RESEARCH PAPER

Role of Bioorthogonal Reactions in Live Cells and *In vivo* Imaging: Principles and Applications

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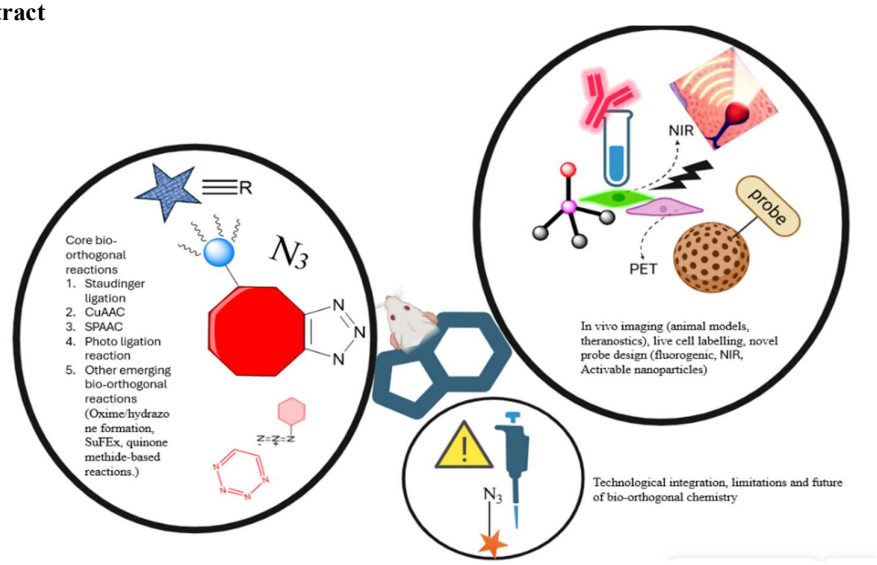
Graphical abstract



Abstract

Bioorthogonal chemistry has emerged as an effective strategy for performing selective in vivo reactions with minimal perturbation of native biological processes. In addition to the Staudinger ligation, reactions such as inverse electron-demand Diels-Alder (iEDDA), strain-promoted azide–alkyne cycloaddition (SPAAC) and copper-catalyzed azide–alkyne cycloaddition (CuAAC) have been widely applied for rapid and efficient bioconjugation in biomedical studies. This review highlights key bioorthogonal reactions and their roles in modern biomedical research. These reactions enable the selective labelling of glycans, proteins, lipids, and nucleic acids, supporting applications such as live-cell imaging, targeted drug activation, antibody–drug conjugates, and in vivo labelling. Recent advances in probe design, including fluorogenic, near-infrared II (NIR-II), and stimuli-responsive probes, have improved detection sensitivity and spatiotemporal resolution in complex biological environments. The integration of bioorthogonal chemistry with advanced imaging techniques has further expanded its applications in biomedicine. Despite these advances, challenges such as reduced efficiency under physiological conditions, potential toxicity, and probe instability remain. Strategies including ligand design optimization, strain-promoted systems, and computational approaches are being explored to address these limitations. Emerging directions include applications in organoid models and data-driven approaches in bioorthogonal chemistry. This review summarizes major developments in bioorthogonal chemistry reported in recent years, with a focus on reaction mechanisms, probe design, and imaging applications.

Keywords: Bioorthogonal chemistry, Click reactions, Copper-catalysed, azide–alkyne cycloaddition (CuAAC), Strain-promoted azide–alkyne cycloaddition (SPAAC), Inverse electron-demand Diels–Alder (iEDDA), *In vivo* imaging and theranostics
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1. Introduction

Bioorthogonal chemistry refers to chemical reactions that proceed selectively within living systems without disturbing native biological environment. Based on the mathematical idea of orthogonality, bioorthogonal chemistry characterizes reactions that take place within biological systems but independent of native biochemistry, resulting in different chemical and biological reactivity spaces. There is no disruption to physiology because these reactions are inert to endogenous biomolecules. By conducting reactions inside living cells and organisms, bioorthogonal chemistry emerged from early developments in selective chemical reactions compatible with living systems in biological settings[1,2].

In contrast to conventional bioconjugation, which targets naturally occurring reactive groups (amines, thiols) and frequently results in non-specific interactions, bioorthogonal techniques provide high yields, minimal disturbance, and greater selectivity. Since then, the discipline has grown quickly, producing new reactions, reagents, applications and for this Bertozzi, Morten Meldal, and K. Barry Sharpless were also awarded the 2022 Nobel Prize in Chemistry[3,4]. The idea started in Bertozzi's glycoscience lab at Berkeley, where innovation was encouraged by the need to investigate glycans, which are important indicators of cancer and other illnesses.

Healthy and cancerous cells have different glycan patterns which present options for diagnosis and treatment. A bioorthogonal handle (X) is metabolically incorporated into biomolecules and then a selective reaction with a complementary probe (Y) produces an XY adduct. Amber codon suppression, expressed protein ligation, metabolic engineering, and suppression tagging were among the early techniques[5–8].

One of the earliest reactions created was the Staudinger ligation that formed amide bonds using an azide-phosphine reaction, but due to the slow kinetics, other faster reactions were developed. The speed and biocompatibility of copper-catalyzed azide–alkyne cycloaddition (CuAAC) and its copper-free version SPAAC were introduced. Due to its remarkable speeds, high yield and compatibility, the inverse electron-demand Diels-Alder (iEDDA) reaction between tetrazines and strained alkenes/alkynes later became a standard[9]. Azide reporters remain widely used due to their ease of metabolic incorporation.

These developments transformed glycan imaging by making it possible to dynamically visualize sialic acids, fucosylated glycans, O-GlcNAc alterations, and mucin-type sugars[10] all of which are essential for researching drug development, immunological signalling, viral entry, and cancer progression. Previous instruments, such as lectins or antibodies offered only static snapshots and were constrained by poor affinity, tissue impermeability, or toxicity[11–13]. These obstacles were overcome by bioorthogonal techniques such as cyclooctyne-based SPAAC which enables live-cell and in vivo research and fluorogenic probes which provide quick, high-contrast imaging.

Beyond glycans, bioorthogonal chemistry enables labelling of proteins, lipids and nucleic acids without interfering with their natural functions. Nucleotides are altered by azide-modified nucleoside triphosphates, proteins by noncanonical amino acids or post-translational tags (SNAP, CLIP, Halo) and lipids by azide/alkyne-modified analog feeding. These techniques are used in biopharmaceuticals such as antibody–drug conjugates (ADCs) including site-specific Aldehyde Tag and HIPS Ligation platforms as well as pulse-chase and metabolic labelling investigations. Tetrazine-functionalized systems are being explored for tumor-specific cytotoxicity through in-vivo prodrug activation, highlighting their translational potential as bioorthogonal platforms[14–16].

Overall, bioorthogonal chemistry enables study and manipulation of biological systems with previously unheard-of accuracy. This review is structured to provide a comprehensive and coherent overview of the field, section 2 outlines the major bioorthogonal reactions and their underlying mechanisms, section 3 examines contemporary probe design strategies, section 4 highlights their integration into advanced technological platforms and applications and last two sections 5 and 6 discuss current limitations and future perspectives within evolving biological and clinical settings. Bioorthogonal chemistry has grown quickly with many reactions designed to improve speed and selectivity but despite progress most of the reactions are tested under lab conditions. This review provides an overview of key bioorthogonal reactions, probe design strategies and their applications in imaging and therapeutic systems.

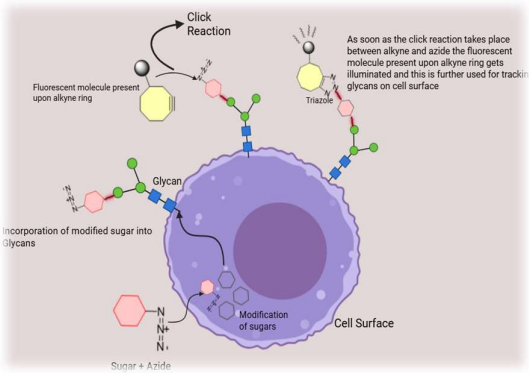


Figure 1. Diagram of metabolic glycan engineering, which uses Bertozzi's method to introduce azide-bearing sugar analogues into cell-surface glycans to provide bioorthogonalhandles for selective labelling and imaging via click chemistry

2. Core bioorthogonal reactions and their mechanisms

2.1 Azide-alkyne cycloadditions

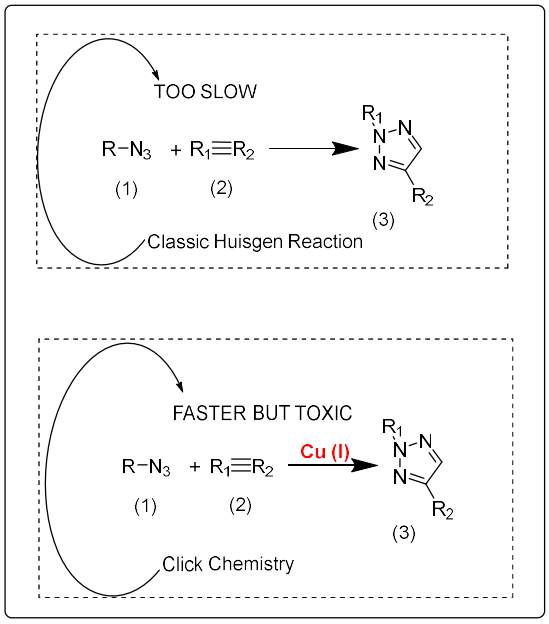
2.1.1 Copper-catalysed (CuAAC)

The copper-catalyzed azide-alkyne cycloaddition (CuAAC) was created in 2002 after Morten Meldal and K. Barry Sharpless independently showed that copper(I) catalysis could significantly speed up azide-alkyne cycloaddition. 1,4-disubstituted 1,2,3-triazoles are produced via this highly site-selective [3+2] cycloaddition between terminal alkynes and azides **(Scheme 1)**[17] Mechanistically, a copper-acetylide intermediate is formed when copper (I) coordinates with the terminal alkyne. This intermediate then interacts with the azide to form the triazole ring through cyclization[17–19]. CuAAC has emerged as a cornerstone of bioorthogonal ligation and a prototypical example of click chemistry.

CuAAC is widely used in chemical biology because of its remarkable orthogonality, which enables it to proceed selectively in the presence of various functional groups without interference. It is widely used for position-specific protein labelling, enabling the coupling of biomolecules modified by azides or alkynes with fluorophores or affinity tags for tracking, imaging, and purification[20]**.** A good example is precise labelling of proteins such as SOD1 in living cells using unnatural amino acids and selective targeting of enzymes like GSTO1-1 through azide-based activity probes with subsequent fluorophore tagging. CuAAC also supports metabolic labelling techniques and activity-based protein profiling which allow visualization of biomolecular processes by incorporating modified sugars, amino acids or nucleotides into live systems[21,22] for instance, metabolic incorporation of azide-functionalized sugars like Ac-4ManNAz into cell-surface glycans followed by fluorescence tagging in systems such as HeLa cells and neurons as well as glycoprotein enrichment and analysis in yeast. The reaction offers a modular platform for creating drug conjugates including peptides, small molecules and antibodies in the drug development process[23]. Conjugation of cyclic RGD peptides with cryptophytic anticancer agents improving targeting efficiency and stability through resilient triazole linkages will be a good example. Beyond its role in biology, CuAAC is extensively employed in materials science to functionalize surfaces, polymers, and nanoparticles, assisting in the creation of materials that are stimuli-responsive and multifunctional[24–26] materials**.** For example, modification of cellulose nanocrystals and clay-polymer nanocomposites to enhance dispersion, thermal strength and overall material performance.

Water-soluble copper-stabilizing ligands including THPTA, BTTAA, and BTTES **(Figure 2)** improve aqueous solubility, stabilize copper (I), speed up reaction kinetics and lessen cytotoxicity and thereby enhancing biocompatibility. Toxicity, catalyst recovery, and scalability concerns are further addressed by heterogeneous CuAAC systems that use immobilized copper catalysts[27,28]. However, copper-induced oxidative damage to biomolecules and disruption of metal-dependent cellular functions continue to limit CuAAC as well as by the necessity of azide, alkyne, and copper species being available simultaneously in living systems[29,30]. To get around these limitations and increase CuAAC compatibility with live-cell and in vivo applications, ongoing initiatives include low-copper systems, nanoparticle-based catalysts, and optimized ligand design[31,32]. CuAAC is highly utilized since it displays high regioselectivity, and the reaction takes place under moderate conditions. As an illustration, 57-91% yield of triazole adducts was achieved in a one-pot process using biomass materials as substrates, with few by-products formed. As seen above it has been very useful in bioconjugation and chemical biology. CuAAC shows a clear contrast in bio-orthogonal chemistry, it works well in chemical systems but has limitations in biological environments. Most current approaches try to reduce copper toxicity or try to mask copper toxicity issue instead of solving the main factor of its incompatibility with living systems. These observations indicate future progress may come from designing reactions that are naturally safe in biological conditions rather than modification.

Azide(1) (organic azide) reacts with terminal alkyne (2) to form 1,4 di-substituted 1,2,3 triazole linkage (3). The uncatalyzed Huisgen reaction is slow and requires elevated temperatures (>80–120 °C). In contrast CuAAC proceeds at room temperature to 37°C using Cu(I) (e.g., $CuSO_4$ /ascorbate or CuBr with ligands such as TBTA/NHC), giving high yields (~90-98%) with second order rate constants of ~ 10^1–10^3 M^{-1} s^{-1}. Buffers such as HEPES buffer (ph7.5) provides optimal kinetics[33–35].



Scheme 1. Illustrating the transition from the slow, uncatalyzed Huisgen cycloaddition to the Cu(I)-catalyzed azide–alkyne click reaction, which yields 1,2,3-triazoles with better kinetics[17].

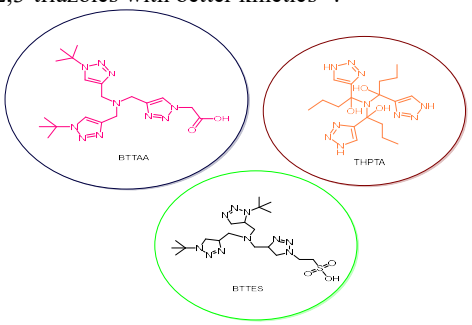


Figure 2. Water-soluble ligands BTTAA, BTTES and THPTA stabilize the Cu(I) catalyst, increase reaction rates in aqueous media, and lessen cytotoxicity in copper(I)-catalyzed azide–alkyne cycloaddition (CuAAC), allowing for effective and biocompatible biomolecule labelling under physiological conditions.

2.1.2 Strain promoted (SPAAC)

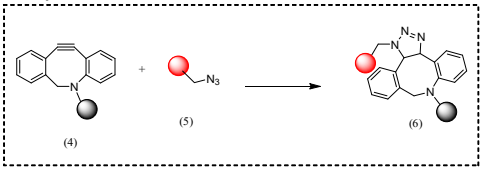
Strain-promoted azide-alkyne cycloaddition (SPAAC) is the most well-known metal-free azide-alkyne cycloaddition techniques designed to address the cytotoxicity of copper catalysts in CuAAC. Under mild, bio-orthogonal conditions, Bertozzi and colleagues initially showed that the intrinsic ring strain in cyclooctynes drives a catalyst-free [3+2] cycloaddition with azides, generating stable triazoles. Even though SPAAC's second-order rate constants are lower than those of Staudinger ligation's, the reaction occurs effectively in live systems with no discernible toxicity, allowing for early applications in cell-surface and in vitro biomolecule labelling[36] for example, live imaging of glycans in zebrafish embryos and cell-surface labelling in mammalian systems. The electronic and structural characteristics of both reaction partners control SPAAC kinetics. Reported second-order rate constants often fall between 0.2 and 2.9 M^{-1} s^{-1}[37] though these rates can decrease in cellular environments due to macromolecular crowding and diffusion limits. The quickest reaction rates are shown by strained cyclooctynes like bicyclononyne (BCN) and electron-deficient azides with BCN often preferred in live-cell studies due to reduced steric hindrance compared to bulky cyclooctynes. Because the alkyne is distorted from linear geometry, the reaction is driven by the release of ring strain, which lowers the activation barrier and permits metal-free cycloaddition. While electron-withdrawing substituents, like difluorination in DIFO reagents, speed up the reaction by stabilizing developing charges, favourable orbital overlap in the transition state further stabilizes the reaction pathway. Electron-donating groups, on the other hand, typically slow down the reaction, underscoring the need of electronic optimization[38,39]. Advances such as difluorinated DIFO derivatives can approach CuAAC-like reaction rates in vivo imaging applications.

Several cyclooctyne derivatives have been created to improve SPAAC efficiency. Various cyclooctyne derivatives including dibenzocyclooctynes (DBCO) and difluorinated cyclooctynes (DIFO) have been developed which increase ring strain and electronic activation, leading to rate increases of up to three orders of magnitude when compared to unstrained cyclooctynes. Because of their enhanced stability, synthetic accessibility, and adjustable reactivity, DBCO derivatives **(Figure 3)** are very appealing and are frequently employed for biomolecule conjugation and live-cell labelling[38,40]. Other strained alkynes and alternative 1,3-dipoles, such as nitrile oxides, nitrile imines, iminosydnones, and nitrones, have been added to the bio-orthogonaltoolkit in addition to DIFO and DBCO to enable similar strain-promoted cycloadditions (e.g., SPSAC, SPSIC, and SPANIC).

Bulky cyclooctyne scaffolds can adversely impact pharmacokinetics, biodistribution, and cellular absorption, especially for small-molecule probes, and SPAAC is inherently slower than tetrazine-based ligations or catalyzed CuAAC, despite its versatility. According to recent research, steric bulk and hydrophobicity can restrict tissue penetration and target engagement[41]. For instance, DBCO derivatives show reduced penetration compared to smaller BCN analogues in vivo. This has led to the development of techniques like PEGylation or the usage of smaller strained alkynes like BCN to enhance in vivo performance for example, PEGylated DBCO-antibody systems show improved labelling efficiency and pharmacokinetics. Furthermore, buffer composition, pH, temperature, and other environmental

conditions might affect SPAAC response rates, making reproducibility across biological systems more difficult with reaction rates increasing at physiological temperature and varying significantly across media such as DMEM vs RPMI. In order to improve predictability and optimize reaction conditions, computational modelling and AI-assisted screening are being used more and more[42]. Overall, the main benefit of SPAAC is its total lack of metal catalysts, which results in remarkable biocompatibility even at modest reaction rates[43]. SPAAC's special place in live-cell and in vivo labelling is defined by this harmony between efficiency and safety as demonstrated in applications such as tumour-targeted imaging and nanoparticle-drug delivery in mouse models. In order to enable advanced imaging, diagnostic, and therapeutic applications, ongoing developments concentrate on creating cyclooctyne derivatives with better water solubility, quicker kinetics, and lower steric burden. They also integrate SPAAC with fluorogenic probes and orthogonal tagging techniques[44]. SPAAC was created to overcome the problem of copper-related toxicity in biological systems. which also made it more suitable for use in living systems and now it's widely used in vivo and in vitro. SPAAC is often considered fully biocompatible but its performance actually depends strongly on the biological environment. Reaction speeds measured in simple lab conditions often do not match what happens inside the cells where crowding and limited movement affect reactivity. This shows the importance of testing reactions directly in biological systems rather than relying only on standard kinetic data.

Strain-promoted azide–alkyne cycloaddition (SPAAC) is the process by which an azide (5) and a strained cyclooctyne (such as dibenzocyclooctyne, 4) combine to generate a stable triazole (6) without the need for copper catalysis. The reaction proceeds under mild physiological conditions, typically at temperature ranging from (25–37 °C in aqueous buffers such as PBS or HEPES). SPAAC exhibits tunable second-order rate constants (0.01–1.2 M^{-1} s^{-1}), broad functional group tolerance, and typical yields of 65–95%[42,45,46]. These features make SPAAC particularly suitable for in vivo bioconjugation and imaging applications where maintaining cellular viability and minimizing toxicity are essential.



Scheme 2. In the absence of copper, bioorthogonal strain-promoted azide–alkyne cycloaddition (SPAAC) creates a stable triazole bond between a strained alkyne (4) and an azide[39] (5).

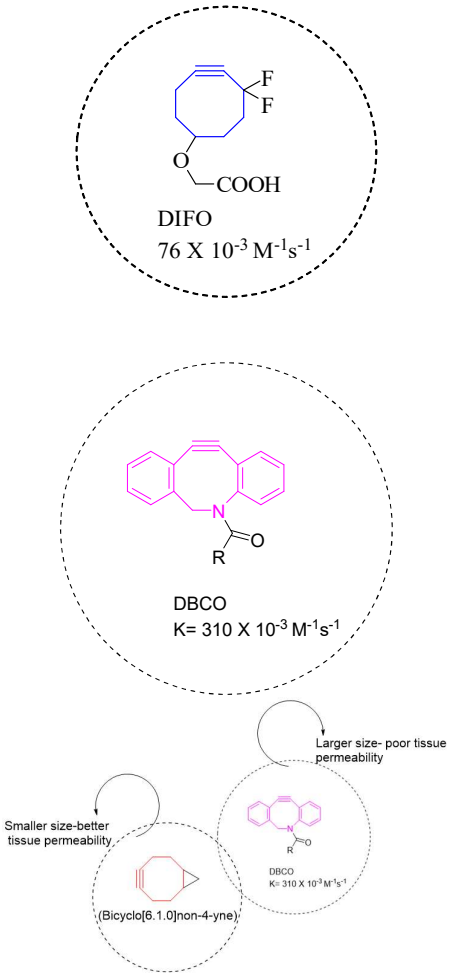


Figure 3. ALO (Alkyne-Ligand Optimized), DIFO (Difluorinated Cyclooctyne), and DBCO (Dibenzo cyclooctyne) are structurally different cyclooctynes employed in SPAAC, where ring strain and electronic substituents modify ligation kinetics and reactivity for biological labelling.

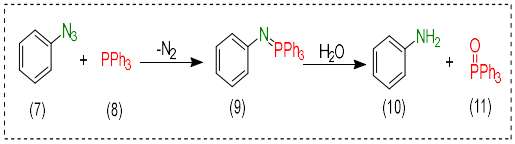
2.2 Staudinger ligation

The famous bioorthogonal reaction known as Staudinger reduction was first described by Bertozzi and colleagues in 2000. It is based on the selective coupling of an azide with a phosphine to create a stable amide bond in an aqueous, metal-free environment. Mechanistically, the phosphine targets the azide's terminal nitrogen, releasing nitrogen gas

and producing an iminophosphorane intermediate **(Scheme 3)**. Staudinger ligation differs from the traditional Staudinger reduction in that, instead of simple hydrolysis to an amine and phosphine oxide, it employs an ortho-positioned electrophilic ester that reacts with the iminophosphorane intermediate to form stable amide bond intramolecularly[47,48]. Because both azides and phosphines are inert toward endogenous biomolecules, the reaction is extremely chemo selective and biocompatible, allowing for selective ligation in a variety of biological settings. This makes Staudinger ligation especially appealing for bioconjugation applications that need stable covalent bonds without the need for metal catalysts. Site-specific protein labelling and in vivo imaging without toxicity or cross-reactivity are made possible by its compatibility with mild physiological circumstances **(Scheme 4)**. Photocaged phosphines enable spatially controlled labelling of azide-modified glycoproteins in live cells and organisms such as zebrafish while metabolic probes have been used to track glucose uptake in cancer cells. However, the reaction is limited by phosphine oxidation and has very slow kinetics ($k_2 = 10^{-3}$ M^{-1} s^{-1}), which can lower efficiency in biological contexts though strategies such as nanocarrier protection and phoshphinothiol variants improve ligation yields and stability in vivo.

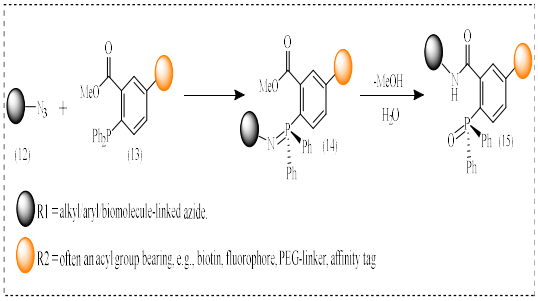
As thoroughly described in Chemical Reviews (2020), significant advancements have been documented to address these issues, such as traceless and non-traceless ligation variations and phosphite-based reagents with improved water stability for instance, traceless ligation enables multi-site conjugation on tumour-targeting antibodies without affecting their biological activity. Phospha-Michael reactions and cyclopropenone–phosphine ligations have been added to phosphorus-based bio-orthogonal chemistry in more recent times[49], providing enhanced stability and reactivity. While nanocarrier-based delivery techniques improve stability, targeting, and accumulation at particular tissues or tumours, strategies like photocaged phosphines provide light-triggered activation with precise spatiotemporal control **(Figure 4)**. Collectively, these developments greatly increase Staudinger ligation's effectiveness, selectivity, and suitability for use in intricate biological systems[50]. Staudinger ligation's slow kinetics (~10^{-3} M^{-1} s^{-1}) and phosphine oxidation make it hard to use in dynamic live imaging, where faster SPAAC or tetrazines work better even though amide bonds are reliable. Biological crowding makes these problems worse by requiring high concentrations that are not possible for sparse targets. Future strategies including, AI-optimized phosphines and hybrid photocaged systems could bring it back to life for precision ADCs and in vivo protein assembly in the future.

In this reaction an azide (azidobenzene) (7) reacts with triphenylphosphine (8) to form an aza-ylide(iminophosphorane) intermediate (9) with the release of nitrogen gas. Subsequent hydrolysis of this intermediate yields the corresponding amine (aniline) (10) along with triphenylphosphine oxide (11). The transformations proceed under mild, metal-free conditions, typically at room temperature to 37 °C in aqueous or mixed solvents and affords high yields (up to ~90% yield, >95% purity)[51] with excellent chemo selectivity.



Scheme 3. An Illustration of the classic Staudinger reduction, in which triphenylphosphine and an organic azide combine to produce an iminophosphorane intermediate that, when hydrolyzed, produces a primary amine and triphenylphosphine oxide[47].

The modified Staudinger ligation uses an ester-functionalized triaryl phosphine (13) that combines with an azide (12) to create an iminophosphorane intermediate. Intramolecular acyl transfer (14) then produces a stable amide product (15) and phosphine oxide in moderate aqueous conditions (typically 25–37 °C). Permanent covalent conjugation with great efficiency (≈90–95% yield, high purity), enhanced stability over the traditional reaction, and outstanding compatibility with biological settings is made possible by this traceless ligation, making it appropriate for in vivo applications[51].



Scheme 4. An illustration of the modified (traceless) Staudinger ligation, in which the azide–phosphine adduct promotes intramolecular acyl transfer, leading to the formation of amide bonds in the end product without any remaining phosphine[48,51].

Staudinger ligation is a process in which a phosphine ester linked to a reporter or imaging probe joins with an azide-functionalized biomolecule. This allows for the selective and biocompatible attachment of fluorescent, affinity, or other functional tags for biomolecular labelling and detection by forming a stable amide bond. (14)[51,52].

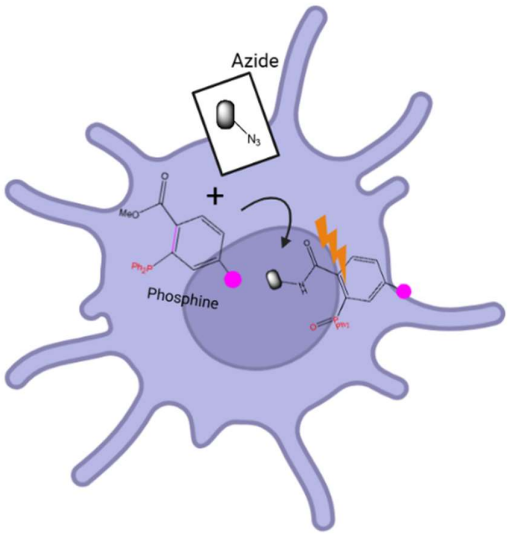


Figure 4. Staudinger Ligation (Tagged and fluorescing cell surface for detection)

2.3 Photo-click and light-activated reactions

Light has become a potent external stimulus for highly selective chemical reactions under mild conditions inspired by natural photochemical processes like photosynthesis. Photo-click reactions combine the exact control over location and timing accuracy of photochemistry with the modularity and efficiency of click reactions under the framework of Sharpless click chemistry thus allowing bond formation to be initiated at specific times and locations, even at the single-cell level[53]. Photo-click chemistry is especially appealing for biological and materials applications because light functions as a clean, non-invasive trigger, reducing off-target reactivity and improving the overall biocompatibility**.**

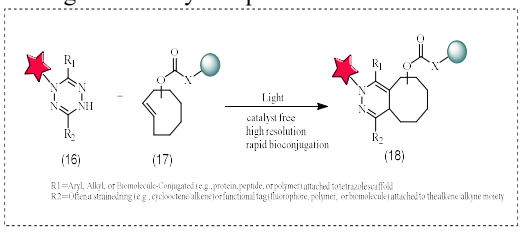
Three main categories can be used to categorize photo-click reactions: (i) catalyst-mediated photo-click processes (ii) photo-isomerization events that produce short-lived reactive intermediates and (iii) direct photo-generation of highly reactive species. Tetrazole-alkene cycloadditions, hetero-Diels-Alder processes and photo-activated azide-alkyne cycloadditions are representative examples that have been extensively used in drug discovery, bioconjugation, polymer chemistry, lithography, and smart material design[54,55]. By using light rather than chemical reductants to produce catalytically active Cu(I) species, photo-mediated CuAAC has revolutionized conventional copper-catalyzed azide-alkyne cycloaddition **(Scheme 5)**. In order to provide spatial control and increased sustainability, early systems used radical photoinitiators, riboflavin, or direct photoactivation of Cu(II) complexes[56]. In order to increase biological compatibility, later developments included photo-deprotection techniques and photoinduced electron transfer (PET) catalysts, which allowed enhanced temporal control and extended activation wavelengths into the visible and near-infrared areas[57]. More modern methods include copper-free photo-click azide-alkyne cycloadditions (PcAAC) which allow regioselective triazole synthesis under visible light and photothermal reduction of Cu(II) utilizing localized heating or hot electrons. Simultaneously, the field of light-regulated bio-orthogonal chemistry has been extended by strain-promoted and photocaged systems. Light-triggered CuAAC is made possible by photocaged alkynes, which are typically covered with nitrobenzyl groups and release terminal alkynes upon irradiation[58,59]. Similar to this, photo-click reactions based on tetrazole and sydnone produce reactive nitrile imines upon photolysis, which quickly conduct cycloadditions with alkenes, alkynes, or thiols. Recent 2025 studies report NIR-II photoacoustic-bioorthogonal hybrid systems capable of imaging tumours deeper than conventional systems(10 mm almost)[55] demonstrating significant improvements in deep-tissue imaging performance and clinical potential.

Beyond CuAAC-based systems rapid, site-specific synthesis of cyclic peptides, glycosides and biomimetic compounds with fine time specific control has been made possible by photo-click reactions such as thiol–ene, thiol–yne, and photo-CuAAC[60]. Applications ranging from peptide stapling and carbohydrate modification to polymer conjugation, surface patterning and hydrogel production are supported by these reactions which proceed quickly and in high yield under mild circumstances. Photolithography, microfluidics, tissue engineering, bioprinting, nanoparticle production and optical materials are examples of advanced uses. Photo-click and light-activated bioorthogonal reactions offer remarkable control over time and location of the reaction for in vivo investigations in chemical biology. Until light exposure produces extremely reactive intermediates, the reagents stay inert, maintaining bioorthogonality and reducing background reactions[55]. With bimolecular rate constants close to 10^2- 10^3 M^{-1} s^{-1}, some photo-activated cycloadditions have incredibly quick kinetics that allow for demanding biological applications.

In order to improve tissue penetration and lower phototoxicity, ongoing studies concentrate on red-shifting activation wavelengths into the near-infrared[61,62]. This will assist applications in fluorescent labelling, PET imaging, targeted medication administration and single-cell analysis. Tetrazoles, tetrazines, diarylsydnones, and photocaged phosphines are examples of optimized reagents that are expanding the arsenal of light-controlled bioorthogonal chemistry and establishing photo-click reactions as revolutionary tools for enhanced imaging and therapeutic approaches[63,64]. Although red-shifted and NIR activation reduces

phototoxicity, limited tissue penetration and light scattering still constrain deep biological applications. Emerging tetrazole and syndone-based systems show promise but remain insufficiently explored in clinical contexts.

Light-Activated Bioorthogonal Ligation, in which light irradiation transforms photocaged dihydrotetrazines (16) into reactive tetrazines (18). These tetrazines provide accurate spatiotemporal labelling of biomolecules in live systems by reacting quickly with trans-cyclooctene (TCO) (17) probes in a regulated "photo-click" fashion. Photoinduced azirine–cyclopropene ligation is a catalyst-free, light-triggered bioorthogonal technique with rapid bioconjugation and superior spatial and temporal resolution. It occurs under mild conditions, allowing conjugation in complex biological settings, with rate constants typically in the range of 10^2–10^3 $M^{-1}s^{-1}$[53]. Following purification, isolated yields range from 80 to 90%, and reported product purities usually surpass 95%. This reaction is developing into a powerful tool for regulated biomolecular labelling and live-cell imaging due to its selectivity and lack of dangerous catalyst requirements[15,18].

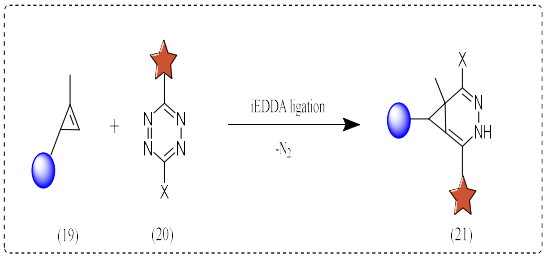


Scheme 5. By using light to change photocaged dihydrotetrazine and trans-cyclooctene (TCO) into reactive tetrazine, light-activated ligation enables high-resolution bioconjugation without the need for a catalyst[7].

2.4 Tetrazine trans-cyclooctene ligation

First described by Blackman et al. and Devaraj et al., tetrazine ligation is a very quick bioorthogonal reaction that produces a dihydropyridazine product by nitrogen emission through a retro-Diels-Alder step after an electron-deficient tetrazine and a strained dienophile undergo inverse electron-demand Diels-Alder (IEDDA) cycloaddition **(Scheme 6).** While simpler strained alkenes serve as model substrates trans cyclooctyne(TCO) derivatives are widely used due to their superior reaction kinetics. Trans-cyclooctene (TCO), one of the most reactive partners, interacts with tetrazines with second-order rate constants as high as 10^3 - 10^6 M^{-1} s^{-1}, which corresponds to reaction half-lives of about 1 s at micromolar concentrations. The IEDDA reaction is especially well suited for in vivo applications at low reagent concentrations or in systems with short circulation times because of its ultrafast kinetics[65]. Notwithstanding its advantages, TCO-based ligation is limited by the in-vivo isomerization of TCO to the less reactive cis-cyclooctene (CCO), a process that is more pronounced in highly strained derivatives and has been associated with interactions with serum proteins that bind copper ions, such as albumin[66]. However, the clever design of TCO scaffolds and tetrazines has made it possible for quick and effective in vivo ligation, which has advanced tumour imaging techniques and allowed for broad application in pre-targeted PET imaging. Through the invention of TCO-conjugated antibodies for colorectal and pancreatic tumours, Lewis and Zeglis' groundbreaking in vivo research demonstrated the clinical potential of TCO-tetrazine pre-targeting, allowing for both Cu-tetrazine PET imaging and Lu-tetrazine radionuclide therapy. Direct F-labelling of highly reactive bispyridyl tetrazines has recently added to the pre-targeting toolset providing probes with better translational potential and pharmacokinetics for clinical PET imaging[67,68]. With reported rate constants close to 10^2 M^{-1} s^{-1}, tetrazine–TCO ligation continues to be one of the quickest and most selective bioorthogonal processes accessible despite persistent difficulties such as TCO isomerization, restricted in vivo stability, and potential off-target interactions. Its remarkable speed and bioorthogonality allow for effective nuclear imaging, prodrug activation and labelling in living systems without interfering with natural biological processes. IEDDA reactions are thus among the fastest bioorthogonal reactions available[69–71]. Their speed has made them very useful in imaging and targeting applications. Although IEDDA reactions are very fast this speed can sometimes make them harder to control in biological systems. Reactions may take place too early before the molecules reach their intended target which can reduce selectivity. Also, the TCO is prone to in vivo isomerization often catalysed by serum proteins such as albumin, which reduces effective reactivity. Recent developments using norberene-based alternatives show improved resistance to isomerization and enhanced biological stability suggesting a shift towards more stable strained alkene systems. These observations suggest future works upon IEDDA reactions should focus on controlling when and where reactions occur instead of only increasing reaction speed.

Strained substituted cyclopropene (19) reacts with tetrazine (20) derivative via inverse electron-demand Diels-alder (IEDDA) ligation to form dihydropyridazine (substituted) product (21) with N_2 extrusion. The reaction proceeds under physiological conditions (RT to 37 °C, aqueous media) with rate constants up to ~10^3–10^6 M^{-1} s^{-1}, affording 78–90% yield and 85–95% purity. This reaction is compatible with physiological circumstances and is frequently utilized for hydrogel functionalization and protein labelling[72].

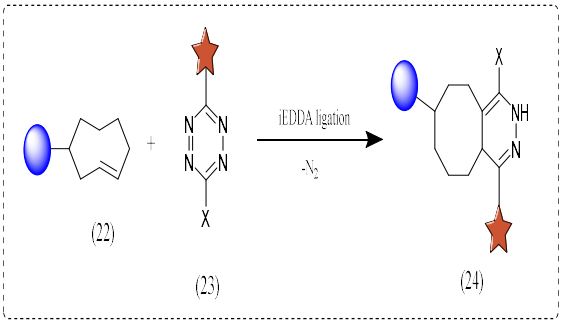


X = strained alkene/alkyne handle linked to a functional group (tag, biomolecule).

Scheme 6. Strain-induced inverse electron demand In the absence of catalysts, the Diels-Alder (IEDDA) ligation between norbornene and tetrazine proceeds rapidly under physiological conditions[73].

Trans-cyclooctene(22) reacts with tetrazine(23) to give dihydropyridazine conjugate(24) TCO-tetrazine adduct with ultrafast kinetics (k up to 10^3-10^6 $M^{-1}s^{-1}$) at RT(37°C) high yields (85–95%), and >97% purity. Biocompatible and catalyst-free, it is frequently used in drug administration, protein modification, and pretargeted imaging.

X (tetrazine side): aryl, heteroaryl, or electron-withdrawing group may carry fluorescent or targeting tags.



Scheme 7. For live-cell labelling, trans-cyclooctene–tetrazine IEDDA ligation provides improved kinetics and bioorthogonalselectivity[73].

2.5 Other emerging ligation strategies

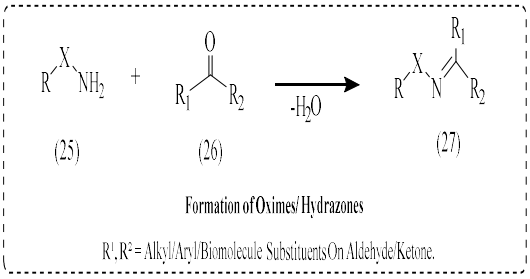
The chemical biology toolset for selective biomolecule modification and labelling in vitro and in vivo has been greatly increased by emerging bioorthogonal ligation techniques such as oxime/hydrazone synthesis, quinone methide–based reactions and sulfur(VI) fluoride exchange (SuFEx) chemistry. Aldehydes or ketones react with aminooxy or hydrazide groups in oxime and hydrazone chemistry to generate C=N linkages. Second-order rate constants under catalysed conditions can reach ~1-10 M^{-1} s^{-1}, allowing for effective conjugation under mild, biocompatible circumstances. Oxime bonds are extremely chemoselective yet they can hydrolyze especially in acidic environments. This reduces long-term stability but might be useful for reversible or dynamic labelling techniques **(Scheme 8).** SuFEx ligation creates incredibly stable S–N or S–O linkages by nucleophilically attacking electrophilic S–F bonds of sulfonyl fluorides with amines, phenols or alcohols. This catalyst-free reaction is especially well suited for in vivo bioconjugation applications since it is irreversible, highly selective, and resistant in aquatic settings **(Scheme 9)[74].** By producing transitory electrophilic intermediates that react specifically with nucleophilic residues on biomolecules, quinone methide-based techniques expand the bioorthogonal repertoire and allow for fine spatial and temporal control in live-cell imaging and therapeutic administration. When combined, these ligation techniques provide quick kinetics, high chemo selectivity, and little disruption of normal cellular functions. They are used in targeted drug delivery, dynamic biological imaging and protein, peptide and nucleic acid labelling

Recent developments have concentrated on bioorthogonal bond-cleavage reactions for regulated payload release in biological systems, going beyond bond creation. Click-to-release techniques use a caged payload reacting with a trigger via bioorthogonal ligation to create a conjugate which then goes through rearrangements like elimination, tautomerization or cyclization to release the active payload. While tetrazine-TCO inverse electron-demand Diels-Alder reactions and transition-metal-mediated processes (such as gold–or ruthenium–catalyzed cleavage or reductive activation of azide or nitroaromatic cages) enable quick and effective activation, redox-responsive systems such as thiol–disulfide and diselenide–disulfide exchange offer reversible control over release kinetics. **(Table 1)** guides selection for particular biomolecular labelling applications by providing a succinct comparison of the main bioorthogonal reactions, describing their reaction types, functional groups, catalyst requirements, kinetics, and practical advantages and limits. Recent advances introduce tetrazine-based activation platforms that enable tumour-selective prodrug release via controlled bioorthogonal reactions, significantly improving therapeutic precision in vivo.

Additional advancements include tetrazine-triggered elimination processes and SPAAC-based caged payload systems, where payload release half-life is determined by both the bimolecular ligation rate (k_2) and the subsequent unimolecular cleavage step (k_1), which determine overall efficiency. The kinetic difficulties associated with click-to-release chemistry are highlighted by the fact that only a small number of systems now accomplish fast (>95%) payload release at physiologically relevant concentrations. Experimental variables including pH, temperature, and solvent environment also have a significant impact on reaction efficiency and repeatability, highlighting the necessity of careful optimization in biological contexts[75,76]. While

reactions such as SUFEx and oxime ligation are efficient and selective issues such as hydrolysis and reversibility limit long-term stability. Click to release systems remain kinetically constrained with few limitations like achieving efficient payload release at physiological concentrations, which highlights the need for in vivo benchmarking.

Aldehydes or ketones (26) react with aminooxy or hydrazide (25) derivatives to make stable oxime or hydrazone (27) linkages when they are in a physiological state. These bioorthogonal reactions don't need a catalyst and happen in water at pH 4.5–7.4 and 25–37 °C. They usually have yields of 70–85% and purity of over 90%. Aniline catalysis or pH change can increase reaction speeds, allowing for reversible bioconjugation and dynamic cellular research[77]

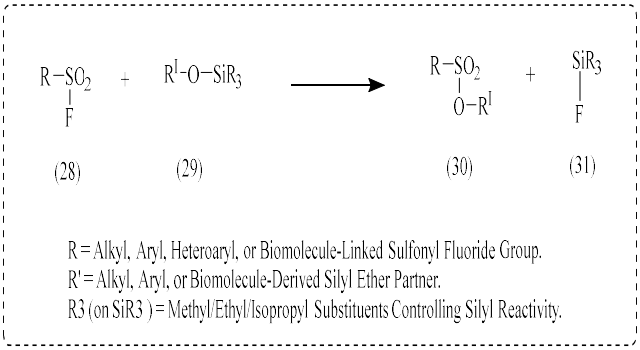


Scheme 8. Oxime/hydrazone ligation between aminooxy or hydrazine derivatives and carbonyl compounds creates stable imine connections[77,74]

Caged Payload (C–P) + Trigger (T) → TC–P → unimolecular reaction → Payload (P) + T

Figure 5. Diagram showing how a trigger molecule (T) and a caged payload (C–P) combine to generate a temporary conjugate (TC–P), which then goes through a unimolecular reaction to release both the trigger and the active payload (P).

SuFEx ligation is the process by which sulfonyl fluorides (28) and silyl (29) ethers react to form stable sulfonate linkages (30) at low temperatures. The reaction takes place in water or a mixture of water and other substances at 20–37 °C, often with base activation. The reaction proceeds with 85–98% yields and >95% purity[78]. Because it is very selective and can handle water, it is useful for bioconjugation and materials applications.



Scheme 9. Sulfur(VI) fluoride exchange (SuFEx) ligation between sulfonyl fluoride and silyl ether forms strong sulfonate bonds, enabling modular and stable conjugation chemistry[78,43]

long-circulating linkers in ADCs and polymeric

Reaction type	Year	Functional groups	Catalyst	Rate Constants ($M^{-1}s^{-1}$)	Strength	Limitations
Staudinger Ligation	2000	Azide + Phosphine	None	$10^{-3} – 10^{-2}$	Biocompatible, selective	Very slow, phosphine oxidation
CuAAC	2002	Azide + Alkyne	Cu (I)	$10^{1} – 10^{3}$	High yield, special triazole	Cu toxicity, *in vitro* preferred
SPAAC	2004	Azide + Cyclooctyne	None	$10^{-2} – 1$	Copper free, live cell compatible	Bulky, slower than CuAAC
Tetrazine-TCO	2008	Tetrazine + TCO	None	$10^{3} – 10^{6}$	Ultra-fast, ideal for *in vivo* labelling	TCO instability, reagent synthesis complexity
Click-to-release	2015	Tetrazine + TCO	None	High	Prodrug activation, controlled release	Still experimental in clinic
Photo-Click Chemistry	2012	Tetrazole + Alkene	Light	Variable	Spatiotemporal control	Needs irradiation, limited tissue penetration
SuFEx	2014	SF + Nucleophile	None	Moderate	Robust, special linkages, chemical diversity	Less explored *in vivo*

Table 1. An overview of the main bioorthogonal processes, emphasizing their kinds, functional groups, catalysts, rates, strengths, and limitations so that efficiency and biocompatibility can be quickly compared[51,79].

Based on the table in **(Table 1)** we can classify reaction according to the application. Staudinger ligation is now limited to niche, low -rate labelling while SPAAC remains useful for antibody and glycan labelling under copper-free conditions. Tetrazine-TCO IEDDA dominates pretargeted PET/SPECT (e.g., ^{89}Zr-tetrazine with TCO-antibodies) enabling rapid in-vivo reactions and improved imaging by separating antibody pharmacokinetics from radiotracer clearance and is expanding into multimodal theranostics. Click-to-release systems are widely used for bioorthogonal prodrugs and cleavable ADC-like platforms allowing controlled activation of cytotoxic agents or gasotransmitters with reduced off-target effects. Photo-click and spatiotemporal cleavage strategies enable tumour-localised, on-demand activation while SuFEx is increasingly applied to build stable, carriers.

3. Design and Optimization of Bioorthogonal Probes

3.1 Fluorogenic and Tetrazine Based Probes

The subject has advanced significantly since Bertozzi introduced bioorthogonal chemistry in , especially with ultrafast tetrazine–TCO ligations with rate constants up to 10^3 M^{-1} s^{-1}. Chemical biology applications are substantially expanded by these reactions which allow fluorogenic "turn-on" imaging with remarkable signal-to-noise ratios and nanomolar sensitivity. Tetrazine-based probes have made it possible to perform deep-tissue near-infrared (NIR) imaging, activatable phototherapies, and multiplexed super-resolution imaging of proteins, organelles and nucleic acids[80]. Despite these developments, limited NIR-II exploitation, a lack of uniform quenching techniques and an imperfect mechanistic understanding impede wider adoption. Dual-targeting systems and AI-assisted probe design are expected to further advance, providing better clinical translatability, sensitivity, and specificity.

3.2 NIR and NIR-II Imaging Platforms

Next-generation techniques for deep-tissue imaging employing long-wavelength light (1000–1700 nm) include NIR-II fluorescence and photoacoustic imaging[81]. While photoacoustic techniques provide deeper anatomical context by turning optical absorption into ultrasonic signals, fluorescence imaging offers high molecular sensitivity. High quantum yield, photostability, solubility, and biocompatibility are all combined in effective probes. Inorganic agents like quantum dots, carbon dots, and rare-earth nanoparticles support multimodal performance while organic dyes like ICG, AIEgens and porphyrins enable tunable emission **(see Figure 9 below)**. Lipid carriers, micelles, microbubbles and protein assemblies are examples of advanced delivery platforms that improve targeting effectiveness and in vivo stability, making NIR-II imaging a potent translational tool[82–84].

3.3 Staudinger ligation in imaging and therapy

A fundamental bioorthogonal reaction, especially in cell-surface engineering is the Staudinger ligation as discussed in section 2. Without interfering with natural biology, this method offered insights into immune recognition, cell-cell communication and disease-associated glycosylation. Such compatibility has enabled the development of fluorogenic probes for real-time metabolic imaging, building on this compatibility[49]. Liang et al., for instance, described a quenched glucose-based probe that showed a 20-fold increase in fluorescence upon intracellular Staudinger ligation, allowing for wash-free monitoring of glucose uptake and differentiation between malignant and healthy cells[85]. Staudinger ligation has made bioorthogonal therapeutic activation possible in addition to imaging. An azide-masked Se-rhodamine photosensitizer was introduced by Liu and colleagues[86]. It was inactive until phosphine-triggered ligation, at which point red-light irradiation produced singlet oxygen and specifically prevented HeLa cell multiplication. This approach minimized harm to healthy tissues while precisely controlling phototoxicity in space and time. Zhang et al. created a Staudinger-triggered NIR probe for nitrosyl (HNO) detection, which achieved quick response and high sensitivity for tracking redox signalling in living cells[49].This chemistry has also been adapted for reactive species sensing. Staudinger ligation is nevertheless useful in situations where biocompatibility and controlled activation are crucial despite its slower kinetics and vulnerability to phosphine oxidation.

3.4 Tumour Microenvironment Imaging

The tumour microenvironment (TME), which is defined by hypoxia, acidosis, ion imbalance and reactive oxygen species may now be dynamically visualized thanks to developments in bioorthogonal imaging. The development of ratiometric fluorescent nanosensors that can detect TME parameters non-invasively and self-calibrate has been prompted by the limitations of traditional imaging methods. These platforms, which are made of organic dyes, quantum dots, and hybrid nanomaterials, allow for the real-time monitoring of metabolic activity and therapeutic response both in vitro and in vivo. **(Figure 6)** shows the principle of ratiometric fluorescence imaging in the tumour microenvironment where a dual-emission probe reacts to abnormal redox conditions and acidic pH that are typical of cancers[15].

3.5 CuAAC and Fluorogenic Probe Development

Ratiometric fluorescence probes are widely used for qualitative imaging and minimizing concentration-dependent variability. The variations in emission intensity ratio lead to different colour changes, thus avoiding problems due to the influence of the concentration of the probe, intensity of stimulation, and environmental conditions and leading to self-calibrating, on-line sensing of reactive oxygen species, oxygen changes, and tumour acidosis[87]. Precision oncology applications benefit from improved photostability and multiplexing through integration into polymer matrices. The creation of fluorogenic probes has also benefited greatly from copper-catalyzed azide–alkyne cycloaddition (CuAAC). For live-cell imaging, conjugation with luminophores such as coumarins, rhodamines, bodipy dyes and AIE systems has produced "turn-on" probes with great brightness and photostability[88]. These techniques have made it possible to visualize cancers, glycans, enzymes, bacterial cell walls and to integrate imaging and photodynamic therapy. CuAAC has also shown translational utility in treatments and diagnostics such as fast bacterial detection employing DNAzyme-based systems suitable with portable devices and in situ probe activation within Alzheimer's amyloid plaques **(Figure 7)**[89].

3.6 BONCAT and Proteomic Labelling

Important bioconjugation techniques like bioorthogonal noncanonical amino acid tagging (BONCAT) which permits selective labelling and proteomic analysis of freshly generated proteins are supported at the molecular level by CuAAC and strain-promoted azide–alkyne cycloaddition (SPAAC).BONCAT (Bioorthogonal Non-Canonical Amino Acid Tagging) enables incorporation of azide or alkyne functionalized amino acids into newly synthesized proteins during translation followed by selective labelling and detection using bioorthogonal reactions. Emerging proteomes can be specifically seen, enriched and analyzed thanks to subsequent bioorthogonal click reactions using fluorescent or biotinylated probes that selectively label nascent proteins while leaving pre-existing proteins unaltered **(Figure 8)**[90]. Altogether, these advancements demonstrate how click chemistry has evolved from a flexible ligation reaction to a clinically useful platform that connects precision

drug delivery, targeted therapy and molecular imaging.

By integrating BONCAT with CuAAC, SPAAC, and tetrazine ligations, recent developments now enable multimodal labelling of developing proteomes in living systems. In complex biological systems, BONCAT (Bioorthogonal Non-Canonical Amino Acid Tagging) facilitates the space time coordinated labelling of newly generated proteins, supporting quantitative proteomics that monitor minute expression changes during illness or therapy. Methionine-tRNA synthetases and methods for difficult environments such microbial communities and mammalian secretomes have been developed in an effort to increase labelling efficiency, decrease false positives and improve cell type specificity[91,92]. Researchers may now analyze tissue repair, cell signalling, microbial activity and viral infection dynamics with previously unheard-of precision by combining BONCAT with high-resolution proteomics and imaging. BONCAT is frequently used for functional heterogeneity characterization, translationally active cell isolation, and secretome profiling **(Figure 8)**.

3.7 Nanotechnology and Theranostic Platforms

CuAAC has extended into nanotechnology, offering space-time regulated reaction control by DNAzyme–click hybrids, mesoporous nanoreactors and copper-containing nanoparticles[93]. These systems enable the use of heat or light to activate fluorophores and prodrugs, expanding the use of CuAAC beyond imaging to include pathogen detection and therapeutic interventions **(Figure 10)**[79]. Ongoing efforts concentrate on safer ligands, stabilized nanoparticles and copper-free substitutes despite worries about copper toxicity. Nanoscale delivery platforms have also proven advantageous for photoacoustic and NIR-II fluorescence imaging. Protein assemblies improve brightness and fine-tune pharmacokinetics, polymeric micelles improve circulation and biodegradability, lipid-based carriers improve payload delivery, stability and biocompatibility and microbubbles allow blood-brain barrier penetration with ultrasound-mediated release, although their mechanical fragility necessitates careful handling[94].

Theranostic probes, such as glutathione-responsive Cu(II) nanoreactors or AIE photosensitizers that glow upon immobilization and generate reactive oxygen species for photodynamic therapy enable selective medication release in tumour microenvironments[95]. While Devaraj created RNA-templated processes and streamlined Tz-fluorophore synthesis for sensitive microRNA detection, Wombacher's bodipy-Tz probes produced nearly 24-fold improvement[87]. In order to provide long-lasting, background-free luminescence appropriate for multicolour imaging and enzymatic activity tests, IEDDA probes have also been expanded to metal complexes and lanthanide systems[96]. Terbium-based probes in zebrafish, iridium–Tz complexes for multicolour labelling and FRET-based iridium–rhodamine sensors for caspase-3 imaging are a few examples. Additionally, metal ions like Mg^{2+} can be seen with organelle-specific labelling thanks to bioorthogonally triggered Tz probes.

3.8 Barriers to Clinical Translation and Future Outlook

Although NIR-II probes enable deep-tissue imaging, inconsistent quenching efficiency limits signal reliability. Aggregation-induced emission (AIE) based probes offer improved performance but challenges in large-scale synthesis and clinical translation remain. Targeting and imaging specificity are improved by complementary functionalization with PEGylation, antibodies, peptides, aptamers or biomimetic membranes especially for tumour and central nervous system administration. Overall, these techniques offer a flexible toolkit for precise, deep-tissue imaging however, more advancements in delivery, stability, and multifunctionality are required.

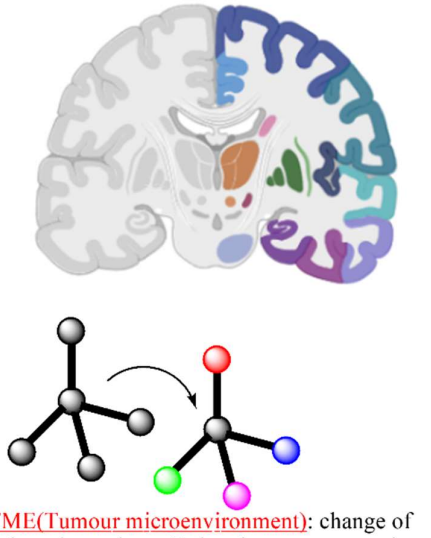


Figure 6. Schematic representation of tumour microenvironment imaging using ratiometric florescence. The probe responds to pH/ ROS variations enabling real-time monitoring.

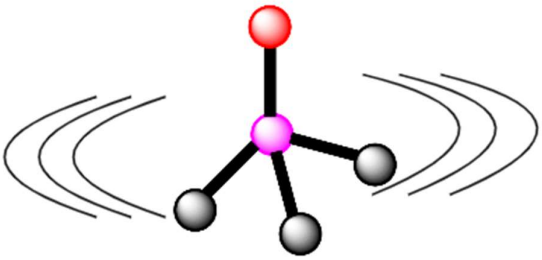


Figure 7. Schematic representation of a bioorthogonal ligation reaction in live cells, highlighting selective labelling under physiological conditions.

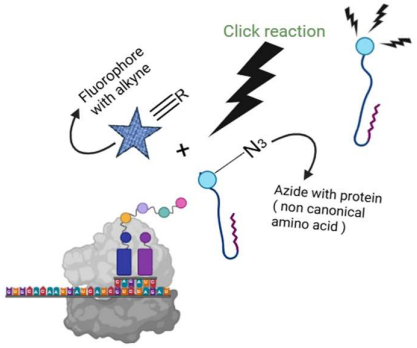


Figure 8. Illustration of an example of Bioorthogonal Non-Canonical Amino Acid Tagging, BONCAT

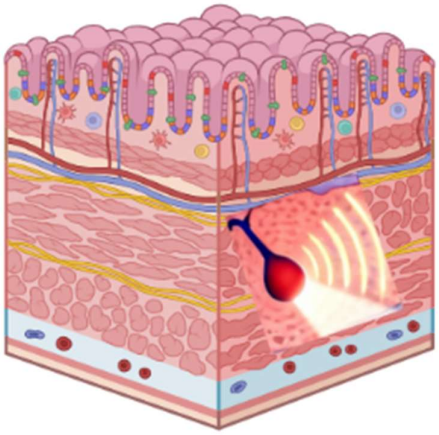


Figure 9. In contrast to traditional optical imaging, the figure highlights how NIR-II excitation allows for greater tissue penetration, less scattering, and crisper vision.

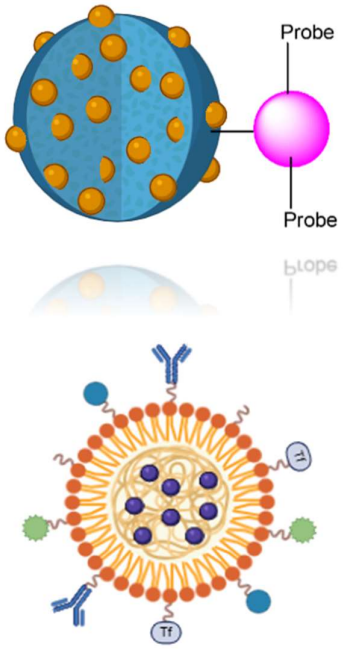


Figure 10. An Illustration of drug delivery systems based on micelles, nanoparticles, and nanospheres in bioorthogonal chemistry. These nanocarriers allow for targeted transport, controlled release, and selective activation at disease locations by encapsulating or conjugating therapeutic chemicals and bioorthogonal probes.

4. Technological Integration

Fluorescence, Raman, MRI, PET, photoacoustic imaging (PAI) and SPECT are examples of advanced molecular bioimaging techniques that have revolutionized biomedical research, diagnosis and treatment. High spatiotemporal resolution and quantitative detection at the cellular and organismal levels make fluorescence imaging unique[15]. Conventional fluorescent probes like emissive dyes and dye-conjugated biomolecules (like antibodies) are constrained by endogenous autofluorescence interference, photobleaching and nonspecific binding. Metal complexes, aggregation-induced emission (AIE) systems, and near-infrared (NIR)-II dyes are examples of next-generation probes that enhance photostability, lower background and allow for deeper tissue penetration[82]. Targeted imaging, biomolecule labelling and in vivo drug delivery have been made possible by selective reactions including CuAAC, Staudinger ligation, IEDDA and CBT click chemistry, since early developments in bioorthogonal chemistry multiple reaction platforms have been introduced. Initially, non-emissive molecules can be "turned on" in situ using bioorthogonally triggered fluorescent probes allowing for wash-free, real-time, highly precise imaging. PET and SPECT visualization provide quantitative tumour imaging while dual-modal agents that combine nuclear and NIR labels enable

both whole-body imaging and fluorescence-guided surgery[97]. Probe building for precision imaging is improved by bioorthogonal processes such as tetrazine–TCO, which enable the clean conjugation of radionuclides and fluorophores[80].

Additionally, bioorthogonal chemistry has improved organoid design and three-dimensional cell cultures. Tetrazine-norbornene or TCO cross-linked hydrogels mimic the characteristics of extracellular matrix (ECM) and allow for the location and time regulation of mechanical and rheological stimuli that impact tissue formation[98]. Dental organoids cultivated in bioorthogonally cross-linked gelatin hydrogels show how scaffold stiffness and crosslink density control cell communication, structural organization and epithelial-mesenchymal interaction[99]. Additionally, dynamic microenvironments for researching illness and regeneration are made possible by these hydrogels. Bioorthogonal chemistry, artificial intelligence and image-based phenotyping work together to speed up cell biology and molecular imaging research. Reactions such as SPAAC and tetrazine ligations provide very selective fluorescent tagging which AI can assess for multiparametric cell, organoid and tissue profiling. When paired with metabolic labelling or noncanonical amino acid incorporation, these probes allow automated segmentation, revealing structural and functional patterns associated with disease or regeneration[100]. Super-resolution microscopy combined with minimal tags or chemical handles enables multicolour imaging of spatial features and dynamics deep within living organisms. Furthermore, long-term monitoring of transplanted cell survival, migration, differentiation, and localization via NIRF or dual-modality imaging is made easier by bioorthogonal metabolic labelling[101].

Live, multiplexed molecular analysis is improved by integration with microfluidic technologies. Microfluidics preserves cellular integrity while enabling the regulated delivery of bioorthogonal probes with little reagent usage. Bioorthogonal processes allow for high-sensitivity, site-specific tracking of biomolecules, nucleic acids, and metabolites when combined with sophisticated live imaging methods like stimulated Raman scattering and multiphoton fluorescence microscopy[102]. Fast biological process analysis and metabolic flux monitoring are made possible by the excellent spatiotemporal resolution and repetitive, non-invasive real-time measurements offered by minimal tags and vibrational probes compatible with microfluidics. These technologies provide high-throughput screening in model organisms and tissue constructions, multicolour orthogonal tracking, and dynamic imaging. The translational impact of bioorthogonal chemistry in integrated microfluidics and live imaging is anticipated to be further enhanced by ongoing advancements in probe chemistry, hardware and reaction kinetics[103,104]. Reaction rates in vivo are often reduced by a massive scale compared to in vitro conditions due to macromolecular crowding and diffusion limitations. Additionally, immunogenic responses associated with tetrazine and TCO scaffolds remain underexplored, necessitating validation in humanized models.

5. Limitations and challenges in Bioorthogonal Chemistry

Strong in vivo and clinical translation of bioorthogonal chemistry is currently constrained by a number of issues despite remarkable advancements. While individual limitations have been discussed in earlier sections, this section provides an integrated overview of key challenges. Because even slight structural alterations in tetrazines, strained alkenes/alkynes or bulky handles can change pharmacokinetics, tissue distribution and protein interactions as well as occasionally cause immune responses or off-target accumulation, reactive motifs and catalysts can introduce toxicity and immunogenicity. Copper-induced oxidative damage and interference with metal-sensitive pathways continue to be concerns for metal-catalyzed systems like CuAAC, which drives the development of encapsulated or quickly cleared catalyst designs[76,105]. Appended tetrazine or stretched alkene handles can boost immunogenicity and promote the development of anti-drug antibodies in pretargeted techniques employing modified antibodies or proteins but systematic, long-term safety evidence in prediction models is still lacking.

Incomplete and context-dependent reaction kinetics are a second significant problem. Steric hindrance, restricted movement, compartmentalization, and low concentrations of reagents in vivo severely impact the efficiency of reactions. Benchmark ligations like IEDDA and SPAAC frequently perform less effectively in crowded, heterogeneous biological environments which can lower labelling density and quantitative accuracy[13,49]. High inherent reactivity can also result in off-target ligation with thiols, nucleophiles or reactive oxygen species which can cause premature prodrug activation, background signal, or non-specific drug release. Although these effects are lessened by caged or conditionally activatable probes, hydrophilic and sterically protected scaffolds, and stimulus-responsive designs, the trade-off between speed and selectivity remains unresolved[106].

Probe stability, delivery and manufacturability are further constraints. The hydrolysis, oxidation, isomerization, aggregation, or non-specific protein binding unintentional reactions of a number of frequently used partners (tetrazines, TCOs, strained alkynes) impair effective in vivo lifetimes and deep tissue performance[104]. While PEGylated and nanocarrier-based systems enhance circulation and

targeting, they also raise concerns regarding long-term biodistribution and clearance, scale-up difficulties and additional synthetic complexity[107]. Regulatory translation is complicated by the disparities in immunity and metabolism between humans and small animal models, and the large-scale, GMP-compliant manufacturing of strained reagents, tetrazines, and multifunctional prodrugs is still expensive and time-consuming. Bioorthogonal reactions are being used to activate drugs at specific sites especially in cancer treatment. This helps rescue unwanted side effects. Even though these strategies are chemically precise enough their success in real systems is limited by the difficulty of bringing both reaction partners together at the same place[48,52]. A central limitation is the delivery and in vivo transport of reaction partners, rather than the intrinsic efficiency of the bioorthogonal reactions themselves[53].these observations indicate ongoing challenges in achieving efficient targeting and delivery in biological systems. Lastly, chemical deterioration, quick renal clearance of small hydrophilic probes, and ongoing accumulation of more lipophilic or nanoparticle-based constructs impede sustained, long-term in vivo tracking, highlighting the need for more efficient yet safely cleared probe architectures and standardized long-term safety assessment frameworks

6. Future Directions and Emerging Trends

Inverse electron-demand Diels -Alder (IEDDA) reactions have become a prominent framework for clinical bioorthogonal chemistry with tetrazine-TCO pretargeting progressing into phase-II colorectal cancer trials. Recent advancements encompass dual-labelled tetrazines attaining elevated tumour to blood ratios (~30:1) and Lu-177-based radioimmunotherapy providing localized doses with negligible off-target effects. Other than IEDDA From Staudinger and CuAAC to SPAAC and ultrafast tetrazine-trans-cyclooctene iEDDA, as well as SuFEx and reversible covalent ligations for stable or dynamic conjugation and click-to-release systems, bioorthogonal chemistry is advancing toward faster, more selective, and more biocompatible ligations that function dependably in complex biological environments[19]. Genetic code expansion and mutually orthogonal reaction sets are important trajectories. Site-specific tagging of biomolecules in cells and entire organisms is supported by the incorporation of noncanonical amino acids and other unnatural substrates bearing azides, alkynes, cyclopropenes, and related handles. This allows multiplexed SPAAC, IEDDA, and boronic acid-type chemistries without cross-reactivity, which is essential for dual/triple labelling, synthetic circuits, and multifunctional therapeutics like ADCs and programmable biomaterials[51]. Further in IEDDA AI-optimised strained alkenes lower TCO isomerization and photocaged tetrazines let us activate things when we want them to with light. IEDDA is also being added to hydrogels to make organoid models and used with SPAAC and photo click reactions to track multiple biomarkers at once. Click-to-release systems now also make it possible to activate payloads quickly in physiological conditions which is useful for targeted therapy and cell-based treatments. IEDDA is a very promising platform for future clinical translation because it has very fast kinetics (about 10^6 M^{-1} s^{-1}) and works well with multimodal imaging

From a technological perspective, with machine learning and quantum chemistry models screening vast libraries and suggesting new ways to jointly improve stability, sensitivity, selectivity, and biocompatibility for imaging and therapy, AI-driven and computational design are becoming increasingly important[108]. To guarantee clinical reliability, complementary mechanistic and translational research highlights the need to balance reaction reactivity with tissue compatibility, pharmacokinetics, scalable synthesis, and blood stability[2]. Organelle- and cell-type-selective labelling is another developing field in which site-specific bioconjugation and suppression of codons (genetic engineering) in mitochondria, lysosomes, nuclei, and specific cell populations is made possible by customized physicochemical characteristics, genetic tags, and targeted delivery[8]. Orthogonal pairs are frequently used for multiplexed subcellular imaging and functional interrogation. These instruments support synthetic biology approaches wherein "smart" therapeutic cells react to bioorthogonal inducers by regulating immunomodulation, drug release, or gene expression.

Driven by SPAAC and tetrazine-TCO platforms, pretargeted nuclear/optical imaging, fluorescence-guided surgery, point-of-care diagnostics, and bioorthogonal prodrug activation are expected to be the first real-world applications on the clinical horizon; success will depend on GMP-compatible synthesis, standardized in vivo kinetic/safety benchmarks, and evolving regulatory frameworks to support routine use in the coming years[52].

7. Conclusion

Bioorthogonal chemistry has emerged as a robust platform for facilitating efficient and selective bioconjugation reactions in complex biological environments. Fast bioconjugations like tetrazine-TCO inverse electron-demand Diels–Alder reactions along with well-studied chemistries such as CuAAC, SPAAC, and Staudinger ligation provide an array of potential applications including in vivo visualization and protein labelling as well as site-specific drug activation and multimodal theranostics. Click-to-release strategies, SuFEx reactions, and photoactivation have been employed in extending spatiotemporal control over chemical reaction in biological systems. Nevertheless, several translational issues must be considered. Reactions in

vivo will not be as efficient because of diffusion problems, molecular crowding, and compartmentalization; therefore, reactions in vivo are slower than in vitro. The instability of probes and off-target reaction as well as the undesirable pharmacokinetics of some probes limit their application. Copper toxicity is one of the major drawbacks of Cu-based probes that could be dangerous to patients' health. Moreover, the immune response to targeted probes is one of the limiting factors.

Moving forward, advancements will rely on attaining an equilibrium between reactivity, stability, and biocompatibility by engineering reaction partners with optimal structures and minimally disruptive chemical handles. Activatable probe formats and secondary triggers like light, pH changes, and enzymes are likely to increase spatial resolution and reduce background noise. Further improvements in the field include the development of delivery methods like degradable nanoparticles, PEGylation, and biomimicry techniques, which will improve target specificity and in vivo utility.

The incorporation of AI technologies and computational methods is anticipated to facilitate faster probe identification by allowing for the optimization of multiple factors at once, including kinetics, selectivity, and pharmacological characteristics. When combined with kinetic characterization, predictive in vivo models, and superior imaging capabilities, these trends suggest that bioorthogonal chemistry will be a crucial technology platform for future advances in diagnostic and therapeutic applications.

List of Abbreviations:

CuAAC: Copper-catalysed Azide–Alkyne Cycloaddition [1]
SPAAC: Strain-Promoted Azide–Alkyne Cycloaddition
iEDDA: Inverse Electron-Demand Diels–Alder Reaction [2]
SuFEx: Sulfur(VI) Fluoride Exchange
DBCO: Dibenzo Cyclooctyne
DIFO: Difluorinated Cyclooctyne
BCN: Bicyclo [6.1.0]nonyne
SPANIC: Strain-Promoted Alkyne–Nitrone [3] Cycloaddition
SPSAC: Strain-Promoted Sydnone–Alkyne Cycloaddition
SPSIC: Strain-Promoted Sydnone–Imine Cycloaddition [4]
PET: Positron Emission Tomography
MRI: Magnetic Resonance Imaging
NIR: Near-Infrared
ADC: Antibody–Drug Conjugate
FGE: Formylglycine-Generating [5] Enzyme
HIPS: Hydrazino-Pictet-Spengler
ncAA: Noncanonical Amino Acid
PBS: Phosphate-Buffered Saline
HEPES: 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic Acid
HPTA: Tris(hydroxypropyltriazolylmethyl)amine
BTTAA: 2- [4-({Bis [(1-tert-butyl-1H-1,2,3-triazol-4-yl)methyl]amino}methyl)-1H-1,2,3-triazol-1-yl]acetic acid
BTTES: Bis(triazolyl methyl)amine-based ligand with sulfonate group
TBTA: Tris [(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine
DIPEA: N, N-Diisopropylethylamine

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Declaration: The authors themselves have generated figures submitted with the manuscript and have not been copied or adapted from other sources. The standard of the English language in this article has been enhanced using LLMs, namely Meta's language model Llama-3.2-1Band, OpenAI's chatbot, Chat Generative Pre-trained Transformer 4.5. We bear full responsibility for the contents of this publication. We have conducted a comprehensive examination, made improvements, and refined all languages generated by the LLM to ensure their precise alignment with our initial research outcomes and interpretations.

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