The IEDDA reaction has emerged as an important tool for probing the mechanism and function of bioactive molecules in living systems. For such applications, the rate constant is of critical importance as very fast kinetics are required for labelling cellular processes that occur on biological time scales. In this regard, the exceptional speed of the IEDDA reaction revolutionised our ability to explore such demanding applications. In Section 2 the key factors that are behind the fast kinetics of the reaction will be discussed. In addition, the increased use of the IEDDA reaction has triggered the development of new tetrazine and dienophile precursors to create more stable and reactive partners. As such, a brief summary of the methodologies recently described for the synthesis of “IEDDA precursors” will be also presented. As the reaction rates depend directly on the reaction partners, Section 3 presents an overview of the most relevant tetrazines and dienophiles, their stability and their reactivity. Another beneficial aspect of using tetrazines as coupling partners is the increase of fluorescence

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Fig. 1 Schematic representation of chemical biology approaches for protein labelling and/or engineering through bioorthogonal chemistry. The first step involves the introduction of a bioorthogonal reporter on a protein either by genetic, enzymatic or chemical methods. In a second step the bioorthogonal reporter reacts with a molecule of interest bearing a complementary reactive handle. This approach has been used to install a diverse array of molecules for (i) fluorescent labelling, (ii) glycosylation, (iii) lipidation, (iv) ubiquitination, (v) PEGylation, (vi) biotinylation, and (vii) drugs.
observed after bioorthogonal reaction, resulting in a “turn-on system” useful for example for cell imaging applications. In this section, we also provide some representative examples of highly fluorogenic tetrazines for IEDDA labelling. Over the years numerous tetrazine and dienophile functionalities have been incorporated into proteins via genetic, enzymatic and chemical approaches. These methods are presented and discussed in Section 4. In Section 5 we present some selected examples where the appealing features of the tetrazine ligation provide a step-change in biological applications beyond the reach of other bioorthogonal chemical reactions. One of the most challenging and important applications of the tetrazine ligation is the selective labelling of biomolecules inside living organisms for imaging and therapy using pretargeting approaches. These applications, which are extensively explored by radiochemists, are summarized in Section 6. The major focus of bioorthogonal chemistry in the past two decades has been largely centred on ‘bond formation’ reactions for protein modification. Just very recently, focus has been placed on reactions that can instead cleave specific bonds under bioorthogonal conditions. Section 7 will focus on recent developments in IEDDA elimination reactions for accurate spatiotemporal control over protein function and drug activity. Section 8 outlines the potential of the IEDDA reactions in the development of biomaterials for applications in biology and medicine such as 3-D cell culture, drug delivery, multimodal imaging and clinical diagnostics.

Finally, we draw conclusions and present a brief prospect of the role of IEDDA reactions in the field of bioorthogonal chemistry.

1. Meeting the candidates: a brief introduction to bioorthogonal chemistry

Arguably the most quoted example of a click reaction is the Cu(I)-catalysed [3+2] azido–alkyne cycloaddition (CuAAC) between an azide and alkyne, which was discovered independently by Sharpless et al. and Meldal et al. (Fig. 2i).16,17 Since these initial findings the CuAAC has been shown to be suitable for protein labelling in living systems, however, the wide use of CuAAC chemistry has been hindered by the potential toxicity induced by copper. In fact, Bertozzi et al. reported that mammalian cells could only survive to low concentrations (<500 μM) of Cu(I), leading to very slow CuAAC reaction rates at such concentrations that are not compatible with biological time scales. In addition, Zebrafish embryos also exhibited a similar sensitivity to Cu(I) limiting its use for in vivo applications.21 To improve the biocompatibility of the CuAAC reactions, the toxicity of Cu(I) was addressed by use of water-soluble Cu(I) ligands that stabilize the metal oxidation state and so, prevent the release of free copper ions that generate toxic reactive oxygen species.22,23 Notably, these biocompatible ligands have allowed the labelling

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**Fig. 2** Representative examples of bioorthogonal reactions for protein modification. (i) CuAAC,22,23 (ii) SPAAC,24 (iii) Staudinger,25 (iv) traceless Staudinger,26,27 (v) hydrazones and oximes formation,29 (vi) photoclick reactions,10,31 (vii) strain-promoted alkyne-nitrone cycloaddition (SPANC) reactions,32 (viii) transition metal catalysis,35 and (ix) IEDDA reactions. Reactive handles installed site selectively into proteins by genetic encoding techniques except for (vii).
of glycans in developing zebrafish embryos.\textsuperscript{23} The Cu(i) catalyst toxicity could be also obviated by use of strained alkyne substrates. The strain-promoted \([3+2]\) azide–alkyne cycloaddition (SPAAC) developed by Bertozzi \textit{et al.} in 2004 proceeds under physiological conditions without the need for a catalyst, however, in relatively slower kinetics when compared to the Cu(i) catalysed reaction (Fig. 2ii).\textsuperscript{18} Further enhancements in the rate constant of SPAAC additions have been reported using novel cyclooctyne analogues (\textit{e.g.} DIBO, DIFO and BCN).\textsuperscript{24} The use of azides as chemical reporters has been also explored by the Staudinger ligation with phosphines (Fig. 2iii).\textsuperscript{25} Whereas azides produce a stable triazole linkage in CuAAC and SPAAC reactions, the Staudinger ligation generates a stable amide bond. Soon after the publication on the Staudinger ligation, Raines \textit{et al.}\textsuperscript{26} and Bertozzi \textit{et al.}\textsuperscript{27} simultaneously reported the so-called traceless Staudinger ligation (Fig. 2iv). Although this click reaction has sufficient biocompatibility to be performed in living systems it suffers from phosphine oxidation and very slow kinetics.\textsuperscript{28} A wider list of bioorthogonal reactions includes other chemical transformations such as aldehyde/ketone-amine ligations\textsuperscript{29} (Fig. 2v), photoclick 1,3-dipolar cycloadditions between tetrazoles and substituted alkenes\textsuperscript{30,31} (Fig. 2vi) and, strain-promoted cycloadditions involving nitrones and alkynes\textsuperscript{32} (Fig. 2vii). These bioorthogonal reactions, however, have been used mainly for \textit{in vitro} bioconjugation because of the reaction conditions (\textit{e.g.} requirement of an acidic pH and a light source in the case of ketones/alkdehydes and tetrazoles, respectively). Recently, transition metal catalysis has been considered a powerful tool for the continued expansion of the bioconjugation toolkit (Fig. 2viii). Several emerging examples for forming new carbon–carbon bonds have been described including Suzuki\textsuperscript{13} and Sonogashira couplings\textsuperscript{34} or olefin metathesis.\textsuperscript{35} This “new class” of bioorthogonal reagents have been explored for labelling of proteins \textit{in vitro} and on the cell surface.\textsuperscript{36}

A breakthrough was achieved with the IEDDA reaction between tetrazines and strained dienophiles (Fig. 2ix), arguably the most optimal bioorthogonal reaction developed to date. A general overview of the features of the abovementioned bioorthogonal chemical reactions is presented in Fig. 3 with a focus on the general utility of each reported bioorthogonal reaction in terms of their reaction rates and labelling efficiency but also their various biological applications. The non-metal nature and physiological pH makes IEDDA more cell-friendly than CuAAC and oxime ligation. Superfast kinetics (10 000-fold

**Fig. 3** Examples of bioorthogonal reactions useful for bioconjugation and general comments about their utility and challenges.
faster than CuAAC) allows IEDDA to proceed with low concentrations, similar to the concentrations of intracellular proteins. This click reaction endows itself an irreplaceable tool to study cellular functions and dynamic processes. Indeed, this reaction meets the need for demanding biological applications, i.e., has high selectivity, is compatible with mild reaction conditions in water and has impressive fast kinetics. Such assets have never been achieved with other site-selective reactions, highlighting the importance of IEDDA ligations in bioorthogonal chemistry.

2. Principles of IEDDA reaction

2.1 Mechanism

Diels–Alder [4+2]-cycloaddition describes the reaction between a diene (e.g. 1,2,4,5-tetrazines) and a dienophile (alkene or alkyne) to form a six-membered ring in a \( \pi_4s + \pi_2s \) fashion (Fig. 4a), via suprafacial/suprafacial interaction of 4\( \pi \)-electrons of the diene with the 2\( \pi \)-electrons of the dienophile (Fig. 4b). In contrast to a normal electron demand Diels–Alder reaction, where an electron-rich diene reacts with an electron-poor dienophile, in an inverse-electron-demand Diels–Alder reaction (IEDDA), an electron-rich dienophile reacts with an electron-poor diene (Fig. 4b). The ability of tetrazines to react with unsaturated compounds was first revealed in 1959 by Lindsey et al.\(^{37}\) This Diels–Alder reaction proceeds via the 1,4-addition of the \(-\text{C}==\text{N}==\text{N}==\text{C}-\) diene system of the tetrazine to an appropriate alkene, yielding a highly strained bicyclic intermediate (Fig. 4a). Upon the evolution of 1 equivalent of nitrogen, the adduct undergoes a retro-Diels–Alder reaction to afford the corresponding 4,5-dihydropyridazine, which either isomerises to the corresponding 1,4-dihydro-isomers or is oxidized to give a pyridazine product (Fig. 4a). Alkyne dienophiles directly yield the respective pyridazine upon reaction.

2.2 Features for reactivity of IEDDA reactants

Based on the frontier molecular orbital theory (FMO), the IEDDA reaction kinetics is governed by the energy gap between the corresponding HOMO and LUMO of the reactants (Fig. 4b). In particular, any pairs of diene/dienophile with a smaller HOMOdienophile–LUMOdiene energy difference would react faster in IEDDA reactions. Since the earlier comprehensive kinetics studies in organic solvents performed by Sauer and Boger, efforts have been made to find fast IEDDA reactants for rapid bioorthogonal reaction in aqueous media. Indeed, by fine-tuning the tetrazine and dienophile pairs considerable rate enhancements and bioorthogonality have been achieved. The different factors tuning the IEDDA kinetics are discussed as follows.

2.2.1 Electronic effects of the substituents in the IEDDA partners. Electron-donating groups (EDG) raise both HOMO and LUMO energy of the cycloaddends, whereas electron-withdrawing groups (EWG) act conversely. Therefore, by electronically tuning the cycloaddends it is possible to manipulate the LUMO\(_\text{dienophile}–\text{HOMO}_{\text{dienophile}}\) energy gap and enhance their reactivity. In terms of the diene partner, electron deficiency lowers the LUMO\(_\text{dienophile}\) energy, accelerating the IEDDA reaction (Fig. 5a).

![Fig. 4](image.png)

Fig. 4 Mechanism of IEDDA reaction. (a) Schematic representation of the reaction between a dienophile and a tetrazine. (b) Frontier molecular orbital of normal and inverse electron demand Diels–Alder reaction. EDG = electron-donating group, EWG = electron-withdrawing group.

![Fig. 5](image.png)

Fig. 5 (a) Dependence of reactivity against LUMO energy of tetrazine. (b) Reactivity of heterocyclic ring bearing various numbers of nitrogen atoms. (c) Electronic effects on regioselectivity. (d) Electronic effects on olefinic and acetylenic dienophiles.
compared to cyclooctene (TCO) as a precursor gave a tremendous rate difference (Fig. 6a). Furthermore, it was observed that the use of trans-cyclooctene (TCO) as a precursor gave a tremendous rate difference compared to cis-cyclooctene and most importantly to the other cyclic alkenes with higher ring strain (e.g. cyclopropene). This higher reactivity is attributed to a ‘crown’ conformation adopted by TCO, which is lower in energy than the ‘half-chair’ conformation of cis-cyclooctene as predicted by ab initio calculations (Fig. 6b). Indeed, computational studies have emerged as an important tool for understanding and predicting the reactivity of IEDDA partners. Studies performed by Houk et al. revealed that strained dienophiles are more reactive due to their pre-distorted conformation towards the transition structures. Therefore, less distortion energies are needed for the dienophile to react. Additional insights on designing more reactive dienophiles were obtained with the help of computers. Based on ab initio calculations, the fusion of a cyclopropane ring with trans-cyclooctene to form s-TCO was proposed as a strategy to induce the formation of a non-crown conformer which was expected to increase the reactivity towards tetrazines (Fig. 6c). The resulting more highly strained, fused TCO was found to have a lower computed barrier, which makes the reaction 160 times faster than the original TCO, as verified by experimental \( \Delta \Delta G^\text{cis-cyclooctene} \) (3.0 kcal mol\(^{-1}\)) and calculated \( \Delta \Delta G^\text{cis-cyclooctene} \) (3.34 kcal mol\(^{-1}\)). Similarly, in order to increase the stability and hydrophilicity, while maintaining the ring strain, a cis-dioxolane-fused TCO (d-TCO) was proposed to display high reactivity towards tetrazines (Fig. 6d). Compared with the original TCO, 27-fold rate enhancement was observed towards diphenyl-s-tetrazine, which is in good alignment with the computationally predicted \( \Delta \Delta G \). Similarly, a high dependence between the degree of ring strain and reactivity is observed for the IEDDA reaction of cyclooctynes and tetrazines. As previously described for cyclooctynes there is a decrease in the activation energy by raising the HOMO of the cyclooctyne-dienophile.
The influence of the stereochemistry is also presented in others dienophiles. The exo-norbornene reacts with tetrazines 3 times faster than the endo one (Fig. 7b).66 The same trend is observed for syn-d-TCO that has a slightly higher rate constant (1.2 ×) compared with the anti-diastereomer (Fig. 7c).60 Finally, the cyclooctyne endo-BCN isomer is slightly faster than its exo isomer in reaction with tetrazines.67

2.2.4 Steric effect. In the dienophile, any substituent exchanging hydrogen, in principle, results in an increase of the steric effects hampering its reactivity. Therefore, terminal alkenes and alkynes dienophiles are more reactive than the corresponding internal ones. Consequently, introducing EDGs into dienophiles needs to compensate for the impeding steric effects, which sometimes leads to only comparable or even lower reactivity.49 The reactivity of tetrazines can be similarly altered via steric modifications. For instance, several groups have reported that mono substituted tetrazines participate in IEDDA reactions with faster kinetics than di-substituted tetrazines, even with strong EWG.40,44,68 This important observation has been used to design mutual orthogonal IEDDA reactants. In a pioneering study Devaraj group elegantly demonstrated that a mono-substituted tetrazine undergoes IEDDA ligation with TCO more than 30 times faster than cyclopropene (Fig. 8a). In contrast, if the tetrazine is substituted with a bulky tert-butyl group, the unfavourable steric repulsion between TCO and the “bulky tetrazine” slows the reaction, leaving the cyclopropene tag with faster reaction rates.69 In a concurrent study, H-tetrazine (H-Tz) was proved to be more reactive than Me-tetrazine (Me-Tz),62 due to electronic effects, and mostly, due to additional distortion energies from the methyl group. Thus, under certain reaction conditions and time scale, Lemke et al. showed that some dienophiles could react quickly with the reactive Me-Tz but not with the Met-Tz. Specifically, it was observed that SCO exclusively reacts with H-Tz, due to steric constrains, while TCO reacts with nearly both tetrazines (Fig. 8b). The authors applied these exclusive reactivities for the dual labelling of cell surface proteins (more details in Section 5).8 Importantly, the orthogonality of this system to azides renders multi-colour labelling of biological systems possible via Diels–Alder and SPAAC click reactions.8 Recent DFT studies revealed that SCO undergoes normal electron demand Diels–Alder reactions with both H-Tz and Me-Tz instead of inverse reactions.62 These results should be highlighted since the type of reaction may switch upon minimal chemical changes, which may have direct consequences for the rational design of orthogonal dienophiles, in this particular case, or in the optimization of the IEDDA reaction.62

2.2.5 Solvent and pH effect. Protic solvents are described to accelerate the IEDDA reaction and this effect is even more pronounced when reactions are performed in water. This effect is attributed to the stabilized interaction between the activated complex and water but also to an enhanced hydrophobic interaction between cycloaddends, which is facilitated in water.70 Additionally, hydrogen-bonding with tetrazines, similar to electron-withdrawing substituents, induces polarity and lowers the HOMO–LUMO energy gap, enhancing the reaction rate.42,54,71,72 It should be noted that the reaction solvent may also affect the product formation in IEDDA reactions. For instance, when ketene acetals are used as dienophiles, increasing polarity of the solvent favours the meta-isomer whereas ethanol exclusively affords the ortho-isomer.24 The formation of stable hydrogen-bonded complexes between tetrazine and protic solvents is also described to guide the reaction route.73 The dependence of reaction rate on pH was found to be minor. A lower pH is expected to accelerate the reaction possibly by protonation of the pyridine rings.40,71

2.3 Synthesis of tetrazine derivatives

Conventionally tetrazines are obtained via the synthesis of dihydrotetrazines by reacting the appropriate precursors
(e.g. aromatic/aliphatic nitriles and imidoesters) with hydrazine, followed by oxidation to afford the reactive tetrazine cores. Since recently, new methodologies based on carbon–carbon bond formation have been explored in the generation of various tetrazines, especially tetrazines functionalized with fluorescent dyes. In the following sections these methods are presented and discussed in detail.

2.3.1 Classic methods for the preparation of tetrazines.

A well-developed method for the synthesis of tetrazines was first reported by Adolf Pinner in 1893.\textsuperscript{74} Typical Pinner conditions involve the reaction of imidoesters with hydrazine, leading to the formation of amidrazone intermediates, which undergo further reaction with excess of hydrazine to give dihydrotetrazines and finally tetrazines by oxidation (Fig. 9a).\textsuperscript{74} Another well-established, and probably, the most widely used method for the synthesis of tetrazines is the condensation of two aromatic or alkyl nitriles with hydrazine (Fig. 9b(i)). Hydrazine, depending on the type of starting materials, is often used as the hydrate or anhydrous forms with THF or ethanol as co-solvents. Although useful, this nitrile-based route is however limited if the aim is to prepare disubstituted asymmetric tetrazines since a statistical mixture of products of difficult purification is obtained (Fig. 9b(iii)). By using a large excess of one of the components, asymmetric tetrazines could be afforded, however, still with poor yields (<22%) and on relatively small scales.\textsuperscript{40} Of note, the condensation reaction of one nitrite and formamidine acetate results in the formation of monosubstituted tetrazines (Fig. 9b(iii)). The addition of sulphur as catalyst is also being extensively explored for the synthesis of both symmetric and asymmetric tetrazines and leads to improved reaction yields (60–70%, Fig. 9b(iv)).\textsuperscript{75,76} Sulphur is described to react first with hydrazine to give NH\textsubscript{2}NHSH, which serves as an active nucleophile towards nitriles, generating a reactive intermediate that eliminates H\textsubscript{2}S to form the dihydrotetrazine core.\textsuperscript{43,75,77} Importantly, although these conditions have been successfully used for the synthesis of aromatic tetrazines, very low yields are reported in the case of alkyl tetrazines. Alternatively, N-acetylsalicylic acid was successfully tested as catalyst for the synthesis of tetrazines possibly through the formation of amidrazone intermediates.\textsuperscript{78}

Recently, new synthetic routes have been explored for the synthesis of tetrazines. Devaraj \textit{et al.} reported the use of Lewis acids as catalysts for the activation of nitriles, including unreactive alkyl nitriles, by coordinating to the nitrile and promoting the nucleophilic addition of hydrazine. It was shown that nickel and zinc triflates allowed the one-pot synthesis of both symmetric and asymmetric dialkyl tetrazines in good yields (Fig. 9b(v)).\textsuperscript{79} Ni(OTf)\textsubscript{2} catalyst was later used for the preparation of asymmetric monosubstituted tetrazines in high yield (~75%) on gram-scale synthesis (Fig. 9b(vi)), whereas sulphur-promoted reaction only resulted in yields lower than 20%.\textsuperscript{80} Importantly, this method was applied in the synthesis of coumarin-tetrazine and BODIPY–tetrazine fluorescent probes.\textsuperscript{81,82} Although high yields were achieved, this one-step synthesis method has limitations of substrate scope. The hydrazine and heating conditions are not compatible with several functional groups that are susceptible to either nucleophilic addition or reduction, such as carbonyls and alkyl halides. To overcome these limitations acylhydrazides have been used for the synthesis of (a) symmetric substituted tetrazines, in a stepwise reaction first reported by Stolle \textit{et al.}, involving the synthesis of 1,2-dichloromethylene hydrazines by treatment with PCl\textsubscript{3} and subsequent condensation with hydrazine and oxidation (Fig. 9c).\textsuperscript{83,84} With this general methodology, both symmetric and asymmetric tetrazines with EWGs and aliphatic substituents were synthesized with modest yields.\textsuperscript{85} Microwave irradiation has also been proved to assist the formation of tetrazine.\textsuperscript{86} A further optimization on the condensation of 1,2-dichloromethylene hydrazine with hydrazine monohydrate by the same group showed that microwave irradiation could reduce the reaction time from 5–16 h to 30 mins with an extra 10–20% yield increase, compared to refluxing conditions.\textsuperscript{42} Another alternative method commonly used for tetrazine synthesis is the nucleophilic aromatic substitution (SNAr) starting from the relevant precursors (e.g. methylthio-1,2,4,5-tetrazine, 3,6-dichloro-1,2,4,5-tetrazine, 3,6-(3,5-dimethylpyrazolyl)-1,2,4,5-tetrazine, Fig. 9d).\textsuperscript{87}
2.3.2 Modern methods for tetrazine synthesis by C–C bond formation. As mentioned, a relatively small number of asymmetric substituted-tetrazines have been reported due to the difficulty of their preparation and purification. To overcome these limitations transition metal-catalysed coupling reactions have been used to prepare tetrazines by C–C bond formation directly onto the tetrazine core or onto a number of different substituents attached to tetrazines (see next section for further details). Both approaches have been particularly useful for introducing multifunctionality on tetrazines such as fluorescent probes or functional groups (e.g. amino groups) for further coupling reactions, which is desirable for biological and material chemistry. The first reported cross-coupling reaction on tetrazines was via the Sonogashira coupling reaction of various chlorotetrazines with acetylene derivatives in the presence of Pd(PPh$_3$)$_2$Cl$_2$ and CuI (Fig. 10a). The starting materials were prepared in moderate to good yields by selective displacement of one chlorine atom from the commercial available 3,6-dichlorotetrazine by different nucleophiles. The Sonogashira coupling products were obtained in low, moderate or good yields (30–65%) depending on the conjugated substituents. Indeed, success of the reaction depends on the electron-donating properties of the ring substituent. More electron-deficient chlorotetrazines tend to result in tetrazine decomposition.

Negishi coupling of chlorotetrazines with hexynylzinc chloride also affords coupling products with 30% yield (Fig. 10b). Under Suzuki-like and Stille-like conditions Guillaumet et al. reported the reaction of 3-methylthio-1,2,4,5-tetrazine derivatives with boronic acids and organostannane precursors (Fig. 10c and d). Yields of 30–70% were achieved under microwave conditions for both reactions. This Pd-catalysed cross-coupling reaction represents the first of few methods to construct asymmetrical tetrazines using vinyl, aryl and heteroaryl substituents. Recently, Pd-catalysed Stille cross coupling reactions were developed for the derivatization of tetrazines at positions C3 and C6 with fluorescent probes (Fig. 10e). Wombacher et al. identified 3-bromo-6-methyl-1,2,4,5-tetrazine as a suitable building block for coupling with protected fluorophore-organotin derivatives, giving yields around 20%. Interestingly, this method is applicable for fluorescein and Oregon green derivatives but not for rhodamine or Si-rhodamine dyes. The replacement of the chlorine on tetrazines by nucleophilic aromatic substitution using alkylolithium is another alternative method developed by Tang et al. (Fig. 10f). A number of tetrazine derivatives have been also prepared by Audebert et al. by nucleophilic substitution of chlorotetrazines with ethynyl compounds in the presence of n-BuLi, although in low yields (~12%).

2.3.3 Coupling reaction on tetrazine substituents. Considering the high need for the preparation of fluorescent-tetrazine conjugates, various cross coupling reactions have been tested for the direct conjugation of 1,2,4,5-tetrazines to fluorescent dyes. Recently, Devaraj et al. introduced an elimination-Heck cascade reaction (Fig. 11a) for such purposes. In the presence of Pd$_2$(dba)$_3$ and a co-ligand (iron complex) alkenyl highly fluorogenic tetrazines were afforded in good to excellent yields bearing popular dyes such as xanthene and BODIPY fluorophores. At the same time, Wombacher et al. also managed to synthesize tetrazine fluorophores by Sonogashira cross-coupling reactions (Fig. 11b). With PdCl$_2$(PPh$_3$)$_2$, and Cu(I) as catalysts, tetrazine ethynyl fluorophores were synthesized in good yield (64% and 69%) and on a relatively large scale. Suzuki- or Heck-type cross-coupling reactions were also used for synthesizing tetrazine-phenoxazine derivatives with reasonable yields (Fig. 11c and d). Recently, Friedel–Crafts alkylation was also developed...
for the preparation of red-shifted tetrazine fluorophores using aldehydes and diarylether or diarylsilane compounds (Fig. 11e). Stille coupling has also been widely explored in several reports of tetrazine synthesis. Different conditions have been examined with reasonable yields (Fig. 11f).

### 2.3.4 Oxidation of dihydrotetrazine

Nitrous gas, generated from sodium nitrate with acid, is the most commonly used oxidant for converting dihydrotetrazine into the corresponding tetrazine (Fig. 12a). A typical protocol involves addition of NaNO₂ solution together with 1 M HCl or glacial acetic acid at 0°C until the oxidation is completed. However, low yields might occur when dealing with intolerant functional groups, such as amino groups. Metal oxides MnO₂ and CrO₃ have also been explored for oxidation of dihydrotetrazines but the moderate yields and toxic chromium waste limit their applications. Other oxidants like peroxy acid, m-CPBA and benzoyl peroxide are being used for oxidation of dihydrotetrazines. DDQ (dichlorodicyanobenzoquinone) has recently been reported to oxidize amino dipyridine–dihydrotetrazines where NaNO₂ fails. However, the product was difficult to separate from the hydroquinone byproducts. A recent systematic study by Fox et al. identified Phl(OAc)₂ as an efficient oxidant and the product could easily be separated from the byproduct iodobenzene (Fig. 12b). The oxidant is amine-tolerant and robust for synthesizing dialkyl- and diaryl-tetrazine bearing and heteroaromatic rings. During this study iodogen and a fluoruous analogue of chloramine-T (F-CAT), were found to oxidize amino dipyridine–dihydrotetrazines with 86% yield and the product could be easily separated through fluoruous solid-phase extraction.

Alternatively, by using photocatalysts, the dihydrotetrazine could be oxidized to tetrazine in quantitative yields with 660 nm LED in the presence of methylene blue within 200 s. Even under ambient light, the photoredox reaction could be afforded in 47% conversion in 2 h (Fig. 12b). Similarly, horseradish peroxidase could also oxidize dihydrotetrazine with fast reaction rates ($K_m = 1.0 \times 10^{-4}$ M, $k_{cat} = 27$ s⁻¹, and $k_{cat}/K_m = 2.7 \times 10^5$ M⁻¹ s⁻¹, Fig. 12c).

### 2.4 Synthesis of dienophiles

For metabolic oligosaccharide engineering and genetic encoding applications, the synthesis of dienophile-bearing sugars and amino acids is usually performed by using commercially available NHS- and p-nitrophenyl-activated dienophiles. Otherwise, multistep synthesis is required where unstrained dienophiles, such as terminal alkenes, are of advantage. Cyclopropenes are usually accessed from TMS-protected propynes via rhodium-catalysed cyclopropanation with diazo compounds (Fig. 13a).

For the preparation of cyclopropene amide-linked derivatives, the TMS-protected ester subsequently undergoes base-catalysed hydrolysis (e.g. potassium hydroxide) to give the TMS-free acid, which is then subjected to the corresponding amine to afford the desired amide (Fig. 13a). For the synthesis of cyclopropenes containing a carbamate scaffold, DIBAL reduction agent is typically used to produce the appropriate alcohol, followed by carbamate formation and TMS removal. Importantly, methylcyclopropenyl methanol cannot be isolated as it is prone to rapid polymerization upon concentration, where a one-pot synthesis is required.

The fast grow of IEDDA reaction in the bioorthogonal chemistry field is closely boosted with the advanced TCO synthesis methodology. A robust synthesis of this IEDDA partner was reported by Fox et al. in 2008 using a one-step reaction via flow photo isomerisation. Recently, in order to improve the trans/cis ratios and reduce the photo degradation of the active trans-cyclooctene, a new synthetic route was proposed in a closed-loop process.

![Fig. 13](https://example.com/fig13.png)  
Common synthetic routes for the preparation of most common dienophiles.
flow apparatus.\textsuperscript{108,109} In this synthesis, the reaction mixture is continuously photo irradiated at 254 nm and pumped through AgNO\textsubscript{3} impregnated silica, which binds strongly with the desired trans-isomer, eluting the cis-isomer to be isomerised again until completely consumed. This direct method was further applied in the synthesis of most TCO derivatives published so far since then.\textsuperscript{108}

Despite the extensively use of TCO in bioorthogonal chemistry, the commercial availability of TCO is still limited. All typical syntheses for TCO derivatives involve the construction of the corresponding cis-derivatives and then trans-isomerisation. Efforts have been made to introduce extra ring strain and functionality at defined positions. For example, rhodium-catalysed cyclopropanation on bicyclo[6.1.0]non-4-yn-3-yl was also used to annihilate the cyclopropane ring to maintain the half chair conformation of bicyclo[6.1.0]non-4-ene (Fig. 13b).\textsuperscript{59} Robillard et al. designed an elegant synthesis route to construct a bis-NHS-activated TCO-bearing linker for antibody-targeted therapy. A methyl group near the carboxylic acid was introduced to prevent epimerisation and to control regioselectivity\textsuperscript{15} (Fig. 13c).

3. The toolbox of bioorthogonal partners for IEDDA reactions

3.1 Dienophiles as IEDDA precursors

As abovementioned, the IEDDA reaction can be tuned to reach rate constants from 1 up to 10\textsuperscript{6} M\textsuperscript{-1} s\textsuperscript{-1} by changing the electron deficiency of the 1,2,4,5-tetrazine precursors, or by manipulating the ring strain and electronic effects on the dienophiles. Significant efforts have focused on accelerating the reaction rate by expanding the scope of dienophiles. The first reported dienophile for protein bioconjugation via IEDDA reaction was the strained TCO introduced by Fox et al. that revealed an unusually fast second-order rate constant towards a dipyriddy-tetrazine precursor (Tz\textsubscript{8}) of \(k_1 = 1140\text{ M}^{-1}\text{ s}^{-1}\) in MeOH and 2000 M\textsuperscript{-1} s\textsuperscript{-1} in 9:1 MeOH/water (Fig. 14a).\textsuperscript{100} In pure water at 25 °C, the equatorial-diaxial isomer of TCO reacts with a water-soluble dipyriddy-tetrazine derivative (Tz\textsubscript{9}) with an increased rate constant of 22 600 M\textsuperscript{-1} s\textsuperscript{-1} while the axial-diastereomer reacts faster with a \(k_d = 80\text{ 000}\ M^{-1} s^{-1}\) (Fig. 14a).\textsuperscript{60} Robillard et al. also reported that the axial diastereomer of 5-hydroxy-TCO is more reactive than the equatorial diastereomer due to instability of the axially substituted ring induced by transannular interactions.\textsuperscript{100} Fox et al. subsequently optimized TCO in terms of reactivity by fusing a cis-cyclopropane onto the cyclooctene moiety to increase the ring strain (s-TCO, Fig. 14b).\textsuperscript{57} When Tz\textsubscript{8} is reacted in MeOH at 25 °C, this strained derivative is 19 times more reactive than the parent TCO (\(k_2 = 22 \text{ 000}\text{ M}^{-1}\text{ s}^{-1}\)).\textsuperscript{59} Later, it was demonstrated that a water soluble s-TCO derivative bearing a PEG linker reacts with Tz\textsubscript{9} in pure water at a rate constant of 3 300 000 M\textsuperscript{-1} s\textsuperscript{-1} (s-TCO, Fig. 14b), which makes this dienophile the fastest to date.\textsuperscript{60} There are some limitations, however, that emerge from the high reactivity of the “TCO dienophiles”. One is the deactivation of TCO by isomerisation in the presence of high thiol concentrations. As an example, Robillard et al. showed that in fresh mouse serum at 37 °C the TCO isomer converts into cis-cyclooctene with a half-life of 3.26 h.\textsuperscript{110} Secondly, the “TCO dienophiles” TCO/s-TCO are not stable enough for prolonged storage. Recently, Johnson et al. introduced a new variant of s-TCO complexed with AgNO\textsubscript{3}, which has improved stability and can be used directly in cells for rapid bioorthogonal reactions with tetrazines.\textsuperscript{111} Alternatively, to address the stability limitations of s-TCO Fox et al. designed a new conformationally-strained dioxalane-fused trans-cyclooctene (d-TCO), which maintains high reactivity towards tetrazines (\(k_3 = 366 000\text{ M}^{-1}\text{ s}^{-1}\) for syn isomer and 318 000 M\textsuperscript{-1} s\textsuperscript{-1} for anti-isomer at 25 °C in pure water with Tz\textsubscript{9}) but displays enhanced stability and is more easily prepared (Fig. 14c).\textsuperscript{60} Stability studies of the syn isomer in human serum at room temperature showed no isomerisation or decomposition after 24 h neither after 4 days (>97% trans isomer).

Bicyclooctynes (bicyclo[6.1.0]nonyne, BCN) were found also to undergo fast IEDDA reactions with tetrazines (\(k_4 \text{ = 45 M}^{-1}\text{ s}^{-1}\) in MeOH at 24 °C with Tz\textsubscript{8}; \(k_5 = 1245\text{ M}^{-1}\text{ s}^{-1}\) in 55% MeOH/H\textsubscript{2}O at 25 °C with Tz\textsubscript{10}; Fig. 14d).\textsuperscript{61,112} Recently, Prescher et al. showed that 1,2,4-triazines participate in IEDDA reactions with strained alkenes (Fig. 14e).\textsuperscript{45} In particular, it was found that 1,2,4-triazines react with TCO, but not with norbornene or cyclop propane.\textsuperscript{45} Considering the latter two undergo efficient
cycloadditions with 1,2,4,5-tetrazines, this suggests an opportunity for sequential dual-labelling. However, this methodology remains unexplored most likely because of the relatively slow kinetics of the 1,2,4-triazine: TCO reaction \( (k_2 = 7.5 \ M^{-1} \ s^{-1} \text{ in } 50\% \ ACN/PBS \text{ at } 25 \ ^\circ C \text{ with Tz 11, Fig. 14e}) \). Importantly, this new class of IEDDA reagents showed enhanced stability enabling its direct use in recombinant protein production (fully stable in PBS and in excess of cysteine for 1 week at 37 \(^\circ C\)).\(^{45}\) A contemporary study showed that 1,2,4-triazines also react with strained alkenes (BCN), however, with even more sluggish kinetics \( (k_2 = 0.38 \times 10^{-3} \ M^{-1} \ s^{-1} \text{ with BCN in acetonitrile}) \).\(^{46}\)

Recently, vinylboronic acids (VBA) appeared as a new interesting class of compounds for fast and water-soluble reactions with tetrazines \( (27 \ M^{-1} \ s^{-1} \text{ with Tz 8; Fig. 14f}) \).\(^{113}\) The use of cyclopropenes as tetrazine reactive partners was also proposed as an alternative to TCO dienophiles (Fig. 14g). The Devaraj group demonstrated that modulating the substituents of cyclopropenes has a dramatic effect on their stability and on the kinetics towards tetrazines. Indeed, it is well described that unsubstituted cyclopropenes are susceptible to both nucleophilic attack and polymerization reactions. To overcome these limitations, Devaraj et al. “protected” the double bond with a methyl group, and the resulting 1-methyl-3-substituted cyclopropene derivatives appeared as fast and \( in \ text{ vivo} \) stable dienophiles with one of these analogues having a second-order rate constant of \( 2.3 \ M^{-1} \ s^{-1} \text{ (in } 50\% \text{ aqueous solutions with Tz 8; Fig. 14g}) \).\(^{69,104,106}\) Faster reaction rates for this dienophile are expected if measured under purely aqueous conditions. Norbornenes, another class of cyclic dienophiles, offer an excellent balance between facile strain-promoted reactivity with tetrazines and overall chemical stability (Fig. 14h). Tetrazine Tz 12 reacts rapidly with norbornene in aqueous buffer with a second-order rate constant of \( 1.9 \ M^{-1} \ s^{-1} \) (Fig. 14h).\(^{114}\) Recently, a new bioorthogonal \( N \)-acylazetine small tag showed equal efficiency as the norbornene for protein modification \textit{via} IEDDA reaction with a rate constant of \( 0.39 \ M^{-1} \ s^{-1} \text{ against Tz 12 (Fig. 14i)} \). Vinylbenzyl groups (styrene) also react efficiently in IEDDA reactions \( (0.05 \ M^{-1} \ s^{-1} \text{ against Tz 8 in } 50\% \text{ MeOH/water, Fig. 14j})^{115,116} \). Other minimal-tags include terminal unstrained alkene-decorated sugars\(^{117} \) \( (0.021 \ M^{-1} \ s^{-1} \text{ against Tz 14}) \) and nucleosides \( (5\text{-vinyl-2'-deoxyuridine, VdU, 0.021} \ M^{-1} \ s^{-1} \text{ against Tz 8, Fig. 14k}) \).\(^{117}\) Recently, our group explored chemical installed S-allyl handles for “IEDDA labelling” with tetrazines (Fig. 14l). Although, this handle reacts significantly slower \( (0.002 \ M^{-1} \ s^{-1} \text{ against Tz 8 in } 50\% \text{ MeOH/PBS}) \) compared to \textit{trans}-cyclooctenes, it was suitable for pretargeting live cell imaging.\(^{118}\)

While most studies of reaction kinetics are performed on small molecule models, it is important to study what is the actual kinetics of these reactions in the protein context. In one example, Chin et al. showed that the second-order rate constant for the labelling of sfGFP bearing a 1,3 disubstituted cyclopropene with a fluorescent analogue of Tz 8 was \( 27 \pm 1.8 \ M^{-1} \ s^{-1} \) (Fig. 15). Importantly, it was shown that the rate constant for this reaction in the test tube and in cells is approximately 10-fold faster when compared to the cyclopropene model \( (2.3 \ M^{-1} \ s^{-1}, \text{ Fig. 15}) \). The differences in the reaction rates should be related with the reaction solvent (water for protein and 50% water/org for small molecule). Similarly, Lemke et al. demonstrated that the tetrazine ligation \( (Tz 12\text{-TAMRA}) \) with a BCN-GFP bearing protein occurs with a reaction rate constant of \( k_2 = 29,000 \pm 7,500 \ M^{-1} \ s^{-1} \) (Fig. 15a).\(^{119}\) Progress of the labelling reaction was determined using Förster resonance energy transfer (FRET) to follow the decrease in GFP fluorescence and the increase of the TAMRA fluorescence upon GFP excitation. Just recently, the Lemke group developed a new hydrophilic TCO analogue (DOTCO; dioxo-TCO) for genetic encoding which showed fast reactions with a Cy5 (cyanine dye) tetrazine when incorporated into GFP \( (6,370 \ M^{-1} \ s^{-1}) \) in pure water with Tz 12-Cy5; Fig. 15a).\(^{120}\) An ideal bioorthogonal reaction requires a rate of \( >10^5 \ M^{-1} \ s^{-1} \text{ inside cells to reach completion in seconds to min at biological concentrations (\muM to nM) of the bioorthogonal target} \).\(^{121}\)

![Fig. 15](https://example.com/image1.png) On protein and in cellular rate constant determination for reaction of GFP bearing dienophiles and tetrazine.
To meet these rates, Mehl et al. developed a tetrazine-modified GFP (GFP-Tet-v2.0) that reacts in cells with s-TCO with a rate constant of 72 500 ± 1660 M⁻¹ s⁻¹ (Fig. 15). The authors predict that this reaction rate allows 95% labelling in <1 min at 1 μM concentrations of Tet-v2.0-protein and s-TCO, meeting the needs of the ideal bioorthogonal ligation. Compared to the “cuvette second-order rate” (87 000 ± 1440 M⁻¹ s⁻¹) the rate constant reduction is justified by the in vivo cellular environment or hampered uptake of s-TCO.

3.2 Dienes as IEDDA precursors – fast kinetics versus stability

Tetrazine partners are also a key factor in the IEDDA reaction rates. Hilderbrand et al. have shown that electron-withdrawing substituents enhance IEDDA reactivity of tetrazines, however, these substituents may also induce their degradation. An optimal balance between reactivity and stability must be achieved for effective labelling in in vivo systems. The disubstituted tetrazines Tz 15–Tz 16 comprising electron-donating substituents show the highest stability (~90% stable), but also suffer from slow kinetics (200–2000 M⁻¹ s⁻¹) (Fig. 16). Hydrogen substituted tetrazines Tz 22 and Tz 23 demonstrate a good balance of stability (~70% stable for Tz 23) and fast reaction kinetics (30 000 M⁻¹ s⁻¹). These compounds are not the most electronically favourable (no electron-withdrawing substituents) but the fastest suggesting these tetrazines may react faster due to less steric interference. The least stable tetrazine, but also one of the fastest, comprises electron-withdrawing groups (R₂ = pyrimidine; Tz 21).

3.3 Fluorogenic tetrazines for IEDDA ligations

In bioorthogonal labelling approaches a large amount of the bioorthogonal fluorescent dye is often required to increase reaction efficiency. In these examples the excess of the fluorescent probe has to be removed using several washing steps in order to reduce background fluorescence of the unreacted probes. One alternative approach to minimize background fluorescence is based on the use of fluorogenic probes, which become fluorescent only upon transformation. Such fluorogenic dyes are extremely advantageous for in vivo labelling applications, since they usually result in highly conspicuous images without requiring washing steps.

3.3.1 Principles behind fluorogenicity in bioorthogonal reactions

In general, there are four main strategies to develop fluorogenic probes and these are based on (1) protonation–deprotonation species (pH sensitive probes), (2) complexation (including direct complexation and competitive displacement), (3) cleavage and formation of covalent bonds, and (4) redox reaction. These strategies have recently been reviewed in detail. In bioorthogonal chemistry, fluorogenic probes are designed to have a functional group that quenches the fluorescence emitted from the fluorophore. Upon reaction, the quencher is transformed/modified, enabling the fluorescence to be restored. Other approaches use the bioorthogonal reaction to trigger the release of either photocaging or quencher moieties. One of the very first examples of fluorogenic dyes activated by click chemistry used azido groups to quench coumarins, which after reaction with terminal alkynes afforded intense fluorescent 1,2,3-triazole products. In another example, Wong et al. developed azido-BODIPY probes, which showed strong fluorescence upon triazole formation (Fig. 17a). The most important feature of fluorogenic system is the enhancement factor (or “turn on” effect) that is conferred by the ratio of the fluorescent intensity or quantum yield of the product and the starting material at a given wavelength. The azido-BODIPY probes were found to have a 52-fold increase in fluorescence quantum yield after CuAAC reaction (Fig. 17a).

There are three major photophysical quenching mechanisms, which involve Förster resonance energy transfer (FRET), through-bond energy transfer (TRET), and photoinduced electron transfer (PET). In the CuAAC reaction the increase in fluorescence quantum yield (52×) upon triazole formation is due to the lowering of the HOMO energy level of the aryl moiety that reduce the PET to the BODIPY acceptor (Fig. 17a). The presence of an alkyne motif can also function as a quencher, although not so frequently. After the pioneering work on terminal alkynes by Farhni et al. and on cyclooctyne-fused coumarins by Bertozzi et al. and Boons et al. reported a dibenzo-cyclooctyne, which exhibited a ~59-fold fluorescent increase after SPAAC reaction with alkynes (Fig. 17b). Fluorescence increase is due to large differences in oscillator strengths of the S₀ ↔ S₁ transitions in the planar C₂v-symmetry of the starting material compared to the nonplanar cycloaddition product. Staudinger ligation has also been exploited for the development of fluorogenic probes (Fig. 17c). In one example, a phosphine-modified coumarin showed a 59-fold increase in fluorescence quantum yield after click reaction (λexc 443, λem 495 nm). Prior to the Staudinger reaction the original phosphine is responsible for the quenching via internal charge transfer (ICT). Upon oxidation to the phosphine oxide, the ICT originating from the phosphorous’ lone pair is no longer possible, therefore the fluorescence is restored. For more information about the applications of click fluorogenic probes the reader is directed to a recent review by Kele et al.

3.3.2 Photophysical quenched fluorogenic probes for IEDDA

Tetrazines have become important for bioconjugation strategies also due to their high fluorogenicity. Since tetrazines are chromophores absorbing light at around 500–530 nm they can act as...
quenchers towards a series of fluorophores. Interestingly, depending on the substituent, tetrazines themselves can also exhibit intrinsic fluorescence. However, due to the low extinction coefficient of the associated absorption band their fluorescence suffers from low intensity.\(^{131,132}\) In 2010 Weissleder et al. showed that a series of tetrazine–fluorophore conjugates (coumarin, BODIPY FL, Oregon Green 488, BODIPY TMR-X, VT680) are efficiently quenched by tetrazines, resulting in a “turn-on” effect up to a 20-fold after destruction of the tetrazine core upon IEDDA cycloaddition.\(^ {133}\) The authors propose that the fluorescence quenching is due to resonant energy transfer between the fluorescent chromophore and the tetrazine (benzylamino-1,2,4,5-tetrazine, Tz\(_{12}\)) which has a visible absorbance maximum at 515 nm (Fig. 18a). This explains the wavelength dependence of the quenching; green- and red-emitting tetrazine dyes (BODIPY FL, \(\lambda_{ex} = 505\) nm, \(\lambda_{em} = 512\) nm, 15-fold increase; Oregon Green 488, \(\lambda_{ex} = 495\) nm, \(\lambda_{em} = 523\) nm, 18.5-fold increase; BODIPY TMR-X, \(\lambda_{ex} = 543\) nm, \(\lambda_{em} = 573\) nm, 20.6-fold increase) showed higher

![image](https://via.placeholder.com/150)

**Fig. 17** Bioorthogonal fluorogenic probes. (a) Fluorogenic CuAAC reaction of azido-BODIPY with alkynes. (b) Fluorogenic dyes activated by SPAAC. (c) Coumarin-phosphine fluorogenic dye activated by the Staudinger ligation. \(\Phi_F = \) fluorescent quantum yield.

![image](https://via.placeholder.com/150)

**Fig. 18** (a) Example of the mechanism of quenching of fluorophore–tetrazine conjugates by Förster Resonance Energy Transfer. As the tetrazine is consumed by cycloaddition the FRET quenching is forbidden and fluorescence is restored. (b) Photophysical properties of the depicted dyes before and after reaction with TCO.\(^ {133}\) The extent of fluorescence quenching depends on the wavelength emission of the fluorophores and the absorption spectra of the tetrazine. \(\Phi_F = \) fluorescent quantum yield.
fluorescence enhancements upon cycloaddition, while near-IR-emitting dyes (tetrazine-VT680; $\lambda_{ex} = 669$ nm, $\lambda_{em} = 687$ nm) were not quenched (Fig. 18b).\textsuperscript{133}

Fluorogenic BODIPY probes. We and the Chin group also showed that tetrazines can quench tetramethylrhodamine (yellow-emitting, $\lambda_{ex} = 550$ nm, $\lambda_{em} = \sim 585$ nm, 4–5-fold increase) but the fluorescence enhancement is less pronounced.\textsuperscript{72,118} In a following work the group of Weissleder proposed an alternative way for designing more efficient turn-on probes, which consisted in the design of conformationally restricted tetrazine–BODIPY conjugates for fluorescence quenching through bond energy transfer (TBET).\textsuperscript{82} TBET systems require the energy donor and acceptor moieties to be connected through twisted, but otherwise conjugated, $\pi$-electron systems. In addition, unlike traditional FRET, TBET quenching does not require overlapping emission and absorption bands of fluorophores and tetrazines, respectively.\textsuperscript{134} This approach resulted in new fluorogenic probes with a turn-on up to 1600 times (Fig. 19a). It was shown that structures with enhanced spatial donor–acceptor proximity resulted in higher magnitudes of fluorescence turn-on. The exceptional fluorogenic turn-on ratios of the new probes compared to the flexible linked tetrazine–fluorophore conjugates (e.g. 80-fold greater than tetrazine–BODIPY TMR-X) suggest that TBET is the most likely quenching mechanism for these compounds. The suitability of this fluorophore for biological applications was demonstrated by imaging EGFR expression with TCO-functionalized antibodies on both fixed and live cells.\textsuperscript{82} Later in 2014 Wombacher et al. reported other rigid tetrazine–BODIPY conjugates, which showed less fluorogenicity upon reaction with TCO (Fig. 19b). This work further demonstrated that the close proximity between the tetrazine and the fluorophore as well as fixation of transition dipoles is crucial to obtain strong quenching.\textsuperscript{94} Indeed, the new probes Tz–BODIPY 4 and Tz–BODIPY 5 show little structural flexibility and identical geometrical layouts concerning their donor and acceptor transition dipoles. However, the BODIPY derivative with a shorter interchromophore (Tz–BODIPY 5) exhibits twice of the fluorescence of the one with a longer linker (Tz–BODIPY 4).\textsuperscript{24}

Fluorogenic coumarin probes. A series of coumarin–tetrazine probes were also prepared and their design included aligned transition dipoles of the coumarin fluorophore and the tetrazine in order to maximize contributions from FRET in addition to TBET (Fig. 20a). These probes, named HELIOS probes (HELIOS = hyperemissive ligation-initiated orthogonal sensing), exhibit the highest brightness enhancements reported for any bioorthogonal fluorogenic dyes with fluorescence enhancements up to 11 000-fold. The fluorescence quenching of the coumarin cores by tetrazines occurs mainly via a TBET mechanism.\textsuperscript{81} Imaging of epidermal growth factor receptor (EGFR) on the surface of cancer cells using a TCO labelled anti-EGFR antibody (Cetuximab) and HELIOS 370H was performed within seconds of dye addition and exhibited no nonspecific signal according to the control experiments (Fig. 20b).

Fluorogenic xanthene probes. Xanthene dyes, such as fluorescein and rhodamine derivatives, are arguably the most popular class of fluorescent probes for cellular imaging and are typically highly soluble in aqueous solutions.\textsuperscript{83} In this regard, Devaraj et al. synthesized tetrazine-conjugated xanthene dyes such as Oregon-Green and tetramethylrhodamine (TAMRA) conjugated to tetrazines via a rigid styrenyl linker.\textsuperscript{93} The Oregon Green probe (Tz–OG 1) underwent a 400-fold fluorescence enhancement upon TCO reaction while the red-emitting TAMRA (Tz–Rh 1) showed a 75-fold enhancement in fluorescence (Fig. 21a).\textsuperscript{91}
Recently, Kele et al. reported new tetrazine-phenoxazine fluorogenic probes. The red excitability and emission properties of these probes ensure minimal autofluorescence, while TBET fluorogenicity reduces nonspecific background fluorescence. Indeed, due to its high lipophilicity phenoxazine is often compromised by high background fluorescence, however, the high fluorogenicity of the new tetrazine-phenoxazine probes (up to 275× turn-on) allowed efficient labelling of live cells (Fig. 21b). 95

To expand on this approach in the context of multicolor imaging, Wombacher et al. synthesized a panel of rigidly-linked green- to far-red-emitting fluorogenic tetrazine probes (Fig. 21c). 99 Once again the authors found that the distance between the tetrazine and the fluorophore, and the fluorophore itself, are crucial for efficient quenching and for the resulting fluorescence turn-on upon conversion by IEDDA reaction.

3.3.3 IEDDA fluorogenicity induced by fluorophore generation. All previously mentioned strategies to design IEDDA fluorogenic bioorthogonal reactions are based on the destruction of the tetrazine moiety that suppresses fluorescence of a fluorophore. Another strategy is based on the simultaneous generation of a fluorophore through a bioorthogonal chemical transformation. In this regard, Guo et al. developed a new approach for fluorogenic bioorthogonal labelling via IEDDA reaction. 135 This unprecedented approach is based on the intrinsic fluorescence of 4-phenyl-3,6-di(4-pyridin-2-yl)-1,4-diarylpyridazine (PDHP), the product from the reaction of styrene and Tz 1 (k2 = 0.078 M−1 s−1) (Fig. 22a). Comparing to some commonly used fluorophores (e.g. fluorescein pH 9, Φ = 0.95, ε = 93 000 M−1 cm−1, Stokes shift = 24), PDHP (ex = 360 nm, em = 465 nm) has relatively low quantum yield (0.011 in MeOH and 0.251 in ACN) and extinction coefficient (4329 M−1 cm−1 in MeOH and 3769 M−1 cm−1 in ACN). 135 On the other hand, PDHP has a large Stokes shift (~100 nm), which could be beneficial (e.g. less self-quenching and/or auto-fluorescence background) in certain imaging applications. Finally, a lysine-derived non-canonical amino acid containing styrene moiety (KStyr) was genetically encoded into an intracellular stress response protein, HdeA, and labelled in living cells after tetrazine reaction. Recently, Vrabel et al. reported a fluorogenic reaction between axial-TCO with tetrazine (Fig. 22b). By screening a series of tetrazines, the photochemical properties of the resulting 1,4-dihydropyridazines could be tuned towards 91-fold fluorescence intensity increase and emissions ranging from 480 to 605 nm. By modifying tetrazines with peptides on resin, the addition of axial-TCO resulted in rapid fluorescent product within 1.5 min. Furthermore, taxol- and triphenylphosphonium-tetrazine derivatives were also synthesized for targeting subcellular compartments and efficient and fast fluorogenic labelling was observed on microtubules and mitochondria. 136

4. Methods for installing IEDDA chemical reports on biomolecules

Selective and site-specific labelling of proteins is essential for studying protein structure, activity, localization and trafficking. During the last decade, a wide variety of IEDDA bioorthogonal reporters have been incorporated into biomolecules through genetic encoding of non-canonical amino acids (ncAAs) during protein synthesis, or through enzymatic and chemical modification of native residues on post-expressed proteins. These methods will be presented and discussed in the following sections.

4.1 Genetic code expansion

The development of strategies to genetically encode ncAAs into proteins has had arguably the greatest impact upon the progress of bioorthogonal chemistry in recent years. In this approach, an aminoaeryl-tRNA synthetase (aaRS)/transfer RNA (tRNA) pair is used to insert an ncAA into the growing protein chain in response to an amber stop codon (UAG) on the mRNA. 137–139 A plethora of ncAAs bearing ketones, aldehydes, azides, alkenes, alkynes, tetrazines, or aryl halides reactive groups have been developed in recent years using this strategy and incorporated into E. coli, yeast, mammalian cells and even animals. 141,142

The vast majority of studies on the incorporation of ncAAs in IEDDA applications so far have used the pyrrolysine synthetase/tRNA pair (PylRS/tRNAUA) from methanosarcinina for three reasons. Firstly, this synthetase, in its native form, incorporates none of the 20 canonical amino acids. Secondly, the high promiscuity of PylRS and the evolutionary mutants allowed...
the incorporation of more than 300 hundreds kind of ncAAs.\textsuperscript{143} Finally, while the use of other synthetase–tRNA pairs for incorporating ncAAs is limited to cells from a particular organisms, orthogonal PylRS variants can be selected for \textit{E. coli} and then used to incorporate ncAAs into yeast, mammalian cells and animals.\textsuperscript{143,144} The enormous progresses made in the field of genetic code expansion over the recent years has resulted in up to 26 ncAAs incorporated into proteins for IEDDA applications (Fig. 23). A summary of the most significant examples is described in the following sections.
4.1.1 Norbornene. As the first published example of a genetically encoded dienophile, encoded carbamate-norbornene derivatives were reported simultaneously in 2012 by the groups of Chin (ncAA 1) and Carell (ncAA 2) (Fig. 23). Using the wild type MmPylRS/tRNA_{CUA} pair (Mm = Methanosarcina mazei) a norbornene-containing Lys ncAA (ncAA 1) was incorporated into an epidermal growth factor receptor-green fluorescent fused protein (EGFR-GFP) in HEK293 and efficiently labelled with minimal background using a fluorogenic tetrazine-based fluorophore. Later, the MmPylRS/tRNA_{CUA} pair was found to be orthogonal to the translation system of Drosophila melanogaster. In order to optimize the genetic introduction and labelling efficiency of various norbornene-Lys derivatives (endo-, exo- and strained-norbornene, ncAA 2–4), a MmPylRS triple mutant (Y306G, Y384F, I405R) was successfully tested for the incorporation of ncAA 2–4 into human carbonic anhydrase II (HCA) in E. coli, indicating the promiscuity of this triple mutant synthetase with norbornene derivatives. Recently, Bertozzi et al. established a robust method for cell surface display of mucins. This has been accomplished by genetically encoding a norbornene chemical handle into the extracellular portion of EGFR membrane proteins using the MmPylRS/tRNA_{CUA} pair. The next step involved the modification of the encoded protein by reaction with tetracyclic end-modified glycoproteptides. Norbornene-containing Tyr ncAAs (ncAA 5–7) were also incorporated into proteins using an evolved Mm PylRS mutant [Y306A/N346A/C348A/Y384F].

4.1.2 trans-Cyclooctene (TCO). A TCO-Lys (ncAA 8) was first incorporated site-specifically into proteins in 2012 using a double-mutant synthetase MmPylRS (Y306A, Y384F). Similarly, a Methanosarcina barkeri PylRS triple mutant (MbpPylRS, Y271M, L274G, and C313A) was later used to incorporate TCO into an EGFR-GFP fusion protein in HEK293 cells, allowing rapid and specific labelling with a tetrazine fluorophore in <2 min. In contrast, under identical conditions, a reaction period of 2 h was required for labelling the corresponding norbornene-modified protein. The fast labelling of TCO-tagged EGFR-GFP with tetrazines was recently explored for studying the trafficking of EGFR. Importantly, the same mutant was used to incorporate s-TCO into the same fusion protein. However, in this case no reaction was observed upon addition of the tetracyclic probe, suggesting the in vivo trans-to-cis isomerisation of the dienophile. Other ring isomers of TCO, namely TCO\(^+\) (ncAA 9) and TCO\(^*\) (ncAA 10), are also tolerated by the promiscuous double-mutant MmPylRS. Importantly, TCO\(^*\), but not TCO or TCO\(^+\), showed a high stability against isomerisation to the non-reactive cis form due to a carbamate bond shielding effect. In a recent study, the superior stability and reactivity of TCO\(^*\) was explored for cross-linking proteins in living cells by reaction of TCO\(^*\)-proteins with peptides bearing tetra-cysteine motifs through a “tetracyclic FlasH linker” (Fig. 24a), in a method called T-CrAsH (Fig. 24b).

Although TCO has been successfully used for labelling genetically encoded proteins, several reports have described that the ncAA TCO-Lys is relatively sticky resulting in high non-specific fluorescence background upon addition of the tetracyclic dyes, even if extra washing steps are applied to remove excess of non-incorporated ncAAs. With this in mind, an hydrophilic TCO-Lys derivative containing exo- and endo-cyclic heteroatoms introduced in the TCO ring was developed in order to increase hydrophilicity. The new hydrophilic ncAA DOTCO-Lys (ncAA 11) showed high incorporation efficiency in both prokaryotic and mammalian cells using a double-mutant pyrrolysyl-tRNA synthetase. Importantly, comparative washout studies revealed that only 5 min are required to remove DOTCO-Lys from cells whereas more than 6 h are needed to remove excess of BCN-Lys and TCO\(^*\)-Lys (Fig. 25). Thus, this hydrophilic DOTCO-Lys could be used to label and track intracellular proteins with rapid turn-over and expressed in low concentrations. Apart from Lys derivatives, TCO-Tyr ncAAs (ncAA 12, ncAA 13) have been also incorporated into proteins in E. coli.

4.1.3 Cyclopropene. Cyclopropene functionalisation has the drawback of slower kinetics in IEDDA reactions in comparison to other stained alkenes, but its relatively small size endows it a suitable candidate for certain biological applications where large ncAAs may interfere with structure and function of the target proteins. Lin et al. first genetically encoded a 3,3-disubstituted cyclopropene handle on proteins for photoclick chemistry with tetrazoles. Later, the Chin group explored 1,3-disubstituted cyclopropene ncAAs (ncAA 14) for IEDDA protein labelling, in particular, for proteome tagging and labelling (more details in Section 5.4). In general, the cyclopropene handle has the advantage of being efficiently incorporated into proteins without suffering from isomerisation and presenting a relatively small size and fast kinetics (labelling reaction on proteins completed within 30 min).
4.1.4 Unstrained alkenes. Unstrained alkenes react with tetrazine with second-order rate constants of more than 0.001 M$^{-1}$ s$^{-1}$, rates comparable to those of the Staudinger ligation and the

Fig. 23 Summary of ncAAs that have been genetically encoded into proteins for IEDDA application.

Fig. 24 (a) Chemical structure of T-CrAsH reagent and the products of the two covalent reactions. (b) General scheme of the T-CrAsH method.

Fig. 25 Comparative washout studies with DOTCO, BCN and TCO ncAAs. Confocal microscopy imaging of non-transfected cells washed for different times before labelling with a tetrazine dye. With DOTCO intracellular non-specific background is easily removed from the cytoplasm after washing for 5 min. From ref. 120, Copyright © 2016 by John Wiley & Sons, Inc. Reprinted in part by permission of John Wiley & Sons, Inc.
copper-free dibenzocyclooctyne–azide cycloaddition. In comparison to strained alkenes, unstrained alkenes provide some advantages such as their small size, easy synthesis and enhanced stability towards cellular nucleophiles. Even though unstrained alkenes have already been successfully incorporated into proteins via genetic code expansion, it was not until 2015 that these terminal alkene-encoded proteins were successfully used for IEDDA applications. Liu group successfully incorporated 9 different terminal alkene-bearing amino acids (ncAA 15–18) into E. coli using mutant PyrRS/tRNA_{CUA} pairs. Four of these ncAAs (with highest reaction rates and stability) enabled the selective labelling of proteins on the surface of live bacterial cells (outer membrane protein X-3 and 8, OmpX-3 and OmpX-8) by reaction with tetrazine dyes, although with poor reaction rates (over 8 h incubation). 157 In a following paper, a terminal alkene-containing fatty acyl lysine (ncAA 19) was genetically encoded into ubiquitin and histone H3 and selectively labelled by reaction with tetrazine dyes. 158 Apart from aliphatic alkenes, a styrene-bearing lysine (ncAA 20) was also recently incorporated into super folded GFP in E. coli using a PyrRS/tRNA_{CUA} triple mutant (L274A, C313S, Y349F). 153

4.1.5 Cyclooctyne. Contrasting with alkenes that form various isomers upon tetrazine ligation, cyclooctynes such as strained cyclooctynes (SCO) or bicyclo6.1.0ynone (BCN) have the advantage of yielding single products after IEDDA ligation, which allows the construction of homogeneous protein conjugates. Bicyclo6.1.0ynone (BCN). BCN-Lys (ncAA 21) was first encoded into sfGFP in E. coli and live mammalian cells by Chin et al. using a MBPyrRS triplet mutant (Y271M, L274G, and C313A). 112 Contemporaneous with this work Lemke et al. reported the incorporation of BCN (ncAA 22) into several other proteins (EGFR, hepatitis D virus, actin, vimentin and FK506-binding protein), via MBPyrRS and MmpyrRS mutants. 151,152,153,160 Recently Chin et al. developed a genetically directed bioorthogonal ligand tethering (BOLT) method and used it for selective inhibition (iBOLT) of protein function. Specifically, BCN-MEK variants (MEK = mitogen-activated protein kinase) and specific MEK inhibitors conjugated to a tetrazine through different linkers were used for rapid and covalent inhibition of MEK isoymes. By introducing an azobenzene linker between the tetrazine and the inhibitor (photo-BOLT), reversible and optical regulation of MEK kinase activity was achieved in live cells by cis/trans isomerisation of the azobenzene. This technique was shown useful for spatiotemporal control of protein function. 161 In another example, BCN encoded sfGFP has been found to undergo IEDDA ligations with tetrazine glycans conjugates both in vitro and in vivo, providing another method for rapidly and homogenous engineering of glycoproteins. 162

Strained cyclooctyne (SCO). First developed for site-specific protein labelling through SPAAC with fluorogenic azides, 163 genetically encoded SCO handles were later used for IEDDA applications. SCO (ncAA 23) was incorporated into GFP via a double mutant synthetase from Mm and labelled with a Cy5 tetrazine dye. Detectable fluorescence was observed only after 2 h with excess of dye that led to significant background staining. 119,149 Apart from Lys derivatives, SCO-Tyr ncAA (ncAA 24) has been also incorporated into proteins in E. coli and modified through IEDDA. 148

4.1.6 Tetrazine. Despite the excellent kinetics displayed by TCO ncAAs, their isomerisation and consequent reduced reactivity limits their utility for in vivo approaches (half-life 3.26 h for TCO in fresh plasma and ~3 h for s-TCO in MeOH with excess of thiol). 1,29,110 A stable and reactive tetrazine-bearing amino acid could be an alternative for site-specific labelling through IEDDA. The first genetic encoded tetrazine amino acid (tet-v1.0, ncAA 25), a derivative of p-aminophenylalanine derivative conjugated to a tetrazine, was incorporated in E. coli via an evolved orthogonal M/TysRS/tRNA_{CUA} pair. A tet-v1.0-GFP conjugate was shown to react with s-TCO, however, only with modest kinetics (k_s ~ 880 M⁻¹ s⁻¹ and ~330 M⁻¹ s⁻¹ under in vitro and in vivo conditions, respectively). 164 In line with these results the same tet-v1.0-GFP construct was later labelled with d-TCO with an in vitro rate constant around 100 M⁻¹ s⁻¹. 160 Due to the synthetically insubstituency and moderate reaction rates of tet-v1.0, a new version of this ncAA, a phenylalaninetezine (ncAA 26), was developed for ideal bioorthogonally labelling. Tet-v2.0 showed high stability (several days) in the presence of thiols in PBS buffer and could be successfully incorporated into GFP with the help of an evolved M/TysRS/tRNA_{CUA} pair. The resulting Tet-v2.0-GFP undergoes IEDDA several orders of magnitude faster than Tet-v1.0 with a rate constant of ~72,500 M⁻¹ s⁻¹ and a half reaction time of 12–14 seconds against s-TCO. This new ncAA enabled the efficient substoichiometric labelling of proteins in live cells, eliminating the need for washing out excess labelling agents. 121

4.1.7 Dual genetic encoding. Substituting two native amino acids in a protein with two ncAAs of interest can be useful for example for dual labelling with distinct probes to study folding and conformational changes of proteins. For this purpose, evolved aaRS/tRNA pairs were used to incorporate two ncAAs into a protein in response to an amber and quadruplet codon. 165 Specifically, ncAAs bearing alkyne and cyclopropene reactive handles were incorporated together into a single protein using the orthogonal PrpRS/tRNA_{CUA} and PrpRS/tRNA_{ACU} pairs. The latter decodes the quadruplet codon with the help of the evolved orthogonal ribosome ribo-Q1. Because CuAAC and IEDDA reactions are orthogonal, quantitative dual labelling of proteins was rapidly achieved by reaction with the corresponding tagged-fluorophores (within 30 min). 166 In another example, due to the slow kinetics between deactivated tetrazines and norbornene, aminophenylalanine-tetrazine and norbornene amino acids were shown not to cross-react with each other even when placed in close proximity within the same protein. Thus, these two amino acids were site-specifically incorporated into calmodulin using the optimized orthogonal translation system.Orthogonal reaction with a BCN fluorophore (for labelling the tetrazine ncAA) and a fluorescent activated tetrazine (for labelling the norbornene ncAA), respectively, led to dual labelling. The orthogonal installation of fluorophores at defined sites enabled monitoring local conformational change of CaM towards calcium ions binding using FRET. 167
Dual genetic encoding has been also described using the promiscuity of tRNA synthetases. Indeed, Lemke et al. showed that a PyrRS/tRNA_{CUA} double mutant from Mm could accept both SCO and TCO*. As a result, simultaneous incorporation of two different ncAAs into proteins was possible via a pulse-chase labelling strategy. By fine-tuning the diene partners, selective labelling of these ncAAs allowed dual colour labelling of biological relevant proteins (Fig. 26). Specifically, Lemke’s lab found that, under certain reaction conditions and timescale of the experiment, TCO* reacts with both mono-substituted (H-Tz) and di-substituted (Me-Tz) tetrazines, while SCO reacts only with mono-substituted variants (Fig. 26). Of note, the reactivity of H-Tz and Me-Tz with other dienophiles was recently studied in detail and to proceed as follows: H-Tz:endoBCN > or ≈ exoBCN > TCO* mix isomers > SCO > norbornene; Me-Tz: TCO* mix isomers ≈ endoBCN > or ≈ exoBCN ≈ SCO > norbornene. This provides guidance for constructing orthogonal reactions and dual labelling systems.67

4.2 Alternative enzymatic methods or enzyme tags for protein modification by IEDDA

An alternative to genetic encoding methods for protein bioconjugation uses engineered enzymes that covalently attach a labelled substrate to a tagged protein of interest by recognizing a peptide sequence or a small fused protein (e.g. trypsiligase, lipoic acid ligase, PFTase, sortase). Another approach involves the use of self-labelling enzymes that have been engineered to attach a fluorescent molecule to one of its own amino acid residues (SNAP/Clip tag, HaloTag).1,3

4.2.1 Trypsiligase. The introduction of the tripeptide recognition sequence YRH at the N- or C-terminal region of a protein of interest (POI) via standard mutagenesis enables site-specific enzymatic conjugation of click handles. Trypsiligase, an engineered trypsin variant, is both able to cleave the YRH peptide bond and to attach diverse moieties to the ensuing terminal amino acid. The N-terminal cleavage of the tag is followed by the introduction of the click handles provided in the form of acyl-4-guanidinophenyl ester derivatives (OGp), whose ester leaving groups mimic trypsin-specific arginyl substrate side chains (Fig. 27a). Addition of the click counterpart leads to the final modified protein. Cleavage of the tag at the N-terminal results in a transient acyl–enzyme intermediate that reacts with Arg-His-containing peptides bearing a tetrazine that could be used for further conjugation (Fig. 27a). Using trypsiligase-catalysed reaction, Bordusa et al. managed to modify peptides and antibodies with norbornene and Tz-moieties at N- and C-terminus respectively. Most reactions are completed in one hour with nearly quantitative product yield, suggesting the high efficiency of the trypsiligase reaction. Subsequent IEDDA enables fluorescence labelling, PEGylation and drug conjugation.168

4.2.2 Lipoic acid ligase. Site-specific protein labelling using PRIME (probe incorporation mediated by enzymes), is based on a mutant lipoic acid ligase from E. coli that covalently conjugates chemical handles to a LpLA acceptor peptide (LAP) fused proteins (Fig. 27b). Ting et al. have shown that an engineered LpLA with a single mutation at Trp37 (LpLA W37V) can efficiently ligate TCO into target proteins (e.g. lipoproteins, neurologin, actin, and vimentin). The TCO-ligated LAP reacts with tetrazine–fluorophores with high second-order rate constants (~ 13-fold increase in fluorescence intensity upon reaction) allowed, for example, the labelling of LAP-tagged low-density lipoprotein receptors in HEK 293T cells (15 min for TCO ligation and 3 min for tetrazine labelling).83 Similarly, by using LpLA W37V, Wombacher et al. successfully ligated a norbornene derivative with a transmembrane protein with an extracellular LAP-tag in HEK293T cells.169

4.2.3 Protein farnesyltransferase. Protein farnesyltransferase (PFTase) has been used to covalently attach farnesyl diphosphate site-specifically at the cysteine residue of the recognizing sequence CAAX (Fig. 27c). Using the promiscuity of PFTase, Distefano et al. synthesized a TCO geranyl diphosphate for PFTase mediated chemoenzymatic bioconjugation. CAAX-box containing peptides and proteins proved to be attached to TCO moieties by the catalysis of PFTase. Despite the bulkiness of TCO, the hydrophobic tag fits the active site of PFTase with only 2-fold difference $k_m$ compared with its physiological substrate. Interestingly, further optimized by site-directed mutagenesis, Y205A mutant of PFTase was proved to enhance $k_{cat}$ by 5-fold when compared to the wildtype, reaching a sub-micromolar $k_m$.170

4.2.4 Sortase. Another method uses sortase (SrtA) to mediate site-specific protein labelling. The enzyme recognizes a 5 amino acid peptide sequence near the C-terminus of its target site, cleaves a specific peptide bond within this sequence, and forms an amide bond between the new C-terminal amino acid and an N-terminal glycine of a polyglycine species (Fig. 27d). By using an optimized penta-mutant sortase A, a single domain antibody VHH bearing LPXTG sequence at its C-terminal was site-specifically modified by (Gly)$_3$ TCO. TCO-modified VHH was then ligated with radioactive aminoxy-tetrazine derivative for PET imaging of pancreatic cancer.171 A Tz-modified VHH...
followed by labelling with a $^{18}$F-TCO derivative has also been used for tumour imaging.\textsuperscript{172} Just recently, Cochran \textit{et al.} engineered a sortase variant 7M (SrtA7M), which could use various small amines instead of polyglycine as substrates.\textsuperscript{173}

4.2.5 Halo tag. The bacterial enzyme haloalkane dehalogenase has been engineered as a self-labelling protein tag (Halo Tag). Indeed, an engineered variant of this enzyme catalyses the formation of a covalent ester bond between an aspartate in the enzyme and the alkyl group of the alkyl halide, enabling the covalent attachment of synthetic labelled substrates (Fig. 27e). By using this Halo Tag protein labelling technology, both dienophiles and tetrazines were incorporated into proteins, followed by IEDDA with a suitable dye-conjugated partner. Intracellular labelling of different Halo tagged histone proteins (Halo-H2B-GFP and Halo-H2B-mCherry) was validated and evaluated in HeLa cells using in-gel fluorescence and fluorescence microscopy.\textsuperscript{111} This two-step strategy was recently applied for visualization of compartmentalized cations.\textsuperscript{174}
4.3 Direct incorporation of unnatural nucleotides into RNA/DNA for IEDDA modification

4.3.1 Solid-phase synthesis of oligonucleotides containing IEDDA bioorthogonal handles. Solid phase synthesis has been used as an efficient approach to incorporate reactive handles into DNA/RNA. Due to the sequential and flexible nature of solid phase approaches, alterations may be introduced on different regions of an oligonucleotide, namely, on the sugar, nucleobases or even backbone. For instance, site-specific chemical incorporation of Nor, TCO, and Tz at internal or terminal positions within DNA or RNA has been accomplished for subsequent IEDDA reactions (Fig. 28a). Importantly, Schorr et al. showed that synthetic RNA oligonucleotides tagged with a norbornene dienophile could be labelled in mammalian cells by reaction with a tetrazine probe. The stability of IEDDA reagents under the conditions required for solid-phase synthesis has, however, been a concern. For instance, the synthesis of a TCO-modified oligonucleotide required the use of t-butyl hydroperoxide as an oxidant due to the quantitative degradation of the TCO moieties using standard aqueous iodine oxidation. Additionally, due to the general instability of tetrazines during oligonucleotide cleavage from the solid support, post synthetic labelling was only achieved on beads. Alternatively, enzymatic approaches have been developed for site-specific incorporation of unnatural nucleotides on RNA/DNA. Current methods used for incorporation of bioorthogonal handles directly into oligonucleotides for click chemistry are represented in Fig. 28.175,176,181

4.3.2 Enzyme-mediated modification of RNA. Functionalisation of nucleotides with small bioorthogonal handles is a versatile approach for transcriptional labelling, however, this approach leads to random incorporation of the clickable moieties into RNA (Fig. 28a). Alternatively, incorporation of modified nucleosides into the RNA strands is possible in vitro during transcription using the well-known 5'-initiation method employing the T7 RNA polymerase (Fig. 28b). This enzyme efficiently initiates transcription using the T7 promoter and a guanosine nucleotide as the +1 base. Jäschke et al. showed that this polymerase could terminally modify RNA, using norbornene- and BCN-modified guanosine monophosphate (GMP) as the initiator nucleotides. A related approach by Royzen et al. showed that a cytidine triphosphate (CTP) analogue with a TCO group at the 5-position of the nucleobase is recognized by the T7 RNA polymerase, and therefore is internally and site-specifically incorporated in a RNA strand. Another alternative method for RNA modification uses an engineered methyltransferase that recognises S-adenosyl-L-methionine analogues containing reactive handles and transfers these handles to a RNA of interest (Fig. 28c). The advantage of selectively targeting a particular RNA class (e.g. tRNA, snoRNA or mRNA) or sequence of interest makes this method a valuable approach for chemoenzymatic labelling of RNA.

Recently, Rentmeister et al. showed that a variant of trimethylguanosine synthase 2 from Giardia lamblia (GlaTgs2-Var1) recognises a novel AdoMet-analog bearing a vinylbenzyl group, allowing the modification of capped RNA structures at the N2 position of the 5' cap (30% yield in 3 h with 10% of GlaTgs2-Var1). Similarly, an allyl modified RNA was prepared in 90% yield. The differences in yields were attributed to steric constraints in the substrate-binding pocket of the methyltransferase. Subsequent IEDDA labelling with a tetrazine-TAMRA

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**Fig. 28** Methods used for incorporation of IEDDA bioorthogonal handles into RNA for subsequent labelling by click chemistry. (a) The promiscuity of various RNA polymerases tolerates the incorporation of unnatural nucleotides, producing modified RNAs. (b) Labelling of RNAs using the 5'-end-labelling approach employing the T7 RNA polymerase. (c) Post-synthetic modification of RNA by chemo-enzymatic labelling of 5'-capped RNAs. A methyltransferase (MTase) variant can be used to modify the mRNA cap with the reactive handle if the respective S-adenosylmethionine (AdoMet) analog is provided. NTP = Nucleoside triphosphate.
derivative showed quantitative conversion of the vinylbenzyl modified 5′-capped RNA, but not on the allyl-modified RNA, possibly due to the relatively low reactivity of the allyl handle. This approach was proven to be efficient for a 106-long capped RNA.116 It has also been shown that a cap (guanine N7) methyltransferase from the microsporidian parasite Encephalitozoon cuniculi (Ecm1) could transfer bulky side chains from AdoMet analogues to 5′-capped mRNAs at position N7 with almost quantitative conversion, which allowed efficient labelling with a tetrazine dye.184 By using the above-mentioned methyltransferases and exploring their regioselectivity, dual 5′ Cap labelling of mRNAs was also achieved.185

4.3.3 Enzyme-mediated modification of DNA. DNA IEDDA labelling has also been achieved using DNA polymerase, reverse transcriptase via primer extension (PEX) or reverse transcription. A number of nucleotides bearing vinyl,186 cyclopropene,187 Nor,186 TCO189,190 and tetrizine187 handles have been successfully incorporated in DNA and labelled (Fig. 29). Work by Jäschke et al. used norbornene-bearing primers to synthesise modified DNA through PCR without concerns over polymerase tolerance.191 Another approach to incorporate functional groups into DNA uses terminal deoxynucleotidyl transferase (TdT) that catalyses the addition of modified nucleoside triphosphate (NTP) to DNA 3′-termini.192

4.3.4 Site-specific DNA functionalisation with alkenes. While PEX, PCR and in vitro transcription can modify DNA with multiple dienophiles, these modifications are usually not selective. Applying the above-mentioned nucleoside derivatives to complex cellular environments results in labelling of all nascent transcripts. Since selectivity is highly desirable, approaches for site-specific introduction of non-native nucleotides are required.

Recently, two artificial base pairing systems have been developed for incorporation of functionalities at defined positions. Instead of H-bonding interactions, these unnatural bases form hydrophobic interactions efficiently and selectively even during PCR amplification. Using an dTPT3-dNaM artificial base pair, a ribonucleoside triphosphate containing either norborne or cyclopropane,193 was introduced into RNA via T7 in vitro transcription using DNA templates with the complementary unnatural nucleobase (Fig. 30).

4.3.5 Unnatural nucleotide. Recently, nucleotides bearing alkene handles were shown to be accepted by cellular machineries, providing an alternative strategy for DNA labelling. A vinyl bearing nucleotide (5-vinyl-2′-deoxyuridine, VdU) was synthesized by Luedtke et al. and selectively incorporated into cellular DNA in Hela cells via endogenous enzymes. VdU treated cells were stained with TAMRA-Tz rapidly in 30 min and dual labelling of cellular DNA was achieved on VdU and EdU (5-ethyl-2′-deoxyuridine) on cells via tetrazine and CuAAC ligation.115

4.4 Metabolically incorporation for glycan modification

The simplest method for chemically labelling relevant biomolecules in living systems is metabolic labelling, in which the endogenous machinery of living cells is used to incorporate reactive handles into the biomolecules. In general, this is accomplished by growing cells or organisms in media in which a specific natural substrate (e.g. amino acid, nucleotide and carbohydrate) is replaced with a close analogue bearing a chemical reporter for subsequent modification with affinity or biophysical tags. Consequently, cells use the chemical analogue instead of the natural substrate to synthesize or modify glycans, nucleic acids or proteins. Numerous bioorthogonal probes have been developed for metabolic labelling including azidohomoanaline for labelling newly synthesized proteins by replacement of natural Met residues, azido sugars for labelling glycoproteins and 5-ethyl-2′-deoxyuridine (EdU) for labelling nucleic acids.195

To the best of our knowledge, IEDDA reactions have thus far been successfully applied for metabolic labelling of glycans, and nucleic acids, but the possibility of using the tetrazine click reaction to label proteins tagged with a dienophile-bearing
ncAA (or vice versa), incorporated using the natural translational machinery, is still to be explored.

Glycans participate in diverse biological processes such as extracellular matrix formation, protein folding, protein stability and function, regulation of the interaction of proteins with one another, etc. In addition, disordered glycosylation is associated with a number of diseases. The ability to monitor glycans could provide fundamental insights into their roles in cell biology.

While azide and alkyne reactive handles have been the most popular for labelling tagged glycans using bioorthogonal chemistry, IEDDA-based approaches are now advancing.

4.4.1 Cyclopropene-modified sugars. The possibility of using alkene-functionalised sugars for metabolic oligosaccharide engineering was first demonstrated in 2012 by Prescher et al. In this work the authors showed that a methylocyclopropene handle, incorporated as part of methylcyclopropene-linked sialic acid analogue (Neu 1, Fig. 31), could meet the stringent steric demands required for metabolic incorporation, allowing the visualization of sialylated glycoproteins on live cell surfaces. Following this work, in 2013 Devaraj et al. demonstrated that methylcyclopropene bioorthogonal handles could be used for metabolic imaging of unnatural mannosamine derivatives (Man 1) on live-cell surfaces. Later, Wittmann et al. investigated the effect of having either an amide or carbamate linker between the glycan and cyclopropene moiety. This work showed that N-acyl-mannosamine derivatives bearing a methylcyclopropene tag attached to the sugar via a carbamate moiety (Man 2) react faster with tetrazines (second-order rate constant of 0.99 ± 0.1 M⁻¹ s⁻¹) and could be efficiently accepted by the cell’s metabolism. In this case rapid membrane staining was achieved with a fluorogenic tetrazine in only 5 min. In a similar approach, Prescher et al. synthesised probes based on glucosamine, galactosamine, and mannosamine (Man 2, Gal 1, Glc 1) with different linkers (carbamates versus amides).

As previously observed carbamate-functionalized cyclopropenes showed improved utility for glycan imaging and profiling. It has also been shown that cyclopropenes outfitted with carboxamides (versus amides) at C-3 react about 100 times faster with electron-poor tetrazines. In order to fulfil enhanced kinetics for in vivo dynamic glycan labelling, Ye et al. compared the stability and reactivity between methyl (Ac₄ManNMCp, Man 1) and unsubstituted (Ac₄ManNCp, Man 3) cyclopropene N-acylmannosamine derivatives. The metabolic incorporation efficiency of Ac₄ManNCp was found to be superior to Ac₄ManNMCp in all cell lines leading to a significant increase in fluorescence intensity after Tz labelling. With almost 10-fold faster kinetics and high stability against excess of thiols when compared to Ac₄ManNMCp, Ac₄ManNCp could rapidly probe in vivo sialylated glycoproteins from splenocytes as well as other tissues. Wittmann et al. showed that cyclopropene-tagged N-acetylgalactosamine (Ac₄GalNCyp, Gal 1) and N-acetylglucosamine (Ac₄GlcNCyp, Glc 1) were successfully incorporated into HEK 293T, Jurkat and HeLa cells and could be used to monitor glycosylation of intracellular glycoproteins. Recently, the same group explored Ac₄GlcNCyp (Glc 1) for probing glycosylation of intracellular proteins (e.g. OGT, Fox01 or p53) fused with GFP by detecting FRET between the tetrazine labelled glycans and the fused protein.

4.4.2 Terminal alkene-modified sugars. Unstrained terminal alkynes handles, due to their relatively small size, stability and easy synthesis are also being explored as chemical reporters for metabolic oligosaccharide engineering. Wittmann et al. developed and metabolically incorporated mannosamine and glucosamine derivatives bearing terminal double bonds with carbamate and amide linkage into live cells. Most sugar derivatives (Man 4–9) show kinetics between 0.01–0.1 M⁻¹ s⁻¹, which are comparable to Staudinger and SPAAC ligations. By varying the length of the alkene side chain, it is possible to fine-tune the balance between kinetics and incorporation efficiency. Specifically, kinetic studies showed that the reactivity of alkene-sugar derivatives increase with growing chain length (from C3 to C6). When applied to metabolic oligosaccharide engineering, all sugar derivatives could be employed to label cell-surface carbohydrates on living HEK 293T cells, however, a mannosamine derivative with a median alkene side chain (ManNBoc, Boc = butenylxoycarbonyl) had the optimal balance between metabolic incorporation efficiency and reactivity for cell-surface labelling.

4.4.3 Norbornene-modified sugars. Aside from these small IEDDA handles, bulkier, but more reactive groups have also been engineered for imaging glycosylation. Two norbornene-bearing mannosamine derivatives (Man 10 and 11) were incorporated in HEK 293T cells with a 1% incorporation rate. Importantly, exo-norbornene-modified mannosamine derivative showed more distinctive cell staining in line with its faster (2×) kinetics (k₂ = 4.6 ± 0.5 M⁻¹ s⁻¹) compared to the endo derivative. Although with comparable kinetics to cyclopropene-modified sugars, the norbornene-mannosamine derivatives required 3 hours of reaction, instead of 15 minutes, to obtain significant staining. This was attributed to low incorporation of the bulky norbornene structure.
4.4.4 BCN-modified sugars. Examples of probing glycosylation in vivo have also been reported in mouse and zebrafish using bioorthogonal sugar derivatives. In one example, Bertozzi et al. developed a bioorthogonal approach for systemic imaging of glycans by metabolically incorporating a BCN-functionalized sialic acid (BCNSia 1) at various development stages of zebrafish, following by IEDDA labelling.

4.5 Unnatural \( \nu \)-amino acids IEDDA reporters

Exogenous unnatural \( \nu \)-amino acids have been incorporated in place of the terminal \( \nu \)-alanine of bacterial peptidoglycans and are being used for remodelling and imaging of bacterial surfaces. Thanks to the promiscuity of transpeptidase, various \( \nu \)-amino acids were incorporated into the cell surface and labelled by reaction with fluorescent tetrazines. A \( \nu \)-norbornene (Dap = \( \nu \)-diaminopropionic acid) showed the best labelling efficiency with a balance between incorporation efficiency and IEDDA reactivity. A tetrazine bearing \( \nu \)-amino acid (\( \nu \)-Dap-Tet-NH\(_2\)) was also tested for metabolic remodelling and subsequent TCO imaging. Despite the expected fast kinetics, labelling efficiency with \( \nu \)-Dap-Tet-NH\(_2\) was lower than with \( \nu \)-Dap-NB-NH\(_2\), presumably due to limited incorporation levels.

4.6 Bioorthogonal IEDDA lipid chemical reporters

Bioorthogonal chemical reporters for lipid labelling and imaging have also attracted widespread interest in recent years. In one example, a cyclopropene tagged phospholipid was used for visualizing phospholipid uptake and distribution in human cells (SKBR3 breast cancer cells). Upon treatment with a fluorogenic tetrazine, rapid membrane staining was observed. Following these studies, a TCO-tagged ceramide lipid and a reactive tetrazine were used for imaging Golgi structures and dynamics in live cells by 3D confocal and stimulated emission depletion (STED) microscopy.

4.7 Chemical introduction of IEDDA bioorthogonal handles

In addition to genetic encoding, the development of reactions that enable the selective installation of reactive handles on relevant biomolecules for subsequent modification/imaging would facilitate the study of the biological function of these targets. Within the last few years few examples were reported for the chemical installation of IEDDA reporters on proteins. Functionalization of proteins with IEDDA partners using NHS-chemistry has been widely developed for conjugation; however, this method is not site-selective resulting in heterogeneous mixtures of conjugates. As example, Weissleder et al. converted TCO into the reactive succinimidyl carbonate for conjugation to amine-containing biomolecules, including antibodies. Cetuximab–TCO conjugate was used for pre-targeted imaging of A549 cancer cells that overexpress EGFR (Fig. 33a). Recently, we demonstrated that S-allyl Cys could be easily chemically installed into apoptotic protein markers through a [2,3]-sigmatropic
5. Selected applications of IEDDA reaction in biological systems: examples where tetrazine ligation is needed

IEDDA ligation has enormous potential for bioorthogonal approaches and thus have been used to get insights into the behaviour and function of a variety of biomolecules such as proteins, antibodies, nucleic acids, glycans, lipids or bioactive small molecules. As an example, the very fast kinetics in biological media at low concentrations make the tetrazine–TCO ligation particularly useful for tracking and imaging fast biological processes.\textsuperscript{167,213} Other applications of the “tetrazine ligation” include fluorescent labelling of low-abundance proteins within living cells for super-resolution imaging,\textsuperscript{152} identification of the targets of bioactive small molecules and proteins in living cells,\textsuperscript{214} protein profiling in living systems,\textsuperscript{155} and sequence-specific detection of DNA and mRNA.\textsuperscript{215} The most exciting and recent examples on these topics are summarized in the next sections.

5.1 Super-resolution imaging of site-specifically labelled proteins

Until today, fluorescent proteins (e.g. green fluorescent protein (GFP)) have been widely used to image and track recombinant proteins in live cells. Although powerful, fluorescent protein tags are limited since their relatively large sizes may potentially interfere with protein function.\textsuperscript{216} In contrast, methods to site-specifically label proteins in their cellular context with small, bright and photostable fluorophores by means of bioorthogonal approaches would substantially advance super-resolution imaging.\textsuperscript{152,218} In fact, coupling fluorophores directly to a protein of interest has, comparing with fused fluorescent proteins, the tremendous advantage of minimizing the distance between probe and target, increasing spatial resolution. This becomes apparent when comparing the resolution achieved in live-cell stimulated emission depletion (STED) imaging experiments of microtubules labelled with SiR fluorophore coupled via a SNAP-tag to a microtubule-binding protein\textsuperscript{217} or directly using a docetaxel-SiR derivative\textsuperscript{218,219} (SiR-tubulin).\textsuperscript{3} In addition, synthetic fluorophores can have more favourable spectroscopic properties (e.g. brightness and photostability) than fluorescent proteins.\textsuperscript{220} Recent advances in genetic code expansion and bioorthogonal chemistry have enabled the efficient labelling of proteins. However, super-resolution imaging has been accomplished only just recently.\textsuperscript{6,152} Two challenges have limited progress in this area: (i) the low efficiency of non-canonical amino acid incorporation that limits labelling density and therefore spatial resolution and (ii) the uncharacterized specificity of intracellular labelling that will define signal-to-noise, and ultimately resolution, in imaging.\textsuperscript{152,221} Optimization of incorporation efficiency, coupling chemistry, and labelling protocols recently opened the way to the application of IEDDA chemistry in super-resolution microscopy. In one example, Chin \textit{et al.} reported the efficient production of cytoskeletal proteins (β-actin and vimentin) containing a bicyclo[6.1.0]nonyne-Lys at a specific site. These functionalized proteins could be efficiently labelled with SiRh tetrazines creating densely labelled cytoskeletal ultrastructures. Super-resolution imaging by stochastic optical reconstruction microscopy (STORM) revealed sub-diffraction features, including nuclear actin filaments (Fig. 34).\textsuperscript{152} Furthermore, actin with distinct morphologies at different cellular locations could be observed with nanometre-scale refined features (Fig. 34).\textsuperscript{152} Even though the super-resolution imaging was performed on fixed samples, which usually show much less background signal than live samples because it is easier to wash out unreacted probe, the experiments underline the potential of...
the combination of genetic encoding and bioorthogonal chemistry for super-resolution microscopy.

Even though ncAA encoding technology and IEDDA reactions have been used for super-resolution imaging of highly abundant cytoskeletal proteins and surface proteins as in the previous example, applications to less abundant proteins are largely obscured by the limited efficiency of incorporation that requests the use of excess of the ncAA. This fact, results in high concentrations of non-incorporated ncAA that can also react with the dyes leading to nonspecific binding (sticking) of the fluorescent dyes. For intracellular labelling, new hydrophilic tetrazine dyes with higher “turn-on” fluorescence upon reaction, which may be washed out easily, would help to achieve higher signal to noise.\textsuperscript{120,221,222} Alternatively, Lemke’s group developed a site-specific click-PAINT super-resolution microscopy method for imaging of low-abundance proteins inside mammalian cells, namely, the protein nucleoporin Nup153, a component of the nuclear pore complex (NPC) (Fig. 35).\textsuperscript{222} A schematic representation of this approach is outlined and the respective imaging data is presented in Fig. 35.

5.2 Study of fast dynamic process in living cells: where speed makes the difference

Although the strategy of combining site-specific incorporation of ncAAs with IEDDA bioorthogonal reactions may be advantageous for the labelling of intracellular proteins, there are several criteria that must be satisfied. One of these criteria is the careful selection of the tetrazine-conjugated fluorophores, as their cellular properties, such as membrane permeability, intracellular distribution, and retention, can affect protein labelling efficiency and specificity.

Recently, Hang et al. performed a systematic evaluation of bioorthogonal IEDDA reactions between site-specifically incorporated ncAAs and various tetrazine fluorogenic probes for fluorescence labelling and imaging of low-abundance intracellular proteins in live cells. In particular, it was reported that the bioorthogonal fluorescence imaging of the intracellular protein interferon (IFN)-inducible transmembrane protein 3 (IFITM3), a small vesicle-associated membrane protein that is involved in host restriction of influenza virus and many other pathogenic viruses. Fluorescent labelling of the TCO-tagged HA-IFITM3 (HA, Human influenza hemagglutinin) with various tetrazine fluorophores resulted in fluorescence puncta that are characteristic to IFITM3-containing vesicles (Fig. 36a).\textsuperscript{213}
Interestingly, it was shown that red dextran particles were internalized into cells and then fused with BODIPY-labelled green IFITM3-TCOK and dextran particles were labelled with pHrodo Red. Images were acquired every 30 seconds. Reprinted in part with permission from ref. 213. Copyright 2016 American Chemical Society.

Fig. 36 Bioorthogonal fluorescence imaging of HA-IFITM3-TCOK. (a) HeLa cells expressing HA-IFITM3-TCOK were labelled with H-Tz–BODIPY–FL, H-Tz–rhodamine, or H-Tz–SiR and then subjected to anti-HA immunofluorescence staining. DAPI (blue) was used to stain nuclei. (b) Time-lapse imaging of the fusion process of IFITM-containing vesicles with dextran particles. IFITM3-TCOK was labelled with H-Tz–BODIPY–FL and dextran particles were labelled with pHrodo Red. Images were acquired every 30 seconds. Reprinted in part with permission from ref. 213. Copyright 2016 American Chemical Society.

Importantly, immunofluorescence microscopy performed in parallel with tetrane labelling showed that tetrane fluorophore signals overlapped with anti-HA fluorescence signals confirming labelling efficiency and specificity (Fig. 36a). Notably, when the imaging of IFITM3 in live cells was performed using N- or C-terminal fusions with fluorescent proteins (e.g. GFP or mCherry), both cellular localization and antiviral activity of IFITM3 were disrupted. To explore the trafficking of IFITM3 in live cells, the authors evaluated the dynamics of IFITM3 with exogenously added fluorescent cargoes that are internalized into endocytic vesicles. Interestingly, it was shown that red dextran particles were internalized into cells and then fused with BODIPY-labelled green IFITM3-residing puncta to yield yellow vesicular structures, demonstrating that IFITM3 traffics to the same location as exogenously acquired cargoes after their internalization in the endocytic pathway (Fig. 36b). Moreover, the time-lapse imaging showed that this fusion process is completed within min. These studies significantly expand the scope of site-specific bioorthogonal imaging of intracellular proteins in live cells, and demonstrated the utility of fast and fluorogenic IEDDA reactions for the localization and real-time dynamic trafficking of fast biological events.

5.3 Identification of bioactive targets of small molecules and proteins in living cells

Monitoring how, when, and where small molecules engage their targets intracellularly is a major challenge in chemical biology and pharmacological research, because it provides insights that can assist the design or more efficient binders and to understand their mode(s) of action. For this purpose, Bantscheff et al. developed a modular strategy that enabled small molecule localization, target identification and target occupancy measurements of unmodified drugs in cells using bioorthogonal ligation reactions. Generally, the strategy uses a click-probe derived from a drug of interest that can be functionalized either with tags for affinity purification of potential target proteins or with fluorophores for high-resolution imaging. The feasibility of the modular probe strategy was demonstrated with noncovalent PARP inhibitors. Using a proteomic assay, the authors showed that dose-dependent competitive titration with the unmodified drug (Olaparib) allowed the determination of target and off-target affinity by quantitative mass spectrometry of the click-probe targets that are also bound to the free drug (Fig. 37(1)). In addition, attachment of a fluorophore to the click-probe allows localization of the probe inside cells by confocal microscopy (Fig. 37(2)).

This approach also allowed the determination of target occupancy by measuring the reduction of the localized fluorescence signal in the presence of unmodified drug (Fig. 37(3)). Of note, the authors systematically evaluated the compatibility and efficiency of a number of bioorthogonal reactions (IEDDA, SPAAC and CuAAC) and identified TCO labelled probes and tetrazine-tagged reporters to be the most efficient bioorthogonal coupling partners. Similarly, a dienophile-modified paclitaxel derivative with identical activity to the parent drug was used for imaging microtubular networks in cells upon IEDDA labelling with tetrazine fluorophore probes. Similarly, TCO-modified kinase inhibitors labelled with tetrazine-tagged fluorescent reporters could be used to track and image kinase activities in live cells.

The detection of protein–protein interactions (PPI) within native cellular environments could provide key insights for drug design. To address this challenging question, minimalist linkers cyclopropene and diazirine were synthetically introduced
within a small molecule bromodomain inhibitor. In this approach, the inhibitor is used for target identification, while the diazirine photo-cross-linker enables covalent linking. Lastly, the cyclopropene handle is used either for bioimaging or affinity-based protein labelling using biotin-tetrazine derivatives. The resulting probes were used for proteomic analysis and resulted in the identification of several hundreds of protein candidates targeted by the bromodomain BRD-4 inhibitor (+)-JQ1 (Fig. 38).

5.4 Protein profiling in organisms

The ability to identify newly synthesised proteins at specific times in cells of interest will facilitate the study of dynamic cellular processes. In this regard, several protein tagging strategies through genetic code expansion have been employed to interrogate several complex cellular processes. In one example, an azide-containing analogue (L-azidohomoalaine, AHA) of methionine (Met) was introduced at Met codons and subsequently labelled through cycloaddition with alkyne-bearing probes. Based on auxotrophic strains, incorporation of the amino acid analogues is often achieved in minimal media under starvation conditions for the amino acid to be replaced. This limits the availability of the natural amino acid and decreases labelling efficiency.

A novel technology that addresses some of the above-mentioned issues was developed – stochastic orthogonal recoding of translation with chemoselective modification (SORT-M) (Fig. 39). SORT-M uses a set of reengineered orthogonal pyrrolysyl-tRNA synthetase/tRNA pairs that enable codon-selective incorporation of a cyclopropene Lys derivative, which can be chemoselectively modified with various tetrazine probes for fluorescence imaging and proteome profiling of engineered bacteria, mammalian cells and in specific cell types or tissues in animals (Fig. 39). Importantly, the method could be performed directly in flies for developmental stage-specific proteomic studies. More specifically, by confining PyRS expression to the ovaries of Drosophila melanogaster using a specific promoter, selective incorporation of a cyclopropene-bearing amino acid into proteins derived from these organs was achieved. This was confirmed by fluorescent labelling of proteins specifically synthesised in the ovary. Protein identification could also be performed combining 2-D in-gel fluorescence electrophoresis and MS analysis. More recently, an improved version of SORT-M called SORT-E (SORT with enrichment) was developed.

In this version, SORT-tagged cellular proteins are reacted with a tetrazine diazobenzene biotin compound and then captured on streptavidin beads. The beads can be washed and enriched proteins may be detected using MS after reductive cleavage of the diazobenzene linker.

5.5 Sequence-specific detection of DNA and mRNA in living systems

As mentioned a beneficial characteristic concerning the use of tetrazines during IEDDA is the “turn-on” effect in case a tetrazine–fluorophore conjugate is used. By using these probes, fluorescence is only observed when the bioorthogonal partners meet and, thus, where exactly the bioorthogonal reaction occurs. This feature has been used in the development of tetrazine smart probes for the sequence-specific detection of DNA and mRNA in living systems. Devaraj et al. first applied template-driven reactions with fluorescent tetrazines for nucleic acid detection. Specifically, a quenched fluorophore-tetrazine and a methyl-cyclopropene group were conjugated to two different oligonucleotides and showed to react by IEDDA ligation only in the presence of the DNA template that can hybridize with both oligonucleotides. The use of the template increases the effective molarity (52–123 mM) by confining the tetrazine and cyclopropene partners in close proximity, and the bioorthogonal reaction rapidly takes place, leading to strongly fluorescent products. This approach allowed detection of template DNA inside living mammalian cells (Fig. 40a).

One limitation of this approach is that the resulting product reacts with a similar affinity for the template as the reactive starting oligonucleotides, impeding reaction turnover and signal amplification. To address these issues, the same research group reported an optimized system using oligonucleotide templates derivatised with a norbornadiene and a highly fluorogenic tetrazine–BODIPY probe. The use of a highly quenched tetrazine probe enables a > 100-fold increase in fluorescence in response to hybridization and subsequent proximity-driven reaction. In addition, the new antisense probes undergo dynamic strand...
exchange, which resulted in high turnover amplification allowing the detection of endogenous oncogenic miRNA target mir-21 \((in \text{ vitro and cells})\) down to low-picomolar levels \((Fig. \ 40b)\). Importantly, this system was able to distinguish single-base mismatches in miRNA templates.

6. IEDDA applications in nuclear medicine

6.1 Overview of click radiochemistry

Nuclear imaging techniques are based on the radionlabelling of specific molecular probes using radioisotopes that are used as contrast agents for imaging of pathologic processes or cellular functions either by single-photon computed tomography (SPECT) or positron emission tomography (PET). A major advantage of SPECT and PET over other imaging techniques (e.g. optical and magnetic resonance imaging) is the high sensitivity that allows the detection of radiotracers in concentrations as low as \(10^{-10}\) to \(10^{-12} \text{ M}\). For SPECT, more than 80% of radiotracers used in nuclear medicine centers are with Technetium-99m, while for PET imaging the most clinically useful isotope is Fluorine-18. Other attractive isotopes for medical applications are Indium-111, Iodine-123, and Thallium-201 for SPECT and Carbon-11, Copper-64, Gallium-68 and Zirconium-89 for PET. Usually, radioisotopes are linked to targeting vectors (i.e., small bioactive molecules, peptides, proteins or antibodies) via a (i) bifunctional chelating agent for labelling radiometals or by (ii) direct or (iii) indirect methods for labelling with non-metallic isotopes (e.g. \(^{11}C\), \(^{18}F\) or \(^{131}I\)).

Over the past decade, bioorthogonal chemistry has gained momentum and has been increasingly applied in the field of radiopharmaceutical chemistry. The chemoselectivity and fast kinetics of such reactions in aqueous media makes them ideal for the radiosynthesis of molecular imaging agents. Marik and Sutcliffe et al. reported the first application of click chemistry for \(^{18}F\)-labelling of peptides through a CuAAC reaction between azido-decorated peptides and various \([^{18}F]\)fluoralkynes groups or \textit{vice versa} \((Fig. \ 41a(i))\). Since these initial studies a variety of \(^{18}F\)-prosthetic groups for CuAAC labelling have been prepared in radiochemical yields (RCY) ranging from 25% to 71% and reaction times of 5–10 min. Some examples of these \(^{18}F\)-building blocks are depicted in Fig. 41a(i) \(([^{18}F]\text{PEG-alkyne})\). The CuAAC click reaction has found applications in the development of PET probes for \textit{in vivo} imaging, with one example \(^{18}F\text{-RGD-K5}\) entering clinical trials in the US for diagnosis of integrin α\(_5\)β\(_3\) expressing tumours. The same click reaction was applied for the design and synthesis of radiometal-based radiotracers; Schibli et al. introduced the click-to-chelate concept to form novel ligand scaffolds containing a triazole group known to efficiently chelate the \({}^{99m}\text{Te(CO)}_3\) core \((Fig. \ 41a(ii))\). Although a large number of radiotracers have been developed using this reaction some limitations have been reported. One obvious disadvantage is the use of a large excess of potential cytotoxic copper, which should be avoided for \textit{in vivo} applications. For such applications, the SPAAC reaction was expanded for quick radiolabelling under physiological conditions in the absence of catalyst and widely used for the incorporation of radioisotopes into biomolecules for PET and SPECT imaging in animals \((Fig. \ 41b(i))\). Typical conditions for SPAAC labelling involves reaction times within 15–30 min, with isolated RCY of 19–98% and specific activities of ~60 GBq \(\mu\text{mol}^{-1}\) \((Fig. \ 41b(ii))\). Another example of the click reaction in radiochemistry is the traceless Staudinger reaction. The first \(^{18}F\) variants for Staudinger radioligation were described by Steinbacha et al. and Gouverneur et al. \((Fig. \ 41c)\). The Staudinger ligation has, however, one major limitation whereby the phosphine precursor is subject to oxidation, abolishing its reactivity. In addition to the classical click chemistry ligation, the reaction of unprotected aminooxy- or hydrazine-modified peptides with \(^{18}F\)-labelled aldehydes or ketones have also been explored for radiolabelling, resulting in the corresponding oxime or hydrazone conjugates \((Fig. \ 41d)\).
however limited due to the large excess of the components required, which results in very low specific activities. In addition, oximes and hydrazones are known to be prone to hydrolysis. The cyanobenzothiazole–cysteine (CBT–Cys) condensation reaction has also been recently established as an efficient, aqueous-compatible, and generally applicable method for radiolabelling with 18F (Fig. 41d(ii)).

Although the mild conditions of the abovementioned click reactions are compatible with radiochemical approaches, they often require large amounts of purified peptide/protein precursor to obtain usable reaction rates for radiosynthesis. This leads to low specific activities (i.e., the ratio of labelled-to-unlabelled molecule), which is an important issue since the in vivo targets of PET/SPECT tracers are often receptors or enzymes that are present in low copy number. The slower reaction rates also exclude the use of these click reactions for in vivo pretargeting applications.

These limitations have led to the search of alternative fast and catalyst-free methods applicable to radiochemistry, i.e., fast and high (radiochemical) yielding reactions that proceed under mild aqueous conditions using low concentrations of precursors. Notably, the IEDDA ligation has shown promising features, which make this click reaction suitable for both radiolabelling and in vivo labelling, i.e., pretargeting labelling. As mentioned, this reaction achieves unusually fast rates with no need for
excess amount of reactants, or catalyst, resulting in radioactive preparations with high specific activities. The reaction is also not dependent on the solvent (tolerates water, organic solvents, cell media and blood plasma without reducing the reaction yields) and is highly selective in the presence of a broad range of functional groups. A pioneering work in this field was first reported by Robillard et al.\textsuperscript{266} In this work an \textsuperscript{111}In-DOTA-tetrazine derivative was used to label \textit{in vivo} an antibody bearing TCO groups. Although first applied for radiolabelling with metals, over the past \~8 years the tetrazine–TCO ligation (Tz/TCO ligation) has instead attracted attention for the construction of \textsuperscript{18}F- and \textsuperscript{11}C-based imaging probes, primarily due to the short half-life of these isotopes that required fast labelling reactions.

6.2 IEDDA applications with \textsuperscript{18}F

The application of Tz/TCO ligation for \textsuperscript{18}F labelling was first published by Fox \textit{et al.} in 2010, where it was demonstrated that Tz 8 reacts with \textsuperscript{[18F]}TCO in an excellent RCY of 98\% at room temperature within seconds, using micromolar concentrations of the precursors (Fig. 42a).\textsuperscript{267} The synthesis of the \textsuperscript{[18F]}TCO precursor was accomplished also in good yields (RCY of 71\% within 15 min) by reacting the corresponding nosylate derivative with \textsuperscript{[18F]}-fluoride/tetrabutylammonium bicarbonate (TBAB) in acetonitrile (Fig. 42a). Further studies on the stability of this precursor have found that it is quickly metabolized \textit{in vivo} (plasma metabolites observed within 5 min). At later time points (240 min post-injection, p.i.) PET imaging and \textit{ex vivo} biodistribution studies showed a significant bone uptake indicating defluorination.\textsuperscript{268} The first \textit{in vivo} imaging approach using a \textsuperscript{18}F-labelled peptide prepared via IEDDA chemistry consisted of the labelling of a tetrazine-RGD derivative with the \textsuperscript{[18F]}TCO precursor (Fig. 42b). The resulting \textsuperscript{18}F-labelled tracer was prepared with a RCY of 90\% within 5 min at room temperature showing a high tumour accumulation, but also a high liver uptake associated to the hydrophobicity of this first-generation system (Fig. 42c).\textsuperscript{269}

In the previous example the tetrazine functionality is introduced through Lys acylation, which results in non-specific labelling of Lys residues that may be important for biologic activity. In this context, Conti \textit{et al.} developed a bifunctional tetrazine–maleimide partner for efficient \textsuperscript{18}F-labelling of free Cys residues on peptides (cRGDyC) and proteins (VEGF-SH).\textsuperscript{270} As an example, labelling of Tz-\textsuperscript{18}F-cRGDyC peptide was obtained in $>95\%$ RCY with a specific activity of 111–222 GBq mmol\textsuperscript{-1}.\textsuperscript{270} More recently exendin-4, a glucagon like peptide-1 receptor (GLP-1R), was also modified via tetrazine–maleimide conjugation, and labelled with \textsuperscript{18}F-TCO for PET imaging of intraportally transplanted islet cells.\textsuperscript{271} Importantly, the \textsuperscript{18}F-labelled prosthetic groups N-[2-(4-\textsuperscript{18}F-fluorobenzamido)ethyl]maleimide (\textsuperscript{18}F-FBEM) and \textsuperscript{[18F]}fluorobenzaldehyde (\textsuperscript{18}F-FBA) have also been used for radiolabelling exendin. In this case the \textsuperscript{18}F-labelled exendin tracers were obtained in low specific activity, which resulted in low binding to the GLP-1R in tissues in comparison with the tetrazine-labelled exendin.\textsuperscript{271,272} The disadvantage of the tetrazine–maleimide approach relates with the occurrence

of known thiol-exchange reactions that the maleimide products undergo in plasma.\textsuperscript{273,274}

\textsuperscript{[18F]}TCO has also been used for labelling poly(ADP-ribose) polymerase 1 (PARP1) inhibitors. \textit{In vivo} studies have shown that \textsuperscript{18}F-AZD2281, one of the labelled molecules, accumulates in tumours overexpressing PARP1. Interestingly, the authors have used magnetic TCO-decorated beads to “pull out” the excess of unlabelled tetrazine-conjugated AZD2281 from the reaction
mixture resulting in high specific activities without lengthy HPLC purifications.275

Toward the development of faster IEDDA 18F-labelling reactions Fox et al. developed a new radiotracer based on s-TCO by treating the corresponding tosylate precursor with 18F-tetrabutylammonium fluoride (18F-TBAF) in acetonitrile at 85 °C for 10 min (~29% isolated yield) (Fig. 43a).276 18F-sTCO is stable in PBS pH 7.4 up to 2 h at 37 °C. In fetal bovine serum 18F-sTCO retains 74% of radiochemical purity, after 1 h. This degree of degradation is not an issue, as the IEDDA reaction takes place within few minutes. In fact, it was shown that 18F-sTCO completely reacts with a RGD-tetrazine in less than 5 min (26–40 GBq μmol⁻¹) and can be used to rapidly assemble probes for PET imaging in human U87MG tumour-bearing mice.277 More recently, a 18F version of d-TCO was prepared by nucleophilic substitution of the corresponding tosyl analogue with 12% radiochemical yield (18F-d-TCO, Fig. 43b).278 In PBS pH 7.4, 18F-d-TCO is stable, with 94% of the compound being intact after 2 h incubation at 37 °C. In rat plasma, 18F-d-TCO slowly isomerises into the corresponding cis-derivative, with 52% and 34% of the intact trans-compound being present after 1 and 2 hours incubation at 37 °C, respectively.

“Hot IEDDA” ligations are not only restricted to the 18F-TCO moiety. For example, Knight et al. used a 18F-norbornene derivative (18F-Norb) for labelling of a tetrazine-bombesin peptide. The authors demonstrated that 18F-Norb is capable of undergoing IEDDA click reactions with tetrazine species with promising reaction rates and high specific activities.279

6.2.1 Tetrazine prosthetic groups for 18F-IEDDA labelling. Generally, 18F can be introduced either into the tetrazine or the dienophile. However, because of the insufficient stability of tetrazines most reports have focused on using an 18F-labelled dienophile. A disadvantage of this strategy is that it requires the attachment of a tetrazine to the targeting biomolecules, which involves complex and cumbersome reaction steps. In contrast, the efficient and straightforward methods available for modification of TCO and norbornene facilitate their bioconjugation to targeting biomolecules. As a result, significant efforts have been made for the direct radiolabelling of tetrazine precursors with 18F, providing higher versatility for the application of IEDDA ligations in radiochemistry. The first attempts to label a 1,2,4,5-tetrazine variant with 18F were described by Fox et al. but the instability of the tetrazine precursors during radiofluorination resulted in residual labelling yields (<1%, Table 1, entry 1).267 After these initial efforts, Mikula et al. reported the successful preparation of a [18F]-3-(3-fluoropropyl)-6-methyl-1,2,4,5-tetrazine (18F-Tz2) by direct fluorination of the tosylated intermediate with a RCY of up to 18% (SA = 9.8 GBq μmol⁻¹, Table 1, entry 2).280

A three-methylene spacer was considered between the fluorine and the tetrazine moiety to avoid the undesired high reactivity of benzyl-type precursors. In vivo studies with 18F-Tz2 revealed

Table 1 Summary of 18F-tetrazine derivatives

| Radiolabelled tetrazine | Yield conditions |
|-------------------------|------------------|
| 1 | <1% MeCN, 85 °C, 15 min |
| 2 | <18% MeCN, 90 °C, 5 min |
| 3 | ~78% MeCN/DCM, r.t., 20–25 min |
| 4 | 96% anilinium acetate buffer pH 4.6, r.t., 10 min |
| 5 | 10% DMF, Et3N, 60 °C, 7 min |
a low degree of metabolic degradation and a highly favourable pharmacokinetic profile indicating the suitability of this radio-labelled tetrazine in vivo applications.280

Despite this progress, the radiolabelling of small tetrazine derivatives was still limited by low RCYs due to the instability of the tetrazine moiety under conditions typically used in direct 18F-fluorination. A more efficient synthesis of a 18F-labelled tetrazine derivative used a [18F]SiFA–OH building block (RCY = ~78%) that effectively reacts with 1,4-dichlorotetrazine to generate [18F]SiFA–OTz (18F–Tz 3). Using a specific activity of 7.0–8.5 GBq mol\(^{-1}\) 18F–Tz 3 reacts quantitatively with TCO in 1 minute (Table 1, entry 3).281 Recently, a new 18F-labelled tetrazine derivative was developed also in high yields and with specific activities of 12–16 GBq mol\(^{-1}\) under mild reaction conditions via conjugation of 5-[18F]fluoro-5-deoxyribose with an aminooxy functionalized tetrazine to give 18F–Tz 4 (Table 1, entry 4). The 18F-tetrazine exhibited sufficient blood circulation time and favourable elimination characteristics.282 More recently Liang et al. labelled a tetrazine with [18F]SFB (RCY = 10%) to give 18F–Tz 5 and successfully constructed PET probes with TCO tagged antibody fragments for tumour imaging (Table 1, entry 5).283

6.3 “Tetrazine radiolabelling” with other relevant isotopes

Few examples of the application of the IEDDA reaction for direct labelling of bioactive molecules with other relevant isotopes have been described. In one example, a modular strategy for antibody labelling was developed. Specifically, norbornene was conjugated to trastuzumab – an anti-HER2 targeting antibody – and reacted with a tetrazine bearing different chelators (DOTA and desferrioxamine, DFO) for labelling with radioisotopes (64Cu and 89Zr) [Fig. 44a]. In vivo PET imaging and ex vivo biodistribution experiments revealed significant and specific uptake of the 64Cu-DOTA and 89Zr-DFO-trastuzumab bioconjugates in HER2-positive BT-474 xenografts (Fig. 44a). The authors showed that a dienophile modified antibody can be reacted selectively with various radiometal chelators facilitating cross-comparisons in the development of antibody-based radiopharmaceuticals since the modified antibodies are synthesized using identical ligation conditions.284 Additionally the use of mild IEDDA conditions avoids the use of high temperatures that are usually required for labelling antibodies bearing coordinating agents. Recently, Weissleder et al. developed a tetrazine conjugate that incorporated the DFO ligand for installation of 89Zr and a BODIPY fluorophore for multimodal imaging. 89Zr–DFO–BODIPY–trastuzumab was prepared within minutes by Tz/TCO ligation and PET imaging showed high tumour conspicuity. In addition, biodistribution studies confirmed high specific probe uptake in BT474 xenografts (HER2+) (237.3 ± 14.5% ID per g in BT474) compared to low, nonspecific probe uptake in BT20 xenografts (HER2−) (16.4 ± 5.6% ID per g) 96 h p.i. (Fig. 44b). Ex vivo fluorescence of selected tissues confirmed target localization and persistence of the fluorescence of 89Zr–DFO–BODIPY–trastuzumab. Similar systems may find utility to identify targets intraoperatively through fluorescence.285 Similarly, Spivey et al. used a tetrazine modified 68Ga-DOTA to construct probes for labelling norbornene-containing biomolecules.286 In another example, Reiner et al. used tetrazine bearing amino acids as building blocks for the synthesis of peptides and performed labelling studies with these peptides applying a 89Zr–DFO TCO derivative.287

6.4 Pretargeting based on IEDDA

The IEDDA reaction between tetrazines and TC0s has been employed in a variety of settings from fluorescence imaging of targeted biomolecules to magnetic resonance imaging with nanoparticles,72,113,288,289 however, the most significant impact of this ligation may lie in in vivo pretargeting imaging applications. For such challenging approaches the bioorthogonal reactant pairs should have high stability towards the amino and thiol nucleophiles (among others) present in biological systems as well as high selectivity to ensure that only the target biomolecules are labelled in vivo. In addition, the bioorthogonal reaction should proceed with fast kinetics even in the case of low concentration of the target protein, as is the case with in vivo pretargeting, where the reacting partners are diluted in the blood pool.290 Among all the existing bioorthogonal reactions the IEDDA ligation is the one with most potential for pretargeting applications.291 In these systems, the radionuclide is administered separately from the targeting protein, most often a tumour-targeting antibody. In the first step, the targeting antibody bearing a strained alkene (or tetrazine) is administered and allowed to accumulate in the specific tissue. Lastly, when the excess antibody has cleared from the circulation, a radio-labelled tetrazine (or dienophile) is administered (Fig. 45). In a general way pretargeting can be regarded as in vivo labelling, since the antibody is radio-labelled at the target tissue after in vivo administration.

Until recently, most pretargeting approaches for radioimmunostaining and radioimmunotherapy were based on the ligation of an antibody and a radionuclide using the avidin–biotin system, exploiting the high specificity and strong affinity.
(k_d = 10^{-15} \text{ M}) of avidin (or streptavidin) for biotin. Indeed, this pretargeting approach has been shown to be unaffected by extremes of pH, high temperatures, organic solvents and biological systems.\(^{292}\) Despite these features, the avidin–biotin system does have limitations, in particular, the high immunogenicity of the (strept)avidin, which cannot be humanized precluding its repeated use. The antibody-based pretargeting systems based on bioorthogonal click chemistry address such limitations. The use of IEDDA in pretargeting was pioneered largely by Robillard et al. In this work TCO was conjugated to anti-TAG72 monoclonal antibody [CC49] via an oligothyleneglycol linker and used as a tagging compound while a DOTA modified tetrazine moiety was used for labelling with \(^{111}\text{In}\). The \(^{111}\text{In}–\text{DOTA}–\text{tetrazine conjugate was injected one day after administration of the TCO–antibody conjugate (CC49–TCO) in mice bearing colon cancer xenografts. Impressively, the chemically tagged tumours reacted rapidly with \(^{111}\text{In}–\text{DOTA}–\text{tetrazine using only a small excess (3.4 equivalents) of the tetrazine radioactive tracer, resulting in pronounced accumulation in the tumour, as demonstrated by SPECT (Fig. 46).\(^{266}\) Ex vivo biodistribution studies confirmed a high tumour uptake (~3.0 ID per g) but a lower tumour-to-blood (T/B) ratio, which was assigned to the reaction of \(^{111}\text{In}–\text{tetrazine with freely circulating CC49–TCO. In addition, although this pretargeting approach enabled straightforward antibody modification the highly strained antibody-conjugated TCO tag was prone to slow \textit{in vivo} deactivation (25% in 24 h).\(^{266}\)

In a follow up study, the same researchers showed that the TCO tag is potentially deactivated \textit{in vivo} through isomerisation to the unreactive \textit{cis}-cyclooctene isomer by interactions with copper-containing proteins.\(^{293}\) By attaching the TCO through a shorter linker to hamper interaction with the copper-binding site in albumin, the deactivation half-life of TCO in circulation in mice was enhanced to 4 days.\(^{293}\) In another study, a pretargeting approach was developed for radiolabelling using a \(^{177}\text{Lu}\)-labelled tetrazine probe and the CC49–TCO antibody.\(^{294}\) It was demonstrated that the use of a less hydrophobic tag (TCO-oxybenzylazide) afforded a CC49–TCO conjugate with a longer clearance half-life, improved tumour accumulation and markedly increased \textit{in vivo} tumour accumulation.\(^{295}\) Very recently, the authors also successfully employed a TCO–tetrazine pretargeting approach with antibody fragments and affibodies.\(^{296,297}\) In addition, a number of other groups, have reported the development of a pretargeted imaging strategy based on the IEDDA reaction. Lewis et al. described an A33 TCO-antibody for \textit{in vivo} pretargeting with a \(^{64}\text{Cu}\)-labelled tetrazine in SW1222 tumour-bearing mice.\(^{298}\) Relatively low T/B ratios were observed caused by the reaction between the labelled tetrazine and the freely circulating A33–TCO.

To circumvent the high blood uptake, tetrazine-functionalized clearing agents were developed (\textit{e.g.} galactose–albumin–tetrazine and polystyrene beads coated with tetrazine-conjugated albumin). These enable rapid reaction with and removal of the TCO-tagged antibody from blood resulting in a significant improvement of the tumour-to-blood (125-fold) ratios (Fig. 47a).\(^{294}\) The utility of the IEDDA reaction for pretargeting extends beyond \(^{111}\text{In}, \, ^{64}\text{Cu} \, \text{and} \, ^{89}\text{Zr} \, \text{metals. Recently, a bisphosphonate-modified variant of TCO was used to pretarget bone and for subsequent bone imaging after \textit{in vivo} labelling with a \(^{99m}\text{Tc}–\text{tetrazine (Fig. 47b). This approach explores the high avidity of bisphosphonates to osteoclasts. In the same study a therapeutic radioisotope was also used (\(^{177}\text{Lu} \, \text{conjugated to a tetrazine}.\(^{299}\))

A robust and modular chemoenzymatic strategy was employed for the preparation of site-specifically modified antibody–TCO immunom conjugates on the glycans present in the heavy chain.\(^{300}\) This approach relies on the enzymatic activity of \(\beta\)-1,4-galactosidase and galactosyltransferase [Gal-T(Y289L)] for the incorporation of azide-modified monosaccharides (GalNAs) into the oligosaccharide chain. The resulting azide-modified antibody was then conjugated to a dibenzocyclooctyne-bearing variant of TCO (DIBO–PEG\(_{12}–\text{TCO}) \text{via SPAAC ligation. The site-specifically labelled huA33–TCO immunoconjugate was used for pretargeting PET imaging of colorectal cancer with a \(^{64}\text{Cu}\)-labelled Tz radioligand (\(^{64}\text{Cu}–\text{Tz–SarAr}).\(^{300}\) The same chemoenzymatic strategy was used to construct a TCO- and fluorophore-bearing immunoconjugate of the huA33 antibody (huA33–Dye800–TCO) for multimodal PET/NIRF imaging of colorectal cancer using a \(^{64}\text{Cu}\)-labelled tetrazine (\(^{64}\text{Cu}–\text{Tz–SarAr}).\) Importantly, simulated tumour resections using a near-infrared fluorescence (NIRF) camera showed its potential for real-time delineation of malignant tissue during image-guided surgery.\(^{301}\)

IEDDA ligation has also been successfully used in the synthesis of radioactive iodine labelled constructs.\(^{302,303}\) For example, Valliant et al. developed a convenient method to prepare radioiodinated tetrazines that could be used for labelling a TCO-anti-VEGFR2 conjugate with iodine-125 for \textit{in vivo} studies. The \(^{125}\text{I}–\text{tetrazine was prepared by employing an oxidative halo distannylation reaction. Under these conditions the tetrazine showed high stability resulting in high iodination yields (80% RCY, 15 min reaction).\(^{301}\) Importantly, the \(^{125}\text{I}–\text{tetrazine labelled antibody was shown to be 10 times more stable to deiodination than the same antibody labelled through direct iodination of for example tyrosines using iodogen.\(^{301}\)

6.4.1 Expanding the scope of IEDDA for pretargeting with short-lived isotopes. The use of the tetrazine–TCO cycloaddition for pretargeted imaging has also been described with short-lived isotopes. For example, \(^{18}\text{F}\) and \(^{68}\text{Ga}\) are inappropriate for labelling intact antibodies because they would have decayed before optimal biodistribution of the targeting biomolecule occurs. Aboagye et al. developed a labelling procedure for imaging EGFR expression on cancer cells using the IEDDA reaction between a TCO-modified Cetuximab and a \(^{68}\text{Ga}\)-labelled tetrazine.\(^{304}\) In addition, Lewis et al. used a TCO-bearing immunoconjugate of the...
anti-CA19.9 antibody 5B1 and an 18F-tetrazine radioligand for imaging of pancreatic cancer xenografts. A PEG11–NOTA chelator coupled to a tetrazine was used for complexation of aluminum-[18F]fluoride. The labelling was performed in 54–65% RCY in high purity (>96%) and a specific activity between 21.4 and 26.7 GBq mol⁻¹ was achieved within 15 min at 90°C. For pretargeted in vivo experiments, nude, athymic mice bearing subcutaneous CA19.9-expressing BxPC3 xenografts were injected with 5B1–TCO 72 h prior to the administration of Tz–PEG11–Al[18F]–NOTA (Fig. 48a). PET imaging showed tumoural activity concentrations of up to 6.4% ID per g at 4 h post-injection and low bone accumulation demonstrating the high stability of the Al[18F]–NOTA complex. Aimed to improve the in vivo labelling reaction in the tumour, Weissleder et al. have used 18F-labelled dextran polymers modified with tetrazines for in vivo pretargeting with a TCO-antibody against A33. The dextran polymer was envisaged to extend the circulation time of the 18F-tetrazine in vivo and improve the labelling of the TCO-tagged tumours (Fig. 48b). In a similar study Devaraj et al. have used 68Ga–DTPA–dextran–tetrazine derivative to target a A33 TCO-antibody for imaging of implanted LS174T xenografts. PET imaging and biodistribution studies revealed a prolonged retention in blood and high liver uptake, possibly due to partial release of 68Ga from DTPA in vivo.

The fast rates of the IEDDA ligation has made this reaction so attractive that even labelling with 11C (half-life of 20.3 min) was explored in pretargeting approaches. The first radio-synthesis of a 11C-tetrazine was reported in 2013 by Kristensen et al. in moderate yields (33% RCY) in 2 min but with a total preparation time of 50–60 min that included HPLC purification. The final click ligation with TCO is completed within 20 seconds, however, imaging, biodistribution, and stability data were not reported using this probe. More recently a new 11C-tetrazine precursor was prepared in 50% RCY and an overall synthesis time of 30 min including purification. The in vivo profile of this tetrazine was investigated by dynamic PET/MR scanning and ex vivo biodistribution studies. These results confirmed this 11C-radiotetrazine as a promising tool for pretargeted PET imaging. To investigate the applicability of the 11C-tetrazine for rapid in vivo pretargeting and PET imaging, mesoporous silica was functionalized with TCO. The TCO-modified silica was shown be localized in the lung by PET after in vivo click reaction with 11C-tetrazine, demonstrating the potential of 11C-labelled tetrazine for pretargeting applications.
7. Tetrazines – an emerging trigger for bioorthogonal cleavage reactions

Together with the explosion of bioorthogonal ligation reactions, using biocompatible reagents to trigger bond cleavage under physiological conditions has recently gained much attention. Instead of attaching additional functionality on biomolecules of interest, bioorthogonal cleavage reactions offer an alternative way to restore function of protected motifs, such as caged-fluorophores, prodrugs or masked biomolecules. In order to induce such a gain-of-function event, not only the caging groups, but the trigger, should be bioorthogonal, non-toxic, stable and reactive in cellular environment.

In this section, we report recent progress in bioorthogonal cleavage reactions drawing particular attention to tetrazine-mediated IEDDA decaging reactions and its application in vitro and in vivo.

7.1 Bioorthogonal bond cleavage chemistry

7.1.1 Photo decaging. Photo irradiation, as non-chemical stimuli, could serve as an orthogonal trigger for cleavage reaction. For example, the well-developed o-nitrobenzyl (ONB) ether derivative undergoes photo-deprotection to yield the free amine under UV light (Fig. 49a). Additionally, Chin et al. have genetically encoded caged Tyr amino acid variants into proteins for protein photoactivation. Even though photodecaging could offer high temporal resolution and potential spatial control, the UV light may however cause photo toxicity and perturb cellular processes. Moreover, the nitrobenzyl-based photocaging proved to be not fully bioorthogonal and stable in cells. Additionally, the poor penetration of ultraviolet radiation also hamper its compatibility in tissue and animal studies.

7.1.2 Metal mediated cleavage reactions. Molecule-based response systems have key advantages compared with other decaging approaches due to better biocompatibility and minimal perturbation of cellular processes. In this context, metal mediated cleavage reactions emerged as a powerful tool for decaging, with rapid turn-over and versatility towards various protecting groups. So far, Ru and Pd catalysts have been the two most explored triggers because of their efficiency and biocompatibility. The first example of the use of an organoruthenium complex to form amines from their respective allylcarbamates (alloc) under physiological conditions and in living cells was published in 2006 (Fig. 49b). Based on this first report, the efficiency of the Ru catalyst was further optimized by ligand design, resulting in higher turnover number (TON) that could be used for decaging of fluorophores and the drug doxorubicin within mammalian cells. Other interesting applications of Ru in this field include the introduction of lysine methylation on proteins by decaging of genetically encoded alloc-methyl Lys and decaging of alloc-protected DNA-binding species to trigger DNA binding events in cells. The same deallylation reaction was applied for supramolecular regulation of intracellular catalysis and catalytic activation of cellular bioluminescence. Notably, this Ru-mediated deprotection could also embed spatial and temporal control with certain ligands and substrates.

Apart from organoruthenium, Pd species have also been shown to be a useful catalyst for O-deallylation (Fig. 49c). Using fluorescent polystyrene microspheres, Bradley et al. showed that Pd nanoparticles could cross cell membrane and cleave allylcarbamate linkages intracellularly on mammalian cells (Fig. 49c). Additionally, propargyl groups (proc) could also be cleaved by Pd catalysts and used to activate anticancer drugs in cells and, most impressively, in zebrafish. In a recent work, Chen et al. managed to restore the function of propargyl-carbamates (proc)-caged proteins by Pd catalysis in vitro and in vivo. They also managed to manipulate cell-surface sugars by depopargylation of caged glycans using in situ-generated Pd nanoparticles. Recently, novel cleavable groups such as an allenyl ether on Tyr and non-terminal allylcarbamate linkages were also developed to undergo decaging reactions catalysed by Pd. Notably, other organometallic complexes, such as mercury, copper, iron and cobalt...
species were also found reactive for bond-cleavage reaction on allyl, propargyl and azide groups under physiological conditions, in living human cells and even in nematodes and zebrafish.

It is noteworthy that even though reaction proceeds efficiently at the small molecule level with 10% catalyst loading, when applied for protein and drug activation in cell studies, large amounts of catalyst is always required due to nonspecific binding of metal to proteins, especially for Pd catalysed reactions. Despite the moderate to low toxicity of these metal catalysts, their use is still limited for in vivo applications. In addition, further studies are needed to understand the cellular uptake of these catalysts and their influence on normal cellular processes.

7.1.3 Non-metal mediated cleavage reactions. Alternatively, non-metal cleavage reactions, mediated by biocompatible chemicals, stand out due to their excellent biocompatibility and enhanced orthogonality (Fig. 49d). Interestingly, these metal-free cleavage reactions mostly derive from their parent ligation reactions. By employing readily developed bioorthogonal handles, reactions could be redesigned to induce subsequent rearrangement and bond cleavage on the unstable ligation product (Fig. 49d). Because the ligation step is usually very fast, the overall kinetics of such cleavage transformations should in principle be also rapid. Accordingly, emerging bioorthogonal cleavage reactions were developed based on IEDDA, strain-promoted 1,3-dipolar cycloaddition and the Staudinger reaction, along with novel reactions such as bond-cleaving reactions between N-oxide and boron reagents. As an example, azide could serve as bioorthogonal protecting group for amines (Fig. 49d). In this system, a biocompatible phosphine reacts with an azide forming an azo–ylide conjugate, which is prone to hydrolysis in aqueous media affording the corresponding unmasked amine. This azide-to-amine conversion was employed for the activation of a doxorubicin prodrug, DNA-templated release of fluorophores and functional molecules, as well as controlled activation of epitopes on the surface. Apart from this bioorthogonal reductive transition, azide could also be decaged to give amines by reaction with TCO (Fig. 49d). Upon strain-promoted 1,3-dipolar cycloaddition, the resulting unstable 1,2,3-triazoline eliminates one molecule of nitrogen to generate an imine that undergoes hydrolysis to yield the free amine in aqueous solution. When combined with the self-immolative linker p-aminobenzyloxy carbonyl, the SPAAC reaction has been applied to chemically trigger the release of a caged doxorubicin as well as the decaging of Lys residues site-selectively on proteins. Among all these chemically-triggered bioorthogonal reactions, IEDDA mediated cleavage reactions outperform other examples due to their fast kinetics, catalyst-free nature and biocompatibility (Fig. 49d). Herein, we will present applications of the IEDDA-triggered cleavage on both TCO and vinyl ether moieties.

7.2 IEDDA triggered “click-to-release” of TCO bearing molecules.

TCO pro-drug activation. Robillard et al. first demonstrated in 2013 that the superfast IEDDA reaction could serve as a cleavage reaction for drug activation (Fig. 50a). By reacting a doxorubicin carbamate-linked TCO derivative with tetrazine, the resulting 1,4-dihydropyridazine product could undergo elimination of doxorubicin and CO₂ if TCO is modified at the allylic position (Fig. 50b). Notably, it was shown that the axially substituted TCO presents 156-fold higher reactivity than equatorial corresponding isomer (Fig. 50c), which presumably derives from the steric interaction between tetrazine and equatorial benzyl carbamate moiety. In a recent example, this tetrazine-triggered reaction was used for pretargeting antibody-drug
conjugate (ADC) therapy. Here, the masked drug is conjugated to a tumour-homing antibody via a TCO linker (CC49-TCO-Dox), which could be readily released in antibody pretargeted tumour cells upon tetrazine decaging reaction (Fig. 50d). Indeed, in vivo studies in mice bearing colon carcinoma xenografts showed efficacious drug release and high tumour retention of doxorubicin as confirmed by ex vivo fluorescence RP-HPLC analysis of tumour homogenates. Of note the TCO linker displays high in vivo stability with a half-life of 5 days, comparable to conventional cleavable ADC linkers. Although some isomerisation of TCO is expected, this only results in loss of reactivity and not in non-specific payload release, and thus potential side-toxicity.

Multivalence of nanoparticles and hydrogels enables the introduction of a high copy number of tetrazine motifs, enhancing activation efficiency and local drug concentration, and thus endow these materials as potential vectors for drug activation. In conventional chemotherapy, high doses of a drug need to be used because of the lack of specificity to the target diseased tissue. This leads to side toxicity and limits the general efficacy of the treatment. Royzen et al. envisioned that by systemic administration of non-toxic tetrazine-bearing magnetic nanoparticles (MNP), enhanced permeability and retention (EPR) effect would guide MNP accumulation in cancerous tissues, minimizing side effects by localized drug release. As a proof of concept, MNPs decorated with tetrazines and fluorescent probes could be used to image in situ prodrug activation of TCO-modified doxorubicin in cancer cells (Fig. 51a). Indeed, the pro-drug was activated completely after...
1 hour incubation of fluorescent tetrazine bearing MNP. This multi-model platform has potential for in vivo image-guided drug release either by fluorescence or magnetic resonance imaging.344 Similarly, a tetrazine-based hydrogel was synthesized for local drug activation. In contrast to systemic administration, the hydrogel could be injected directly into the tumour site, which allowed release of drug upon subsequent intravenously injection of the TCO modified drug (Fig. 51b).345 Animal studies revealed an increase in the efficacy of doxorubicin, minimizing systemic toxicity (no signs of toxicity, including weight loss, changes in coat texture and concentration of reticulocytes).

**Protein gain-of-function.** The potential of the bond cleaving IEDDA reaction to chemically activate caged enzymes was first reported by Chen et al. in 2015. In this work a TCO-bearing Lys analogue was genetically incorporated into the active site of firefly luciferase where the catalytic Lys is necessary to convert the non-luminescent luciferin to a highly luminescent product (Fig. 52a). The cleavage reaction with tetrazine was shown to restore the enzyme activity in both cells and animals (Fig. 52b).311,346 As previously reported by Robillard et al. it was observed that the axial isomer showed faster kinetics relative to the equatorial isomer.65,346 Tetrazines could also trigger gain-of-function to rescue the catalytic activity of a kinase in living mammalian cells.311 TCO decaging has not been limited only to drug release and protein gain-of-function studies. In another example, a tetrazine triggered approach has been described for the HPLC-free solid phase synthesis of RNA.347 Additionally, a new strategy for bioorthogonal carbonyl sulphide and hydrogen sulphide (COS/H2S) donation initiated by tetrazines has also been achieved (Fig. 53).348

**Optimized tetrazine derivatives for faster TCO decaging.** The electron properties of the substituents of tetrazines have proved essential for the efficiency of the decaging process (Fig. 54). Using a fluorogenic assay based on a TCO-coumarin derivative, it was possible to evaluate and optimize substituents of the tetrazine structure in order to achieve a superior decaging efficiency. By screening a range of tetrazines, it was found that EDG substituted tetrazines led to poor decaging due to slow cycloaddition. On the other hand, EWG, which are expected to accelerate the reaction due to the lowering of the LUMO energy
of tetrazine suppress the elimination step by trapping the cycloaddition intermediate [Fig. 54]. Based on these observations it was proposed that asymmetrical tetrazines with EWG and small-sized non-EWG could accelerate the cycloaddition and thus facilitate the elimination step. Remarkably, two pyrimidine substituted tetrazines showed excellent yields and very fast decaging efficiencies in vitro and in live cells. More than 90% decaging occurred after 5 min whereas the previously used dimethyl tetrazine gave only 58% decaged yield. The tetrazine TCO decaging reaction also outperformed the photo-decaging reaction in living cells, with 90% decaged protein after 4 min whereas only 38% decaged GFP was observed after 4 min UV irradiation.

7.3 Vinyl-ether for function reactivation by IEDDA ligation

Apart from TCO derivatives, vinyl ethers could also serve as a masking group for alcohols. Our group recently reported a vinyl ether decaging reaction on a cytotoxic drug for activation of a halogen-bearing duocarmycin derivative via a Winstein spirocyclization (Fig. 55). In cells the double prodrug showed similar cytotoxicity to the active drug. The broad applicability of this reaction on other chemotypes, including the protected amino acids serine and tyrosine, an 1,6-anhydro sugar, and a fluorophore, expand the scope and potential of this reaction for applications on proteins and glycans.

Furthermore, based on the vinyl ether decaging chemistry, a tetrazine responsive self-immolative cleavable linker was developed by Bradley et al. to achieve molecular triggered drug delivery (Fig. 56a). Accordingly, the vinyl ether cleavable moiety was embedded on a PEG polymer and upon addition of tetrazine, the decaged prodrug could trigger 80% cell death with 48 h incubation. Also recently, Devaraj et al. explored the vinyl ether decaging for mRNA detection. In particular, a caged near-infrared (NIR)-emitting cyanine dye was shown to restore the fluorescence by up to 70-fold after tetrazine reaction (Fig. 56b). Further this NIR fluorogenic probe was applied for mRNA detection in vitro and in cellular context (Fig. 56c).

8. IEDDA reactions in materials and nanomaterials with biological applications

The aqueous compatibility of IEDDA reactions without involving any toxic catalyst or solvent makes it an optimal reaction for bio- and nanomaterial applications. Most importantly, due to its modular nature, IEDDA reactions allow the facile cross-linking of building blocks and thus enable an alternative way for the construction and modification of molecular structures. Another major advantage of IEDDA reactions is the facilitated purification since none of the conjugation partners has to be used in excess to reach fast reactions. Based on these favourable characteristics, the IEDDA reaction has been explored for biomaterials and nanomaterials preparation for applications in biology and medicine. Some examples of these applications are presented in the following sections.

8.1 Polymer hydrogel formation via IEDDA bioorthogonal reaction

Due to their high-water content, excellent mass transport properties, and tissue-like elasticity, ionically crosslinked......
hydrogels have been used extensively to encapsulate cells and bioactive molecules (e.g. 3D cell culture and drug delivery in vivo) as well as for tissue engineering but so far, these polymers have been largely limited by low crosslinking efficiency and poor integrity of the resulting materials. To overcome these limitations, IEDDA ligation, along with other bioorthogonal reactions, have been proposed as an alternative method for the synthesis of biocompatible hydrogels. In one example, Joshi et al. reported the synthesis of alginate hydrogels via tetrazine–norbornene bioorthogonal chemistry. By using tetrazine/norbornene alginate derivatives, the hydrogel could be prepared in 1 hour displaying tunable mechanical and swelling properties depending on the use of different tetrazine/norbornene alginate ratios (Fig. 57). Compared with the ionically crosslinked hydrogel, IEDDA-clicked gels showed higher cell viability and cell metabolic activity. Minimal inflammatory response and high stability was also observed after in vivo injections.

Furthermore, IEDDA combined with thiol–ene chemistry could serve as a robust reaction for further functionalization. Indeed, post-gelation modification could be introduced by functionalizing excess norbornene units with Cys-containing RGDS bioorthogonal chemistry. This construct showed 3-fold increase in cell adherence compared with unmodified gels. In another example, Anseth et al. demonstrated that hydrogels formed by multi-functional PEG–tetrazine units could be functionalized with norbornene-containing peptides (e.g. an ECM mimetic cell degradable peptide, a RGDS peptide or a short peptide bearing a thiol group) for 3D cell culture. This hydrogel network formulated to have pendant thiols could be subsequently used for functionalization with fluorescein and rhodamine fluorescent norbornene-BSA proteins by photoinitiated thiol–ene click reaction with excellent pattern fidelity (Fig. 58).

In order to develop a dual marking hydrogel system, Weissleder et al. encapsulated an X-ray radiography insoluble contrast agent and a pigment into a gelatin hydrogel, which was prepared by IEDDA reaction between tetrazine- and norbornene-modified gelatines. In vivo studies showed good performance for in vivo CT imaging and ex vivo visual localization of the radiopaque tinted hydrogel. Double network (DN) hydrogels have also received much attention because of its high-water percentage (ca. 90 wt%) and high strength. In another work, Dove et al. reported the synthesis of a DN hydrogel in one-step under physiological conditions and within 3 min by using tetrazine–norbornene click reaction for formation of the loose network (norbornene-functionalized chitosan and a linear PEG ditetrazine) and thiol–ene chemistry for formation of the dense network (PEG–tetraalkyne and a linear PEG–dithiol). The resulting hydrogels exhibited excellent mechanical properties and display high cytocompatibility (> 99% cell viability after 48 h).

Due to its ability to coordinate with metal ions, PEG polymers terminally functionalized with bipyrdyltetrazines could form multi-functional supermolecular gels in the presence of metal ions (Fe²⁺ and Ni²⁺). This platform was explored to prepare metallo-hydrogel scaffolds for photo-triggered drug release (Fig. 59). This approach is, however, limited by slow photo release rates due to the competitive absorption from the dark-coloured gel, and thus, the development of new systems...
with alternative metals or ligands with desirable optical properties is demanded.360

The IEDDA reaction has also emerged as an important tool for producing microsphere hydrogels by interfacial crosslinking using a tetrazine-hyaluronic derivative (HA-Tz) and bis-TCO cross-linker (Fig. 60a and b). Covalent tagging with diffusible Alexa-TCO yielded shell- or core-labelled microspheres. Alternating the presence and absence of TCO-dye afford onion-like structures with 3, 5, and 7 layers (Fig. 60c). These 3D microspheres were tested for 3D culture of prostate cancer cells with good cell proliferation and viability (98% viable at 5 days). Notably, cells aggregated to form clusters instead of homogeneous dispersions.361

8.2 Application of IEDDA reaction in polymer formation and modification

Tetrazine bioorthogonal reaction is increasingly being applied for polymer modification or polymer-polymer coupling. In 2011, O’Reilly demonstrated firstly the suitability of the IEDDA conjugation method for efficient synthesis of block copolymers.362 Importantly, the norbornene–tetrazine combination was found to be compatible with the trithiocarbonate group in RAFT (reversible addition–fragmentation chain transfer polymerisation) chain transfer agents (Fig. 61).
Another application by Jia et al. describes the synthesis of multiblock copolymers through rapid tetrazine–TCO bio-orthogonal polymerization (Fig. 62a). Further modification of the copolymer fibres with a fibronectin-derived cell adhesive peptide promotes the attachment and alignment of fibroblasts with various shapes and orientations, whereas when incubated with peptide-free fibres, cells remained round and unbound.363

In a related paper, Fox et al. discovered that dihydrotetrazine could undergo photocatalytic oxidation to tetrazine thereby turning on its reactivity towards s-TCO (Fig. 62b). Interfacial polymerization between tetrazine-bearing monomers and bis-s-TCO monomers conjugated to dihydrotetrazine moieties (DHTz) produced dihydrotetrazine fibres (Fig. 62b), which could be further reacted with TCO bearing proteins, peptides and dyes (Fig. 62c) under irradiation with sensitizers. Notably, the fibres conjugated to a cell adhesive RGD peptide exhibited a dramatically increased ability to mediate contact guidance of cells (Fig. 62d).103

8.3 Application of IEDDA reaction in the synthesis of nanoparticles for biomedicine

Due to a large number of interesting properties (e.g. multivalent targeting capability, high payload, etc.), imaging and therapeutic nanoparticles have been widely used in nanomedicine research.364 Quantum dots (QDs) are highly fluorescent nanoscale crystals with size-dependent emission spectra and excellent photophysical properties (e.g. high quantum yield, low photobleaching) that have been demonstrated to have several advantages over fluorescent organic dyes for cell imaging.365 The first proof of concept study using “bioorthogonal QD” was reported in 2010 by Bawendi et al. In this work norbornene-coated QDs were used to target cancer cells previously decorated with tetrazine-modified epidermal growth factor antibody (Fig. 63).366 Similarly, Duda et al. conjugated tetrazine-modified antibodies to quantum dots modified with norbornene. Using these QD/antibody conjugates, the authors target a rare cell population in bone marrow at the single cell level in live animals using multiphoton microscopy.367

Beyond QDs Weissleder et al. described the use of magneto-fluorescent nanoparticles (MFNPs; fluorescent iron oxide nanoparticles) in different configurations to improve binding efficiency and cell detection for clinical diagnostics. This technique was referred to as ‘bioorthogonal nanoparticle detection’ (BOND).
The rational for the development of this new methodology was that magnetic based sensing schemes benefit from high sensitivity, low cost, and the ability to perform measurements in samples that are not optically transparent. Their study compared two BOND assays to determine the ability to specifically target cancer cells. Cellular labelling was performed by exposing cells directly to antibody–MFNP conjugates (one step BOND-1 strategy) or by incubating cells first with TCO decorated antibodies, followed by tetrazine–MFNPs (two steps strategy: BOND-2) (Fig. 64). The authors demonstrated that BOND-2 consistently yielded higher nanoparticle binding to cells by a factor of 10 to 15 compared to direct immuno-conjugates obtained from BOND-1 method.

Importantly, a similar two-step targeting strategy using avidin/biotin, although exceeds the direct “BOND-1 conjugate”, falls behind BOND-2, which was attributed to the large size of avidin proteins blocking neighbouring biotin binding sites and potential constraining binding configurations. Using this system, different biomarkers of human circulating tumour cells were detected and evaluated via μ-nuclear magnetic resonance on clinical samples. Alternatively, the Hall effect of MNP enables rapid and sensitive high-throughput screening on mammalian cells and bacteria. Other medical applications of BOND-2 technique are on-chip detection of microvesicles liberated into the blood circulation from glioblastomas, detection of microvesicle urinary markers, and detection of mammalian and bacteria cells for diagnosis of infectious disease. BOND-2 was also evaluated for cell tracking by spatiotemporally labelling with photoactivatable fluorophores, and for targeting intracellular biomarkers in permeabilized and fixed cells by confocal fluorescence imaging and diagnostic magnetic resonance. In one attempt to improve BOND-2 assay sensitivity and robustness, multiple consecutive conjugation steps of fluorescent nanoparticles containing TCO and tetrazine groups were used to amplify the corresponding signals. Detection sensitivity could be increased up to 2 orders of magnitude over current method.

There have also been many advances in the development of nanoparticle systems for in vivo radionuclide imaging. Liposomes are attractive nanoparticle vesicles considered promising systems for controlled drug delivery. Reiner et al. introduced in...
Conclusions

Owing to its high selectivity, unparalleled fast kinetics, and free catalyst nature, the IEDDA reactions have emerged during the last decade as the state-of-the-art approach for selective modification of proteins in live cells and notably in animals. Herein we reviewed recent advances in this field with a focus on examples that highlight the utility of IEDDA ligations over other bioorthogonal reactions. The superfast kinetics and unique fluorogenicity makes IEDDA particularly suitable for super resolution imaging and tracking fast dynamics process in cells with minimal background. In addition, with the deepening understanding of IEDDA reactivity, researchers have realized that by choosing a fine-tuned tetrazine-dienophile pair, two parallel IEDDA reactions can be orthogonal to each other, which allows fast simultaneous multicolour labelling. Although recent developments are very promising a number of key challenges can be envisaged that must be addressed in the future. The fastest IEDDA ligations usually involve relatively large reactive groups, leading to higher chances of influencing biological processes. In addition, the hydrophobicity of large reporting groups could also be disadvantageous in certain circumstances, affording high nonspecific labelling. To circumvent this, a challenge lies in the development of small, reactive and hydrophilic dienophiles that possess at the same time high stability and permeability to achieve fast and selective labelling with minimal background. In this regard, the troublesome synthesis of the tetrazine and dienophiles partners, combined with their limited commercial availability, is still restraining IEDDA as a widely used tool.

In terms of pretargeting, the development of clearing agents introduced before administration of the radiolabelled probe results in improved tissue to non-tissue ratios with pronounced localization of radioactivity in the tumour. However, the next challenges lie in the modulation of the pharmacokinetics of the Tz/TCO components to reduce in vivo nonspecific accumulation of the IEDDA radio ligands. More attention should be also given to the therapeutic potential of pretargeted radioimmunotherapy. It is envisaging that the use of radioligands with optimum pharmacokinetic profiles combined with clearing agents would substantially reduce accumulation in healthy tissues, maximizing the therapeutic efficacy and minimizing side effects. Regarding future perspectives, we anticipate the use of IEDDA reactions will go beyond the straightforward attachment of a given functional moiety to a protein of interest. There are already published work supporting that tetrazine decaging reactions will emerge as efficient tools for study for example the effect of PTMs on protein function and bioorthogonal drug activation. In vivo examples of ADCs containing a dienophile chemically linked to a prodrug could be already used for direct release of the drug after tumour binding triggered by the IEDDA reaction. Future studies will aim to optimize the therapeutic effect of this class of ADCs for pretargeting tumour treatment. In resume, the potential of IEDDA reactions are enormous and suggest a rich future of this bioorthogonal reaction in the field of chemical biology.

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